Table 2 Respondents with usable answers by disease and drug used.

Frequency		Total	DPI-LABA	MDI-SABA	DPI-CS	MDI-CS	MDI-anticholinergic	TP-LABA
Total		1470	523	567	279	132	42	470
Asthma	Children*	171	28	105	3	4	0	88
	Adults	465	149	212	159	67	0	116
	Elderly	411	124	174	117	61	0	154
COPD†	Adults	241	123	51	0	0	31	55
	Elderly	182	99	25	0	0	11	57

*Children: patients aged 0–12 years (their patients answered the questionnaire). Adults: patients aged 18–64 years. Elderly: patients over 65 years of age.
†COPD patients include those diagnosed with chronic bronchitis or pulmonary emphysema.

transdermal drugs. The level of statistical significance was set at $p<0.05$.

Results

Background factors of participants

Usable answers were obtained from 1470 respondents, including 876 adult patients with asthma, parents of 171 asthmatic children, and 423 patients with COPD. Of these respondents, 523 provided answers about DPI-LABA, 567 about MDI-SABA, 279 about DPI-CS, 132 about MDI-CS, 42 about MDI-anticholinergic, and 470 about TP-LABA (Table 2).

Table 3 shows the characteristics of the patients who provided useful answers. There were 655 males and 815 females, including 171 children aged 0–12 years, 706 patients aged 18–64 years, and 593 patients over 65 years of age, and there were 1047 asthmatic and 423 COPD patients. The most common levels of severity of asthma and COPD as determined from the patients' self-evaluation of symptoms, were mild, intermittent asthma in 58.0% of adult asthmatic patients, mild, intermittent asthma in 56.1% of asthmatic children, and BMRC Dyspnea Scale Grade 1 COPD in 36.6% of COPD patients.

Table 3 Attributes of respondents to the survey.

	Number of patients	Ratio (%)
Gender		
Male	655	44.6
Female	815	55.4
Age		
0–12 years	171	11.6
18–64 years	706	48.0
65 years or older	593	40.3
Range	1–76	
Mean	47.8	
Current disease		
Asthma	1047	71.2
COPD	423	28.8
Severity of asthma in adults		
Mild intermittent	508	58.0
Mild persistent	135	15.4
Moderate persistent	62	7.1
Severe persistent	171	19.5
Severity of asthma in children		
Mild intermittent	96	56.1
Mild persistent	30	17.5
Moderate persistent	25	14.6
Severe persistent 1	1	0.6
Severe persistent 2	19	11.1
Severity of COPD		
Grade 1	155	36.6
Grade 2	136	32.2
Grade 3	79	18.7
Grade 4	23	5.4
Grade 5	16	3.8
Grade 6	14	3.3

Adherence to treatment

Comparison by drug formulation (Fig. 1)

Among asthmatic patients, the percentage of those who selected "taking as prescribed" was 52.7% for inhalant users and 83.2% for TP-LABA users . Among patients with COPD, the corresponding percentages were 54.7% and 86.6%. The difference in compliance between the inhalant and TP-LABA users was significant in each group ($p<0.01$).

Comparison of adherence to treatment according to the agent and formulation prescribed (Fig. 2)

The percentage of users taking the drug as prescribed was 53.7% for DPI-LABA, 64.6% for MDI-SABA, 38.7% for DPI-CS, 39.4% for MDI-CS, 31.0% for MDI-anticholinergic, and 84.0% for TP-LABA . The rates of adherence to treatment with DPI-LABA and MDI-SABA were significantly higher than those to DPI- and MDI-CS ($p<0.01$), and the rate of adherence to treatment with MDI-SABA was significantly higher than that to DPI-LABA ($p<0.01$), though there was no difference in rate between DPI- and MDI-CS. Rate of adherence was significantly higher for TP-LABA than that to any of the inhalants ($p<0.01$).

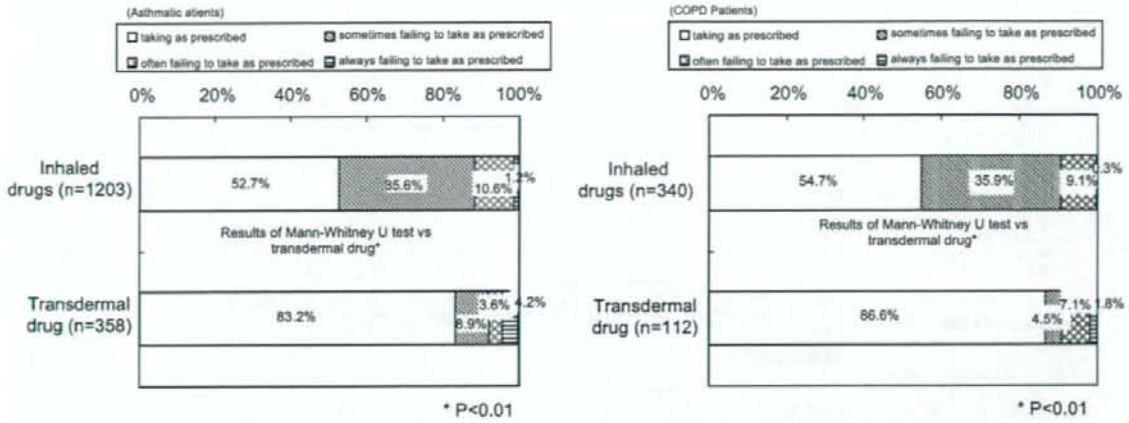

Figure 1 Adherence with regimens (asthmatic patients/COPD patients) by drug formulation. Q1. Are you taking drugs currently prescribed for treatment of asthma/COPD as directed by your physicians? Please provide an answer for each drug.

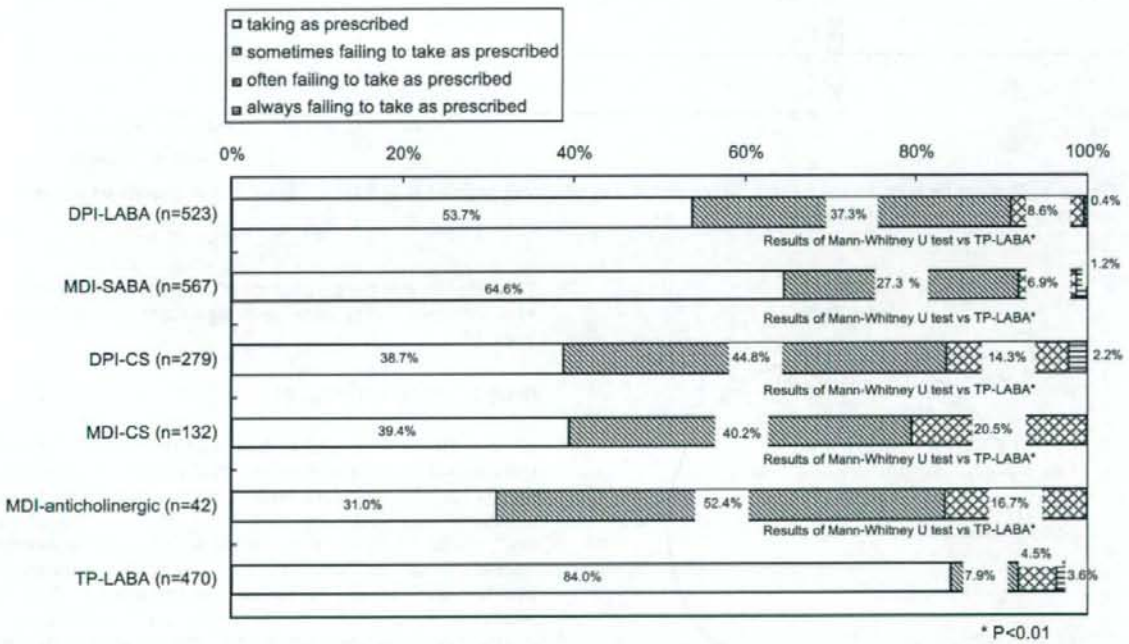

Figure 2 Adherence with regimens by indication. Q1. Are you taking drugs currently prescribed for treatment of asthma/COPD as directed by your physicians? Please provide an answer for each drug.

Factors influencing adherence to treatment

As shown in Fig. 3, for all users, the most common reason for poor adherence among the four choices was "frequency of administration". Although the percentage of users of inhaled formulations who selected "frequency of administration" was more than double that for each of the other three reasons, the percentage of TP-LABA users who selected "frequency of administration" and "timing of administration" was low, at 7.2% and 5.1%, respectively. The percentage of TP-LABA users who selected "frequency of administration" was significantly less than those of the five inhalant users as determined by Z-test ($p<0.01$).

Preferred frequency of administration

Fig. 4 shows the results for the question on preferred frequency of administration; 83.2% of the patients preferred the once-daily administration.

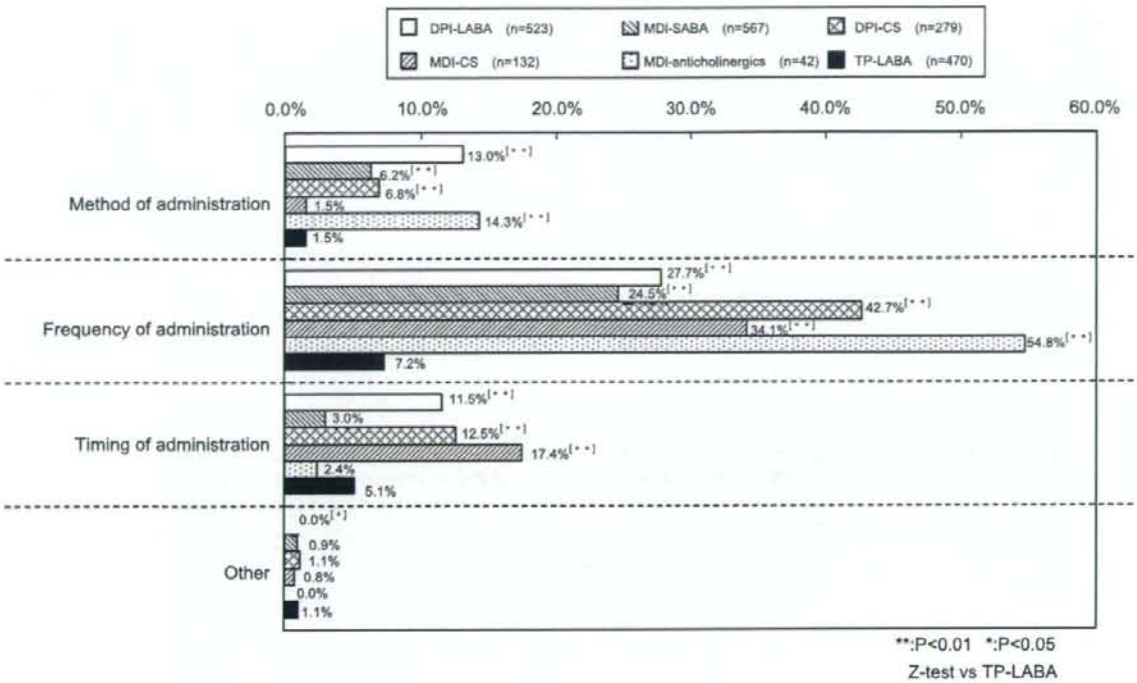

Figure 3 Reasons for poor adherence. Q2. Which part of the treatment regimen is difficult to follow? Please provide an answer for each drug (please select all that apply).

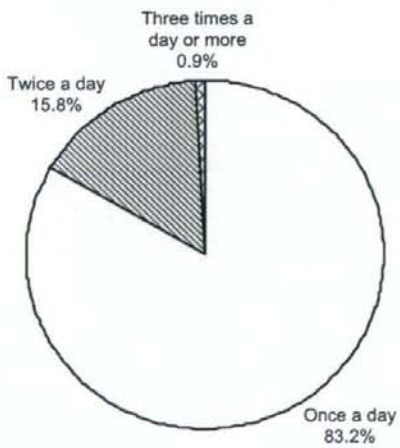

Figure 4 Preferred frequency of administration. Q3. Please indicate the most preferable frequency of administration for your asthma/COPD drugs.

Convenience of individual drugs

As shown in Fig. 5, among inhalant users, 42.7% of those with asthma and 32.1% of COPD patients rated inhalants as "very easy" to use, while 79.3% of patients with asthma and 73.2% of those with COPD rated the patch as "very easy" to use. The difference in the percentage of inhalant and patch users who answered "very easy" was significant in both groups ($p<0.01$).

Drug formulation (Fig. 6)

When patients who used or had used both inhalation and transdermal formulations were asked whether they wished to replace their inhalant with a transdermal drug, 18.0% answered "definitely yes" and 37.7% answered "probably yes", while 11.5% did not wish to switch, i.e., answered "definitely not" or "probably not" . When they were asked whether they wished to switch the transdermal drug to inhalants, 4.1% answered "definitely yes" and 9.4% answered "probably yes", while 49.6% did not wish to switch. The transdermal drug was undoubtedly preferred to inhalant drugs ($p<0.01$).

Discussion

In the present study, among patients with asthma and COPD, more than 80% of TP-LABA users selected "taking as prescribed", indicating higher rates of treatment compliance compared with the inhaled drugs. These findings suggest that TP-LABA, a drug featuring good adherence to treatment for both types of airway diseases, can be expected to be as effective in routine clinical practice, as in carefully controlled clinical studies in which patients are requested to take study drugs in

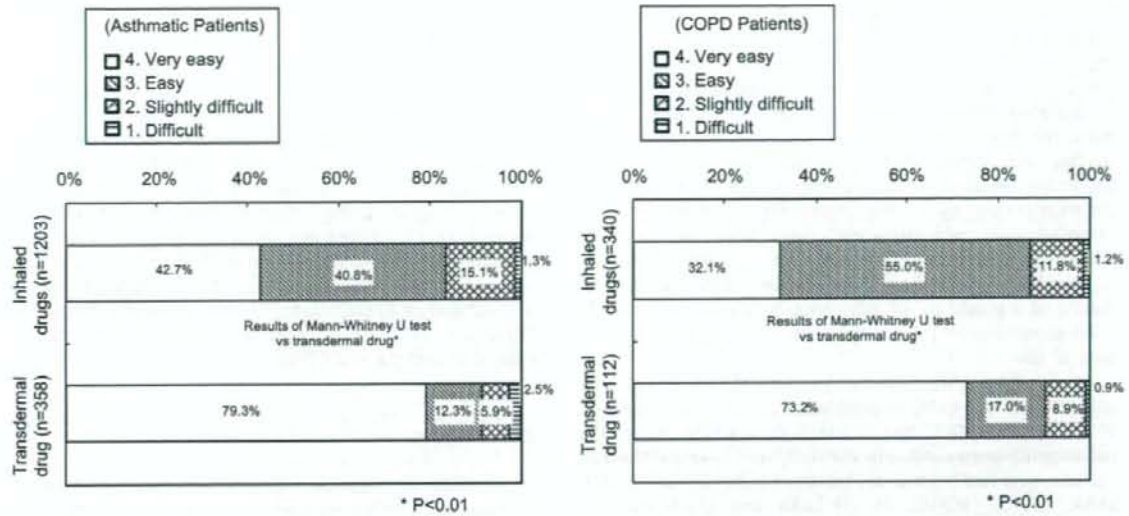

Figure 5 Convenience of drugs (asthmatic patients/COPD patients). Q4. Please rate the convenience of use of your drugs for asthma/COPD using the following rank scale.

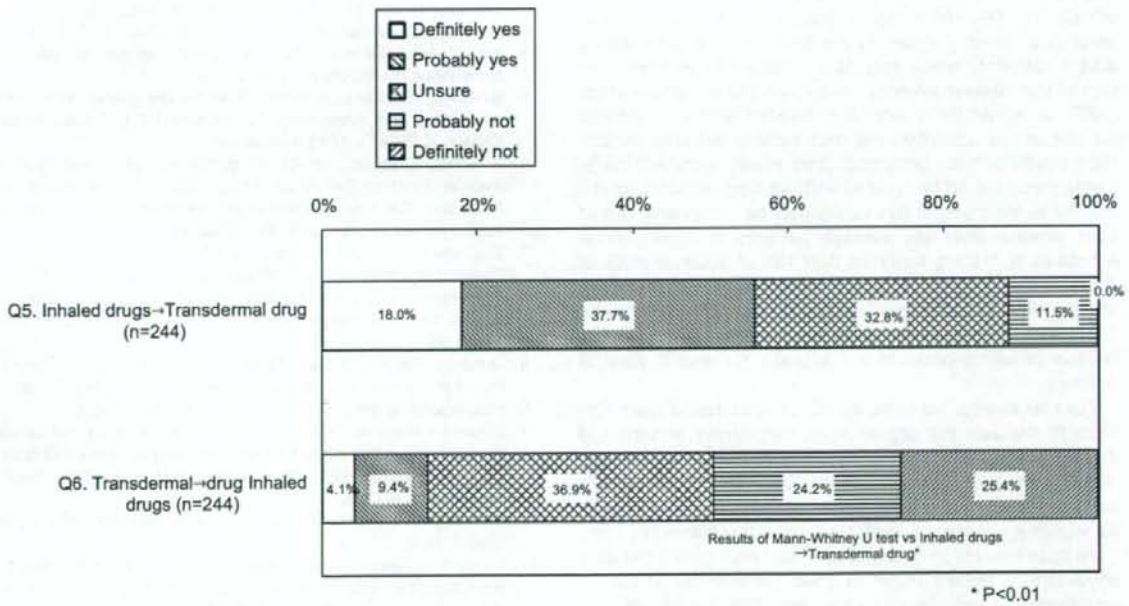

Figure 6 Willingness to replace drug formulation. The following questions are for patients who use or have used both inhaled drugs and transdermal drug. Q5. Would you like to replace inhaled drugs with transdermal drug? Q6. Would you like to replace transdermal drug with inhaled drugs?

accordance with study protocols. In addition, at least 55% of patients who have used both inhaled drugs and TP-LABA wished to switch from inhaled drugs to TP-LABA, and many patients preferred transdermal to inhaled formulations. We thus believe that TP-LABA is an ideal long-term controller which can be taken by patients without fail. We therefore think that physicians should consider TP-LABA to treat patients with asthma and COPD, especially those with poor adherence to treatment with inhaled drugs.

The results of this survey are consistent with the low rate of adherence to treatment with inhaled drugs pointed out by physicians.[1–4,11,12] Some of them have reported that this low rate appears to be due to the need for special instructions to ensure appropriate inhalation and the complicated procedures required for inhalation.[1,13] However, we suggest that special and complicated instructions are not the only important reasons for poor adherence, since we found that the most commonly selected reason for poor adherence was "frequency of administration", and since the rates of adherence to treatment with DPI-LABA and MDI-SABA were significantly higher than those for DPI- and MDI-CS. Our finding of a good rate of adherence to treatment with TP-LABA appears to be related to its once-daily application and ease of use.

As shown in Table 3, more than half of the respondents with asthma had mild, intermittent asthma, and about 70% of those with COPD had Grade 1 or 2 COPD. Since the participants were randomly selected from among asthmatic patients who had been under treatment with DPI-LABA, MDI-SABA, DPI-CS, MDI-CS, or TP-LABA and COPD patients treated with DPI-LABA, MDI-SABA, MDI-anticholinergic, or TP-LABA, all the patients evaluated in this survey were regularly receiving drugs for airway disease. The fact that some patients who reported having mild, intermittent asthma received MDI-SABA together with DPI-LABA, DPI-CS, MDI-CS, or TP-LABA suggests that patients reported the severity of their disease on the basis of symptoms during drug treatment, which may have resulted in underestimation of true disease severity. Given that the severity of their condition would be severer if evaluated before treatment for the actual severity, and that certain patients control their condition with bronchodilators alone, more asthmatic participants should be treated with inhaled corticosteroids. But the participants in this survey may be more conscious of their disease than are average patients in Japan, since Adachi et al.[14] have reported that 18% of adults and 8% of children with asthma used inhaled corticosteroids in Japan. This survey may also reveal a discrepancy, which needs to be addressed, between the guidelines for treatment and the fashion in which patients are actually treated in clinical practice.

For this survey, we selected an Internet-based questionnaire to evaluate the actual use of transdermal and inhaled drugs by patients with either asthma or COPD. We thought that an Internet-based questionnaire would have certain advantages in evaluating adherence to treatment with various drugs, since it is useful in eliminating biases resulting from false reports by patients who may wish their attending physician to regard them as good patients by virtue of maintaining their dosing schedules. The results of our Internet-based survey may thus reflect treatment adherence in clinical practice, unlike those obtained in carefully controlled trials. Usage of the Internet was reported to be correlated with household income in "A survey on use of communication services in Japan" (http://www.stat.go.jp/data/joukyou/2003ni/index.htm) conducted by the Ministry of Internal Affairs and Communications of Japan. Since it has also been reported that adherence to treatment is higher in case of patients with higher household income,[15,16] participants in the present survey may show better treatment compliance than do average patients in Japan. In addition, patients with better adherence may have responded to this survey faster than those with poor adherence to treatment. These considerations suggest that in clinical practice, treatment compliance may be lower than suggested by the results of this survey.

In summary, to obtain in clinical practice the same degree of efficacy as in carefully controlled trials, health care professionals should improve adherence to treatment with inhaled drugs by further educating their patients about the importance of inhaled agents. In addition, inhalant users selected the "frequency of administration" as the primary reason for their poor adherence, which suggests the reason why adherence in once-daily TP-LABA users was the highest. Therefore, TP-LABA appears to be useful for long-term control of asthma and COPD.

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An IL-1 Cytokine Member, IL-33, Induces Human Basophil Activation via Its ST2 Receptor[1]

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Basophils are thought to play pivotal roles in allergic inflammation through rapid release of chemical mediators in addition to sustained production of Th2 cytokines, including IL-4. A newly identified cytokine, IL-33, has been recognized as one of the key cytokines enhancing Th2-balanced immune regulation through its receptor, ST2. The present study was conducted to elucidate whether IL-33 acts directly on, and affects the functions of, human basophils. Real-time PCR analysis showed that basophils express transcripts for ST2. The expression levels were significantly higher compared with eosinophils and neutrophils, and treatment with IL-33 significantly up-regulated basophil ST2 mRNA expression. Expressions of IL-4 and IL-13 mRNA were also up-regulated by IL-33, and there was also enhanced secretion of IL-4 protein. IL-33 increased the surface levels of basophil CD11b expression and enhanced basophil adhesiveness. Although IL-33 failed to directly induce degranulation or attract basophils, it exerted priming effects on basophils. It enhanced degranulation in response to IgE-crosslinking stimulus and also enhanced basophil migration toward eotaxin without changing surface CCR3. Also, IL-33 synergistically enhanced IL-4 production and CD11b expression by IL-3-stimulated basophils. Neutralization using Ab specific for ST2 significantly diminished the enhancing effects of IL-33 on both basophil CD11b expression and migration toward eotaxin, indicating that IL-33 signals via ST2 expressed on basophils. This study revealed that IL-33 potently regulates migration and activation of human basophils. IL-33 may be a key cytokine in the pathogenesis of Th2-dominant inflammation by acting not only on lymphocytes but also on effector cells such as basophils. *The Journal of Immunology,* **2008, 181: 5981–5989.**

Since first having been described by Paul Ehrlich (1), basophils have been increasingly recognized as one of the important effector cell types in allergic inflammation although they constitute only <1% of circulating leukocytes. A hallmark aspect of basophils is the abundant expression of a high-affinity receptor for IgE, FcεRI, on their surface. When surface-bound IgE is cross-linked by specific Ags, basophils rapidly release potent vasoactive mediators such as histamine that are stored in their cytoplasmic granules. In addition, basophils synthesize cytokines such as IL-4 and IL-13 as well as lipid mediators such as leukotriene (LT)[3] C4. Through the release of these proinflammatory mediators, basophils are thought to play pivotal roles in allergic inflammation.

Increased numbers of basophils have been demonstrated in exudates from the upper (2, 3) and lower (4) airway and in the skin (5) several hours after Ag challenge. Recent immunohistochemical studies have also shown tissue infiltration by basophils in allergic inflammation of various organs (6–9). Furthermore, Mukai et al. demonstrated in a murine model that basophils play critical roles in the pathogenesis of very late allergic reactions several days after Ag challenge (10). Through these studies, basophils are increasingly recognized as active effector cells that are attracted to, and activated in, inflammatory sites in allergic diseases.

Several lines of cytokines have been reported to regulate basophil functions. Especially IL-3, IL-5, and GM-CSF, which are known as important basophilopoietins, are strong activators of mature basophils. We and others have demonstrated that these cytokines potentiate peripheral blood basophils by prolonging their lifespan (11), upregulating certain surface receptors, and enhancing their degranulation (12, 13), adhesion (14), cytokine synthesis (15), and migration (11, 16, 17). Our knowledge concerning the regulatory mechanisms of basophil functions continues to expand.

Schmitz et al. recently identified a new cytokine, IL-33, which belongs to the IL-1 family (18). This cytokine binds to the ST2 receptor (also called DER4, Fit-1, or T1), which has high homology to IL-1 receptor (18). ST2 receptor is known to be expressed on mast cells (19) and Th2 cells (20), but not on Th1 cells, and it was initially recognized as a serum-induced gene in fibroblasts (21, 22). Administration of anti-ST2 receptor Ab enhances Th1 responses in mice, and neutralization of ST2 inhibits allergic airway inflammation (20, 23). Based on those findings, ST2 has been considered to mediate the biological action of its ligand, IL-33, which can cause Th2-biased allergic inflammation. IL-33 can exert significant biological effects both in vivo and ex vivo (18). For example, IL-33 enhances production of Th2-associated cytokines by

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[3] Abbreviations used in this paper: LT, leukotriene; HSA, human serum albumin; MESF, molecules of equivalent soluble fluorochrome unit; PI, propidium iodide.

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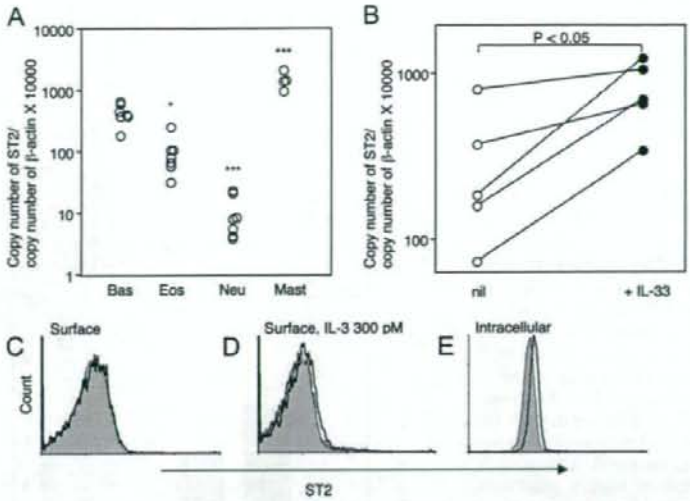

FIGURE 1. Real-time quantitative PCR and flow cytometric analysis for ST2. *A*, cDNAs from highly purified basophils ($n = 7$), eosinophils ($n = 7$), and neutrophils ($n = 7$) were tested. The data are expressed as the following ratio: copy number of ST2 gene/copy number of β-actin gene $\times$ 10,000. *, $p < 0.05$ and ***, $p < 0.001$ vs the ratio of basophils. Data for the positive-control ST2-expressing cells, i.e., cultured mast cells, are reported in our recent article (27) and included in this figure ($n = 4$). *B*, Highly purified basophils were incubated with and without IL-33 at 100 ng/ml for 4 h before RNA extraction ($n = 5$). The calculated ratios for the same donor are connected with a solid line in the graph. *C*, Highly purified basophils were stained for surface-expressed ST2. Cells stained with control Ab are shown as a shaded area. *D*, Purified basophils were cultured with 300 pM IL-3 for 18 h and then stained for surface-expressed ST2. The cells incubated in medium alone are shown as a thin line, and the cells cultured with IL-3 are shown as a thick line. The cells stained with control Ab are shown as a shaded area. *E*, Intracellular ST2 staining of freshly isolated basophils. The cells stained with control Ab are shown as a shaded area. All the flow cytometry data are representative of three separate experiments using cells from different donors and showing similar results.

in vitro polarized Th2 cells. In vivo, treatment of mice with IL-33 induces expression of IL-4, IL-5, and IL-13, resulting in severe pathological changes in mucosal organs such as infiltration by inflammatory cells (18). In addition, Iikura et al. (24) very recently demonstrated that IL-33 enhanced the survival of human umbilical cord blood-derived mast cells and promoted their adhesion to fibronectin as well as their production of IL-8 and IL-13. IL-33 is now recognized as a potentially important cytokine that enhances Th2-balanced immune regulation.

However, to date, there have been no reports regarding possible direct effects of IL-33 on allergic effector cells such as basophils. We thus conducted analyses of IL-33-induced human basophil activation in vitro. In this report, we show that IL-33 potently activates various arrays of basophil functions via ST2 by enhancing basophils' CD11b expression, adhesiveness, migration toward eotaxin, IgE-dependent degranulation, and cytokine generation. Our findings suggest that IL-33 may be an important regulator acting on effector cells, including basophils.

Materials and Methods

Reagents

The following reagents were purchased as indicated: human recombinant IL-33 (Adipogen); human IL-18 (MBL); human recombinant IL-1β (WAKO); human eotaxin/CCL11, human recombinant VCAM-1 and ICAM-1 (R&D Systems); Percoll (Pharmacia Fine Chemicals); PBS and RPMI 1640 medium (Life Technologies); and PIPES and fibronectin (0.1% solution) (Sigma-Aldrich). Human IL-3 was donated by the Kirin Brewery.

The following Abs were purchased as indicated: mouse anti-IL-4 mAb (IgG1, clone 8D4–8) and biotin-conjugated rat anti-IL-4 mAb (IgG1, clone MP4–25D2) (eBioscience); FITC-conjugated goat anti-human IgE Ab (Biosource International); mouse neutralizing anti-ST2 mAb (IgG1, clone 97203) (R&D Systems); rabbit anti-IL-33 pAb (Adipogen); mouse anti-ST2 mAb (IgG1, clone HB12) (MBL); control mouse IgG1 (MOPC21) and mouse IgG2a (UPC10) (Sigma-Aldrich); mouse IgG2b mAb (MOPC 195) (Cappel); and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). Anti-CCR3 mAb (IgG1, clone 444) was donated by Dr. H. Kawasaki (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Anti-human FceRI α-chain mAb (CRA-1) was provided by Dr. C. Ra (Nihon University, Tokyo, Japan).

Cell preparation

Leukocytes were isolated from venous blood obtained from consenting volunteers with no history of atopic diseases. Basophils were semipurified by density centrifugation using Percoll solutions of two different densities (1.080 and 1.070 g/ml) (17). The purity of these Percoll-separated basophil preparations was usually 5–15%, and the yield was $\sim 2.4 \times 10^4$ basophils/ml of peripheral blood. For some experiments, Percoll-separated basophils were further purified by negative selection with MACS beads (Basophil Isolation Kit, Miltenyi Biotec) according to the manufacturer's instructions (purity: 97–100%).

Eosinophils were purified by density gradient centrifugation followed by negative selection using anti-CD16-bound beads as previously described (purity: 97–100%).

Human neutrophils were separated by density gradient centrifugation followed by positive selection using anti-CD14-bound micromagnetic beads (Miltenyi Biotec) (purity: 97–99%).

Real-time quantitative PCR analysis

Real-time quantitative PCR analysis was performed as previously described (17). In brief, total RNA was extracted from highly purified cell preparations from separate donors using RNeasy Mini Kit (Qiagen). For some experiments, MACS-separated basophils were treated with and without IL-33 at 100 ng/ml in RPMI 1640 medium with 0.3% human serum albumin (HSA) for 4 h before RNA extraction. Real-time PCR was performed using a 7500 Real Time PCR System (PE Applied Biosystems). The primers and the probes for ST2 and IL-4 and IL-13 were designed by PE Applied Biosystems. Standard curves were constructed with serial dilutions of specific PCR products, which were obtained by amplifying peripheral leukocyte cDNA as previously described (17).

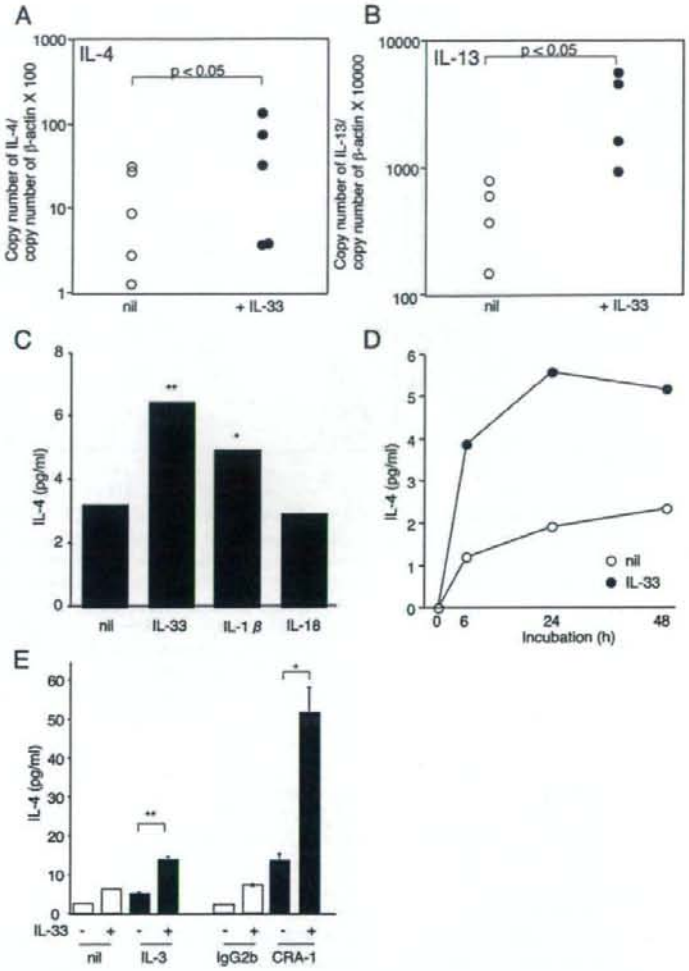

FIGURE 2. *A* and *B*, IL-33 up-regulates IL-4 and IL-13 production by human basophils. Highly purified basophils were incubated with (●) and without (○) IL-33 at 100 ng/ml for 4 h, and then IL-4 (*A*) and IL-13 (*B*) mRNA expression was analyzed by real-time PCR. Data are expressed as the ratio to the copy number of β-actin. *C*, Highly purified basophils (5×10^5/ml) were treated with and without IL-33, IL-1β, or IL-18 at 100 ng/ml for 24 h, and then the IL-4 in the supernatant was measured. Error bars represent the SEM (n = 5–6). *, $p < 0.05$; **, $p < 0.01$ vs medium alone (nil). *D*, Highly purified basophils (5×10^5/ml) were cultured with (●) and without (○) IL-33 at 100 ng/ml, and then the IL-4 in the supernatant was assayed. Experiments were performed in duplicate. Data are representative of two separate experiments showing similar results. *E*, Highly purified basophils (5×10^5/ml) were cultured with and without IL-33 at 100 ng/ml and with IL-3 at 300 pM or CRA-1 or control IgG2b at 10 ng/ml, and then IL-4 in the supernatant was measured. Error bars represent the SEM (n = 5). *, $p < 0.05$; **, $p < 0.01$.

Flow cytometric analysis of surface-expressed molecules

Highly purified basophils were used for flow cytometric analysis of ST2 expression. Basophils were incubated for 30 min at 4°C with 10 μg/ml of either anti-ST2 mAb (MBL) or control Ab and then stained with PE-conjugated goat anti-mouse IgG at 10 μg/ml for 60 min at 4°C. For intracellular staining, the cells were fixed with PBS containing 4% PFA at 4°C for 30 min followed by permeabilization in PBS containing 0.1% Tween 20 at 4°C for 30 min. The cells were then stained and analyzed by flow cytometry.

CD11b expression experiments were performed using Percoll-separated basophils as previously described (25). Following stimulation in PIPES buffer containing 25 mM PIPES, 119 mM NaCl, 5 mM KCl, 2 mM Ca^{2+}, 0.5 mM Mg^{2+}, and 0.03% HSA, basophils were incubated with 10 μg/ml of either PE-conjugated anti-CD11b mAb or PE-conjugated control mouse IgG1 at 4°C and then stained with FITC-conjugated anti-human IgE Ab at 10 μg/ml. Cells showing strong positive staining for IgE were considered to be basophils and were further analyzed for their PE fluorescence. The median values of fluorescence intensity for the basophils were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESF), as previously described (25). Surface receptor levels were semi-quantified using the following formula: ΔMESF = (MESF of cells stained with anti-CD11b mAb) − (MESF of cells stained with control IgG).

MACS-separated basophils were used to analyze surface CCR3 expression as previously described (13, 25). In brief, cells were incubated for 30 min with 10 μg/ml of either anti-CCR3 mAb or control mouse IgG1, and then stained with FITC-conjugated goat anti-mouse IgG Ab before flow cytometric analysis.

FcεRI expression was analyzed using MACS-separated basophils. Cells were incubated with 5 μg/ml of CRA-1 mAb or control mouse IgG2b and stained with FITC-conjugated goat anti-mouse IgG Ab.

Chemotaxis assay

Basophil chemotaxis experiments were performed using Percoll-separated or MACS-separated basophils and Chemotaxicell (Kurabo) as previously described (25). After incubation for 2.5 h at 37°C, cells which had migrated into the lower chamber were collected, stained with FITC-conjugated goat anti-human IgE, and counted by flow cytometry. Experiments were performed in duplicate or triplicate. Migration was expressed as a percentage of the inoculated cells after subtracting the spontaneous migration.

Degranulation assay

Basophil degranulation was assessed using Percoll-separated basophils, as previously described (25). The released histamine was measured using an automated fluorometric technique. Experiments were performed in duplicate. Histamine release was expressed as a percentage of the total cellular histamine after subtracting the spontaneous release (consistently <5%).

Adhesion assay

Culture plates (96-well; IWAKI) were coated overnight at 4°C with 100 μl of BSA (20 mg/ml), fibronectin (20 μg/ml), ICAM (100 ng/ml), or VCAM (100 ng/ml) dissolved in PBS. The coated wells were washed twice with blocking buffer (2% BSA in PBS) and incubated with 100 μl of this buffer

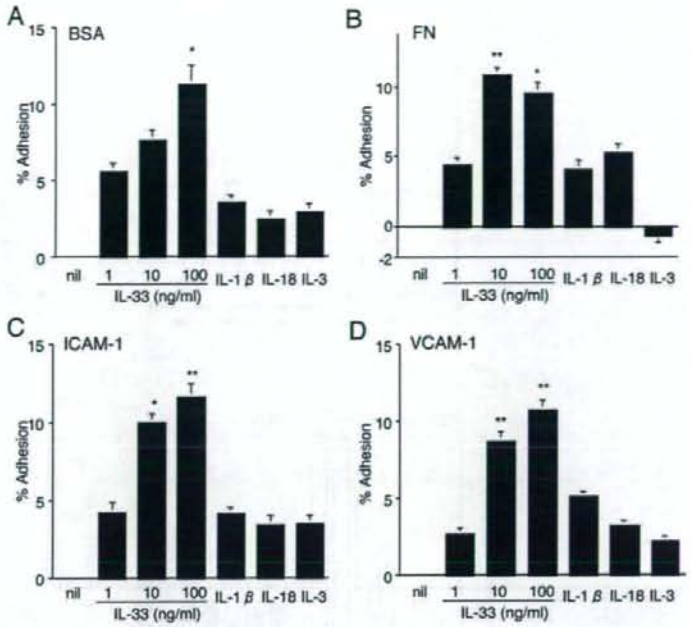

FIGURE 3. IL-33 enhances adhesiveness of human basophils. Percoll-separated basophils were preincubated with and without IL-33 (1, 10, 100 ng/ml), IL-1β (100 ng/ml), IL-18 (100 ng/ml), or IL-3 (300 pM) for 45 min and then dispensed into BSA- (*A*), fibronectin- (*B*), ICAM-1- (*C*) or VCAM-1- (*D*) coated plates. Percentages of adherent cells were calculated based on the histamine content of the cellular histamine content. Error bars represent the SEM ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ vs medium alone (nil).

for 1 h at 37°C. The wells were ready for use after washing twice with RPMI 1640 medium containing 0.3% HSA.

Approximately 3×10^4 Percoll-separated basophils were added to each well with and without cytokines in RPMI 1640 medium containing 0.3% HSA, and the plates were incubated at 37°C in 5% CO_2 for 45 min. After incubation, the wells were gently washed twice with RPMI 1640 medium to remove nonadherent cells. Then 2% perchloric acid was added to each well, and the plates were held overnight at 4°C. Basophil adherence was quantified by measuring basophil-derived histamine. Adherent basophils were expressed as a percentage of the total histamine content of the total basophils placed in each well.

Cell culture and assay of basophil-secreted products

For ELISA assay, 5×10^5 cells/ml of highly purified basophils were cultured at 37°C with cytokines in RPMI 1640 medium containing 0.3% HSA, and the supernatant was collected after centrifugation. Cell lysates were obtained by addition of 0.5% Nonidet P-40 (Sigma-Aldrich) to the cell pellets. Samples were stored at −80°C until assay.

Immunoreactive IL-4 was quantified using a modification of a double-ligand immunoassay. In brief, samples and standards were incubated at 4°C overnight in flat-bottom 96-well microtiter plates (Maxisorp; Nunc) precoated with a mouse anti-IL-4 mAb (eBioscience). After washing, biotin-conjugated rat anti-IL-4 mAb (eBioscience) was added to the plates and reacted for 3 h. The plates were then washed, followed by addition of HRP-conjugated streptavidin (Amersham Biosciences) and incubation for an additional 2 h. The plates were developed with a 3,3′,5,5′-tetramethylbenzidine microwell peroxidase substrate system (Kirkegaard & Perry Laboratories), and the reactions were stopped with 2 N H_2SO_4. Absorbance was measured at 450 nm, and a standard curve was generated for each assay. The ELISA method detected IL-4 concentrations of >0.69 pg/ml.

Immunoreactive LTC4 was measured using an ELISA kit for LTC4 (Cayman Chemicals; detection range: 10–1000 pg/ml) by following the manufacturer's instructions.

Survival assay

Highly purified basophils were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich). Measurement of apoptotic and live cells was performed using an MEBCYTO apoptosis kit (MBL) and flow cytometer as previously described (26). In brief, early apoptotic cells were identified by their ability to bind annexin V and exclude propidium iodide (PI). Cells stained with PI were considered to be necrotic cells. Cells not stained by either annexin V or PI were judged to be alive.

Statistics

All data are expressed as the mean ± SEM. Differences between values were analyzed by the one-way ANOVA test. When this test indicated a significant difference, Fisher's protected least significant difference test was used to compare individual groups.

Results

Human basophils express ST2

In the first series of experiments, we studied the expression of mRNA for the IL-33 receptor, ST2, in basophils and other granulocytes. Cells were purified to apparent homogeneity (>98.5%), and the expression levels of transcripts for ST2 were quantified by real-time PCR (Fig. 1*A*). Basophils clearly expressed ST2 mRNA. The ST2 mRNA expression level by basophils was significantly higher than the levels expressed by eosinophils and neutrophils: judging from the copy number ratio vs β-actin, the expression level of ST2 mRNA by basophils was 2–4-fold higher than by eosinophils and >10-fold higher than by neutrophils. It has been reported that human mast cells are clearly positive for ST2, and they possess abundant ST2 mRNA (19, 24). We very recently analyzed the ST2 mRNA levels using cultured human mast cells (the detailed methods are described in our previous manuscript; Ref. 27), and the data are shown in Fig. 1*A*. The levels of ST2 mRNA in basophils were significantly lower than those in human mast cells. Next, we examined the effect of IL-33 on ST2 expression, since certain cytokines have previously been reported to regulate the expression of their respective receptors (28). Interestingly, treatment with IL-33 at 100 ng/ml for 4 h significantly up-regulated the ST2 mRNA expression by basophils ($p < 0.05$) (Fig. 1*B*).

Next, using highly purified basophils, surface and intracellular ST2 levels were analyzed by flow cytometry. Expression of ST2 on the surface of freshly isolated basophils was hardly detectable, as shown in Fig. 1*C*. However, following incubation with IL-3 at 300 pM for 18 h, ST2 protein was clearly detectable on the basophils' surface (Fig. 1*D*), a finding that is consistent with a recent

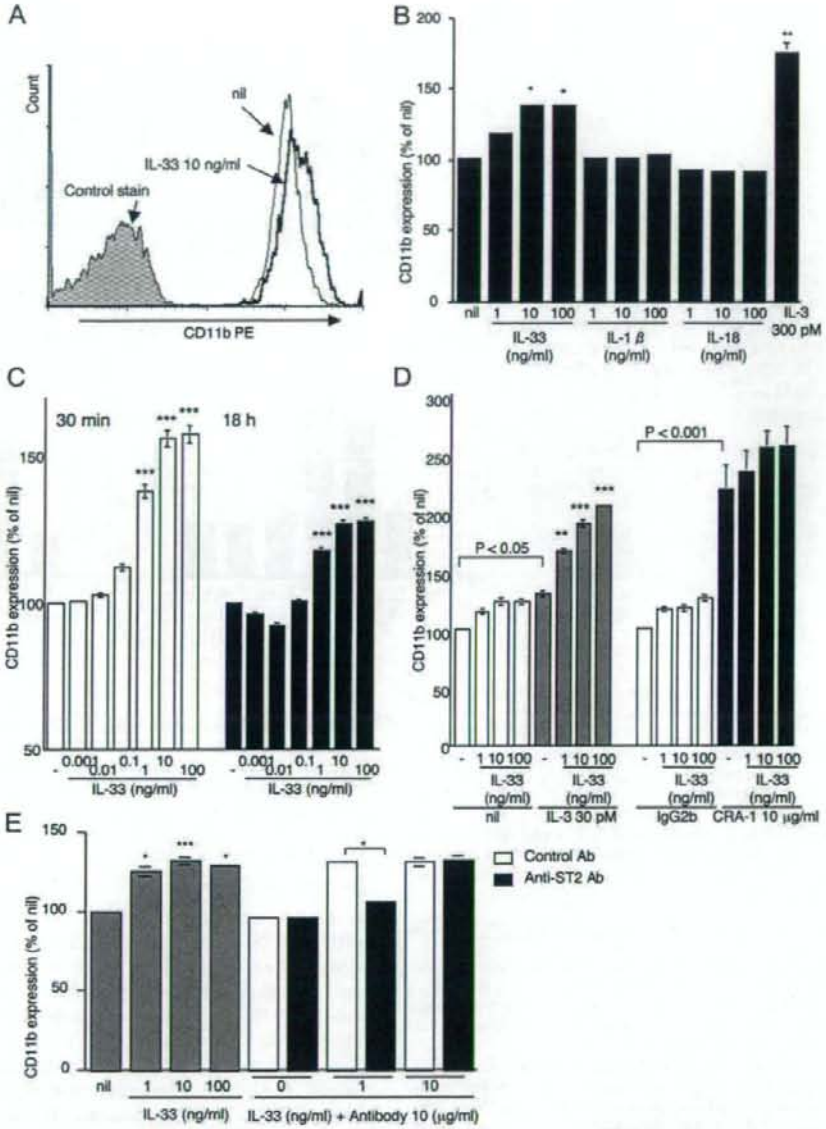

FIGURE 4. IL-33 enhances basophil CD11b expression through their surface ST2 receptor. *A*, Percoll-separated basophils were incubated with medium alone (thin line) or IL-33 at 10 ng/ml (thick line) for 30 min. Then the surface CD11b expression level was assessed by flow cytometry. Data are representative of three separate experiments yielding similar results. *B*, Percoll-separated basophils were incubated with and without cytokines for 30 min, and then the levels of surface CD11b expression were analyzed. The data are expressed as the percentages of MESF values of basophils treated without any stimulus (nil). Error bars represent the SEM ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ vs nil. *C*, Basophils were incubated with the indicated concentrations of IL-33 for either 30 min (□) or 18 h (■). Then the surface CD11b expression level was assessed. Error bars represent the SEM ($n = 5$). ***, $p < 0.001$ vs nil. *D*, Basophils were incubated with IL-33 plus either IL-3 (▨) or CRA-1 mAb (■) for 30 min, and then the expression level of CD11b was quantified. Error bars represent the SEM ($n = 3$–6). **, $p < 0.01$; ***, $p < 0.001$ vs corresponding values of cells cultured without IL-33. *E*, Basophils were preincubated with medium alone (▨), control IgG (□), or neutralizing anti-ST2 Ab (■) at 10 μg/ml for 60 min and then treated with IL-33 at indicated concentrations for 30 min. Error bars represent the SEM ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$ vs nil.

report (29). In addition, intracellular staining clearly showed the presence of ST2 in fresh basophils (Fig. 1*E*).

IL-33 induces cytokine production by human basophils

Basophils are one of the major sources of Th2 cytokines such as IL-4 (15, 30) and IL-13 (31–33). We next examined the effect of IL-33 on cytokine synthesis by human basophils. Using cDNA from IL-33-stimulated basophils, real-time PCR was performed to detect mRNA for IL-4 and IL-13. As a result, transcripts for IL-4 were significantly enhanced by treatment with 100 ng/ml of IL-33 (Fig. 2*A*). IL-13 mRNA was also significantly up-regulated by IL-33 (Fig. 2*B*). ELISA found that the supernatants of IL-33-stimulated basophils contained significantly greater amounts of IL-4 after 24 h (Fig. 2*C*). Another cytokine of the IL-1 family, IL-1β,

also significantly induced IL-4 secretion by basophils. As shown in Fig. 2*D*, time-course analyses showed a gradual increase of IL-4 in the supernatants of IL-33-stimulated basophils, reaching a plateau at 24 h. Furthermore, we assessed whether IL-33 affects IL-4 secretion from basophils stimulated by IL-3 or IgE-crosslinkage. As shown in Fig. 2*E*, IL-33 enhanced IL-4 secretion from basophils stimulated with IL-3. IL-4 production from basophils stimulated with CRA-1 mAb was also potently augmented by IL-33. We also tested for basophil LTC4 synthesis in response to IL-33, but only weak LTC4 secretion, below significant levels, was observed in IL-33-treated basophils (data not shown).

IL-33 enhances adhesiveness of human basophils

Next, we analyzed the adhesiveness of human basophils using Percoll-separated cell preparations. Plastic plates were coated with BSA and tested for basophil adhesion. As shown in Fig. 3*A*, in the presence of IL-33 at 100 ng/ml, a significantly increased number of basophils adhered to the plates compared with the baseline level of adhesion. Unexpectedly, the adhesion-inducing effect of IL-33 was much stronger than that of 300 pM IL-3. Similarly, IL-33 at 10–100 ng/ml significantly induced adhesion of human basophils to fibronectin-, ICAM-1- and VCAM-1-coated microplates, and, again, those effects were more potent than those of 300 pM IL-3 (Fig. 3, *B–D*). On the other hand, IL-1β and IL-18 failed to affect basophil adhesion to plates coated with fibronectin, ICAM-1 or VCAM-1.

IL-33 up-regulates CD11b expression on human basophils

Percoll-separated basophils were used to study the effect of IL-33 on basophil CD11b expression (Fig. 4, *A* and *B*). Consistent with previous reports (14), CD11b expression was markedly up-regulated by IL-3 at 300 pM (74 ± 8.7% above baseline, $p < 0.01$). IL-33 also significantly enhanced surface CD11b expression by basophils, although this enhancement was slightly weaker than that by 300 pM IL-3. This effect of IL-33 was dose-dependent, and the EC50 of IL-33 in terms of enhancement of basophil CD11b expression was approximately 1 ng/ml, which corresponds to 33 pM on a molar basis. The effect reached a plateau at 10–100 ng/ml of IL-33 (Fig. 4*C*). In the next experiments, we compared the effects of IL-33 incubation for 30 min and 18 h. The longer, 18-h incubation was not as effective at enhancing CD11b expression as the shorter, 30-min incubation (Fig. 4*C*). We next tested whether IL-33 affects the level of basophil CD11b expression induced by other well-known stimulants such as IL-3 and FcεRI-crosslinkage. As shown in Fig. 4*D*, IL-33 synergistically augmented surface CD11b expression on IL-3-treated basophils. IL-33 also showed slight enhancement of CD11b levels on anti-FcεRI mAb-treated basophils, but this effect was small and seemingly additive rather than synergistic. Neutralizing Ab for ST2 diminished the enhancement of basophil CD11b expression by IL-33, as shown in Fig. 4*E*. Pretreatment of basophils with anti-ST2 Ab at 10 μg/ml significantly suppressed the effect of 1 ng/ml IL-33 on CD11b expression, indicating that IL-33 regulates basophil CD11b expression by signaling through its receptor, ST2. However, IL-33 at 10 ng/ml or more seemed to be too high for anti-ST2 Ab to efficiently block the IL-33-induced up-regulation of CD11b (Fig. 4*E*).

IL-33 enhances basophil migration toward eotaxin

In vivo local administration of IL-33 was reported to attract inflammatory cells to inflammatory sites (18). Therefore, we investigated whether IL-33 regulates human basophil migration. IL-33 was added to the lower chamber of Chemotaxicell at 10–100 ng/ml, but no induction of basophil migration was observed (Fig. 5*A*). However, when added to the upper chamber with the cells, IL-33

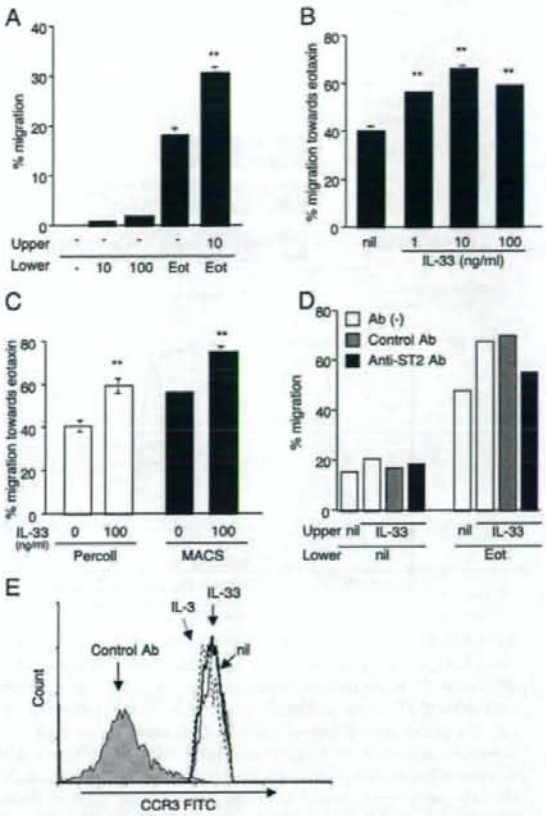

FIGURE 5. IL-33 enhances human basophil migration toward eotaxin. *A*, Two × 10^4 Percoll-separated basophils were added to the upper chamber. IL-33 at 10 or 100 ng/ml or eotaxin (Eot) at 10 nM was added to the lower chamber. Cells mixed with IL-33 at 10 ng/ml were also tested for migration toward eotaxin. The percentage of migrated cells was calculated by subtracting the spontaneous migration (9.6 ± 0.4% for medium only). Error bars represent the SEM ($n = 5$). **, $p < 0.01$ vs spontaneous migration in medium alone. *B*, Percoll-separated basophils were mixed with the indicated concentrations of IL-33 and then tested for migration toward eotaxin at 10 nM. The percentage of migrated cells was calculated by subtracting the spontaneous migration (16.8 ± 0.5% for medium only). Error bars represent the SEM ($n = 3$). **, $p < 0.01$ vs basophil migration toward eotaxin in the absence of IL-33. *C*, Both Percoll-separated (□) and MACS-separated (■) basophils were used for the migration assay. Basophil preparations with and without IL-33 at 100 ng/ml were placed in the upper chamber, and eotaxin at 50 nM was added to the lower chamber. The percentage of migrated cells was calculated by subtracting the spontaneous migration (16.8 ± 0.8% for Percoll-separated and 12.3 ± 1.2% for MACS-separated preparations). Error bars represent the SEM ($n = 3$). **, $p < 0.01$ vs migration of the corresponding basophils without IL-33. *D*, Effect of neutralizing Ab for ST2 on IL-33 enhancement of basophil migration. Percoll-separated basophils were mixed without Ab (□) or with control IgG at 20 μg/ml (▨) or anti-ST2 Ab at 20 μg/ml (■). IL-33 at 10 ng/ml was then added to the cells; eotaxin at 10 nM was added to the lower chamber. Data shown are mean values of an experiment performed in duplicate. Another experiment using basophils from a different donor yielded similar results. *E*, Effect of IL-33 on CCR3 expression by human basophils. Highly purified basophils were incubated with (bold line) and without (thin line) IL-33 at 10 ng/ml for 1 h at 37°C. The dotted line indicates basophils that were incubated with IL-3 at 300 pM. Cells stained with control Ab are shown as a shaded area. Data are representative of two separate experiments using cells from different donors and showing similar results.

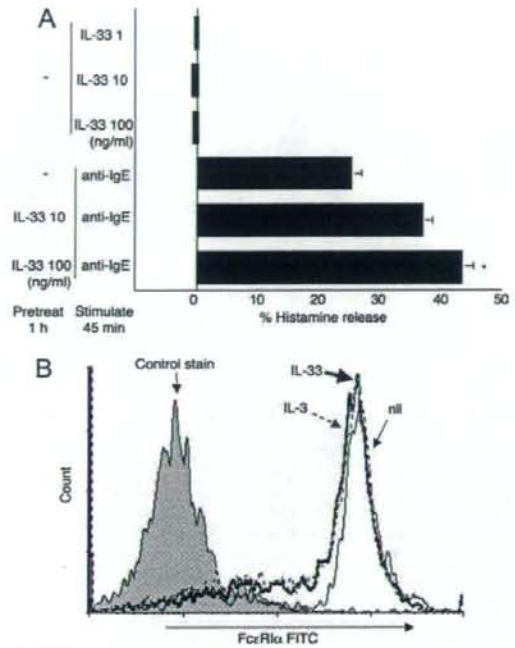

FIGURE 6. IL-33 enhances basophil degranulation following IgE cross-linkage. *A*, Percoll-separated basophils were incubated with and without IL-33 at the indicated concentrations for 1 h and then stimulated with either IL-33 at the indicated concentrations or anti-IgE Ab at 14 μg/ml. The percentage of release was calculated based on the total cellular histamine content. Error bars represent the SEM ($n = 3$). *, $p < 0.05$ vs corresponding release without IL-33 pretreatment. *B*, Effect of IL-33 on FcεRIα expression by basophils. Highly purified basophils were incubated with (bold line) and without (thin line) IL-33 at 10 ng/ml for 1 h at 37°C, and then the surface expression of FcεRIα was analyzed by flow cytometry. Basophils incubated with IL-3 at 300 pM are shown with a dotted line. Basophils stained with control Ab are shown as a shaded area. Data are representative of two separate experiments using cells from different donors and yielding similar results.

at 1–100 ng/ml enhanced basophil migration toward eotaxin (10 nM) (Fig. 5, *A* and *B*). Moreover, we found that IL-33 enhanced chemotaxis of highly purified basophils toward eotaxin (Fig. 5*C*), indicating that possible effects from contaminating cells can be ruled out. Furthermore, when neutralizing Ab for ST2 was added to the upper chamber, the effect of IL-33 on basophil migration toward eotaxin diminished, as shown in Fig. 5*D*, suggesting that IL-33 affects basophil locomotion via the ST2 receptor. As shown in Fig. 5*E*, treatment with IL-33 did not alter the surface level of CCR3, a receptor for eotaxin, on the human basophils, suggesting that IL-33 affects eotaxin-induced intracellular signal(s) downstream of CCR3.

IL-33 enhances degranulation of human basophils

Next, using Percoll-separated basophils, we studied the effect of IL-33 on basophil degranulation. As shown in Fig. 6*A*, freshly isolated basophils did not degranulate in response to IL-33. We next tested IL-33 for basophil priming. Importantly, pretreatment with IL-33 at 100 ng/ml for 15 min significantly enhanced degranulation of basophils stimulated with anti-IgE Ab. We confirmed that the expression level of surface FcεRI remained the same even after IL-33 pretreatment of basophils, as shown in Fig. 6*B*.

IL-33 does not alter survival of basophils

Finally, we analyzed the effect of IL-33 on the viability of highly purified basophils. Although IL-33 is known to enhance the survival of eosinophils (27), this cytokine induced no change in the number of viable or apoptotic basophils compared with basophils cultured in medium alone (data not shown). We next assessed whether IL-33 affects the viability of IL-3-cultured basophils, but it did not show any effect (data not shown).

Discussion

In this study, we demonstrated that human basophils express transcripts and protein for ST2, a receptor for IL-33, and neutralization studies showed that basophil ST2 is functional. IL-33 affected several arrays of basophil functions: this cytokine up-regulated CD11b expression on the cell surface of basophils, enhanced eotaxin-directed chemotaxis, induced Th2 cytokine IL-4 secretion, and augmented the IgE-mediated histamine release reaction. This is the first study to identify the roles of IL-33 and its ST2 receptor in the functional regulation of basophils. Importantly, basophil adhesion was potently enhanced by IL-33, and this action of IL-33 was greater than that of IL-3, a well-known basophil-active cytokine.

The IL-1 cytokine family is known to regulate various inflammatory reactions; among its members, IL-1β and IL-18 are especially potent proinflammatory substances. However, our knowledge regarding the effects of these cytokines on basophil functions is limited. To date, IL-18 has been demonstrated to induce cytokine production by basophils (34, 35), and IL-1α and IL-1β have been demonstrated to potentiate IgE-mediated histamine release from human basophils (36, 37).

IL-33 is a new member of the IL-1 family of cytokines. Schmitz et al. demonstrated that IL-33 has biological activities such as driving Th2-polarized cells to produce Th2 cytokines such as IL-5 and IL-13. In addition, in vivo studies revealed that administration of IL-33 induces histological changes in the mucosa, including eosinophilic infiltration, increased mucus production, and epithelial cell hyperplasia and hypertrophy (18). Thus, locally produced IL-33 may act as a potent inducer of Th2-dominant inflammation. IL-33 is produced by various cells, including epithelial cells and smooth muscle cells (38). Greater knowledge regarding the biological effects of IL-33 on basophils might shed light on the interplay between tissue structural cells and inflammatory granulocytes. In our present study, IL-33 potently enhanced basophil adhesiveness and surface CD11b expression, and these actions of IL-33 were by far the strongest among the tested IL-1 family members. Furthermore, the finding that IL-33 induced IL-4 secretion by basophils implies that this IL-1 family member may strengthen local Th2 dominance through effects not only on Th2 lymphocytes and mast cells but also on basophils, since IL-4 can exert multiple effects causing exacerbation of inflammation (39–41). In addition, our study indicates that transcripts for another Th2 cytokine, IL-13, are also increased by IL-33 in basophils. We further found that IL-4 secretion by basophils stimulated with IL-3 or FcεRI-crosslinkage was potently enhanced by IL-33. IL-33-stimulated basophils may thus be an important cellular source of Th2 cytokines in the pathogenesis of Th2-biased allergic inflammation.

IL-33 was recently identified as a biologically active ligand for ST2 (18), a Th2-associated receptor expressed on Th2 cells and mast cells. Before the ligand was identified, ST2 had been shown to function as an important effector molecule for Th2 responses in experimental models (20, 23). In addition, in the clinical setting, elevated ST2 protein expression was reported in the sera of patients suffering asthmatic exacerbation (42). Thus, ST2 is believed

to have strong relevance to the pathogenesis of Th2-associated diseases. Consistent with a recent study by others (29), we found that human basophils express ST2 protein. We also found that the surface ST2 levels on basophils are changeable, and that the ST2 receptor is functional in basophils. Real-time PCR revealed that the expression level of ST2 mRNA by basophils was lower than that by mast cells but significantly higher than that by eosinophils and neutrophils. We found that the levels of surface ST2 protein on freshly isolated basophils were very low, if any, but culture with IL-3 clearly increased the surface ST2 protein levels on those cells. Furthermore, neutralization experiments showed that ST2 plays a key role in many of IL-33's effects on basophils: anti-ST2 Ab inhibited up-regulation of CD11b expression on basophils and enhancement of basophil chemotaxis toward eotaxin. In our experiments analyzing cell adhesion (Fig. 3), we could not conduct ideal neutralization studies since the added IgG itself augmented basophil adhesion. Nevertheless, we think that ST2 may also be involved in regulation of basophil adhesion. Interestingly, the basophil expression level of ST2 mRNA was enhanced by IL-33 itself. Certain cytokines have previously been reported to regulate the expression of their respective receptors, and similar enhancement has been demonstrated in the case of IL-3 and its IL-3Rα receptor on eosinophils (28). The up-regulation of ST2 expression by its own ligand, IL-33, may contribute to long-term maintenance of IL-33's effects on basophils.

We have shown in this study that IL-33 augments basophil adhesion and CD11b expression. Basophils have previously been reported to express both β1 and β2 integrins on their surface (14), and in earlier studies we demonstrated that β2 integrin represents the first line of adhesion molecules that are involved in basophil transendothelial migration (16) and trans-basement membrane migration (17). Furthermore, basophil CD11b expression is up-regulated by IL-3, resulting in enhanced adhesion to the endothelium (14). Thus, the enhanced adhesion induced by IL-33 may be due at least in part to augmented expression of β2 integrin, and it will lead to increased accumulation of basophils at inflammatory sites.

It is increasingly recognized that basophils and eosinophils share important characteristics such as their growth factors, receptors, cellular functions, and secreted mediator profiles (43, 44). In our recent experiments analyzing the actions of IL-33, we found that this cytokine also activates human eosinophils. However, the precise action of IL-33 on eosinophils differs somewhat from that on basophils: IL-33 failed to enhance migration and degranulation of eosinophils but it suppressed eosinophil apoptosis (27), whereas basophil apoptosis was not affected by IL-33. The different spectrums of IL-33's effects on basophils and eosinophils may in part account for the different behaviors and fates of these effector cells in the pathogenic mechanisms of allergic inflammation. It will thus be important to analyze the extent to which IL-33 regulates the effector functions of basophils (and other cell types) in clinical settings. Further elucidation of the details of the involvement of IL-33 and its receptor, ST2, in the pathogenesis of allergies will enable us to evaluate their potential as useful targets in the therapeutic strategies for allergic diseases.

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Disclosures

The authors have no financial conflict of interest.

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Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils

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Eosinophils are important effector cells in allergic diseases, but the mechanisms regulating their biological functions remain obscure. Interleukin-33 (IL-33) is a recently identified cytokine of the IL-1 family, and it reportedly accelerates the production of Th2-associated cytokines and promotes tissue inflammation. However, the action of IL-33 on effector cells such as eosinophils has remained unclear. In this study, we investigated the effects of IL-33 on eosinophil activation, assessed in terms of the cells' adhesiveness, expression of CD11b and apoptosis. Adhesiveness was quantified by measuring eosinophil peroxidase content of adherent eosinophils, and expression of CD11b was measured by flow cytometry. Apoptosis was determined by flow cytometry based on the ability of cells to bind annexin V. Real-time PCR analysis showed that eosinophils expressed mRNA for ST2, a putative receptor for IL-33. IL-33 at 1–100 ng/ml enhanced the adhesiveness and CD11b expression of eosinophils even more potently than IL-5. IL-33 maintained the viability of eosinophils. Treatment with neutralizing antibodies to ST2 eliminated the effects of IL-33 on eosinophil CD11b expression and cell survival. However, IL-33 did not elicit degranulation or leukotriene C4 synthesis in eosinophils. These findings indicate that IL-33 potently induces eosinophil adhesion and CD11b expression and enhances eosinophil survival. The IL-33-ST2 pathway might be an important regulator of eosinophil biology in the pathogenesis of Th2-biased allergic diseases.

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Blood and local tissue eosinophilia is an outstanding feature of allergic diseases and other disorders such as helminthic parasitic infections and various neoplasms.[1] In the allergic inflammation observed locally in bronchial asthma and atopic dermatitis, eosinophils constitute the major line of effector cells. They possess the capacity to generate and release a wide array of preformed mediators such as major basic protein and eosinophil peroxidase (EPO), as well as newly synthesized mediators including leukotriene (LT) C4 and platelet-activating factor.[2,3] These mediators can cause tissue destruction, modify the smooth muscle tone and vascular permeability and also attract and activate other inflammatory cells. Eosinophils are thus thought to be an important source of proinflammatory mediators. However, only little has been known about which factors are primarily responsible for the accumulation and activation of eosinophils *in vivo*.

ST2, also called DER4, Fit-1 or T1, is a member of the IL-1 receptor family originally identified as a serum-inducible secreted protein in murine fibroblasts.[4] ST2 cDNAs have been also cloned from humans[5] and rats.[6] This receptor is expressed in both soluble and membrane-bound forms as a result of differential splicing,[7] although the expression pattern of ST2 protein differs between humans and mice. ST2 is stably expressed on mouse Th2 cells, but not on mouse Th1 cells.[8] For this reason, this molecule is considered to be a stable cell marker on murine Th2 effector cells. On the other hand, ST2 is inducible in human Th2 cells, and human Th2 cells express ST2 on their cell surface and secrete ST2 following activation.[9] Several lines of evidence suggest that

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the ST2 expressed on Th2 cells[8] and mast cells[10] is linked to important Th2 effector functions.[8] Exogenous administration of soluble ST2 has been demonstrated to effectively neutralize the putative ligand, resulting in alleviation of inflammation by abrogating Th2 cytokine production and induction of the eosinophilic inflammatory response.[11] Moreover, mice deficient in ST2 did not develop a Th2 response to *Schistosoma* egg antigen.[12] In addition, although the ligand for ST2 had not been known for years, elevated levels of the soluble form of ST2 were reported to be present in the circulation of patients with various inflammatory diseases.[13] These results have collectively suggested that ST2 may be an important receptor mediating various inflammatory reactions.

Schmitz *et al*[14] recently identified a new cytokine, interleukin-33 (IL-33), which mediates its biological effects through ST2 and accelerates production of Th2-associated cytokines by *in vitro* polarized Th2 cells. Furthermore, *in vivo* experiments have revealed that exogenous administration of IL-33 markedly increased expression of IgE, IgA, IL-4, IL-5 and IL-13 in the serum and led to obvious pathological changes including eosinophilic and mononuclear infiltration of arterial walls, lungs and intestinal tissues, increased mucus production and epithelial cell hyperplasia and hypertrophy. A very recent study showed that IL-33 induces IL-8 secretion and autocrine production of IL-13 in human cultured mast cells.[15]

Although these novel findings seem to imply that IL-33 potentiates the effector functions of Th2 cells and mast cells, there have been no report showing whether IL-33 acts directly on allergic inflammatory granulocytes such as eosinophils, or whether these inflammatory cells possess functional ST2. Therefore, we for the first time conducted a series of analyses designed to detect IL-33-induced eosinophil activation *in vitro*. In this report, we demonstrate that ST2 is expressed on eosinophils, and that IL-33 affects the viability, increases the adhesiveness and upregulates CD11b expression of human eosinophils.

MATERIALS AND METHODS

Reagents

The following reagents were purchased as indicated: human recombinant IL-33 (Adipogen Inc., Seoul, South Korea); human recombinant IL-18 (MBL, Nagoya, Japan); human IL-1β (Wako, Osaka, Japan); human IL-5 (Peprotech, London, UK); human recombinant VCAM-1 and ICAM-1 (R&D, Minneapolis, MN, USA); Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden); PBS and RPMI 1640 medium (GIBCO, Grand Island, NY, USA); FCS and fibronectin (0.1% solution) (Sigma, St Louis, MO, USA).

The following antibodies were purchased as indicated: mouse anti-human ST2 neutralizing mAb (IgG1, clone 97203), mouse anti-IL-4 neutralizing mAb (IgG2b, clone 34019.111), mouse anti-IL-5 neutralizing mAb (IgG1, clone 14611) and mouse anti-GM-CSF neutralizing mAb (IgG1, clone 3209) (R&D); mouse anti-CD18 neutralizing mAb (IgG1, clone L130) (BD Pharmingen, San Diego, CA, USA); control mouse IgG1 (MOPC21) and mouse IgG2a (UPC10) (Sigma); mouse anti-ST2 mAb (IgG1, clone HB12) (MBL); FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA); mouse anti-CD29 neutralizing mAb (IgG1, clone 4B4), FITC-conjugated mouse anti-CD16 mAb (IgG1, clone 3G8), PE-conjugated mouse anti-CD11b mAb (IgG1, clone Bear 1) and PE-conjugated mouse IgG1 (clone 679.1Mc7) (Coulter Immunotech, Marseille, France).

Isolation of Eosinophils, Neutrophils and Culture of Mast Cells

Leukocytes were isolated from venous blood obtained from consenting volunteers with no history of atopic diseases.

Eosinophils were purified by density gradient centrifugation. In some experiments, eosinophils were further purified by negative selection using anti-CD16-bound beads (Miltenyi BioTech, Belgisch-Gladbach, Germany) as previously described.[16] After this negative selection, the eosinophil purity was >99%.

Human neutrophils were separated by density gradient centrifugation followed by positive selection using anti-CD14-bound micromagnetic beads (Miltenyi BioTech).[16] The purity of neutrophils was approximately 96–99% after the selection.

Human cord blood-derived mast cells were obtained by culturing cord blood CD34-postive cells in the presence of stem cell factor (100 ng/ml) and IL-6 (50 ng/ml) for more than 10 weeks.[17] Purity of mast cells assessed using Toluidine blue stain was >99%.

Real-Time Quantitative PCR Analysis

Real-time quantitative PCR analysis was performed as previously described.[16] In brief, total RNA was extracted from MACS-separated eosinophils, neutrophils and mast cells from separate donors using RNeasy Mini Kit (Qiagen, Hilden, Germany). Real-time PCR was performed using the 7500 Real Time PCR System (PE Applied Biosystems, Foster City, CA, USA). The primers and the probes for ST2 were designed by PE Applied Biosystems. A standard curve was constructed with serial dilutions of specific PCR products, which were obtained by amplifying peripheral leukocyte cDNA as previously described.[18]

ST2 Protein Expression

Highly purified eosinophils were used for flow cytometric analysis of ST2 expression. Eosinophils were incubated for 30 min at 4°C with 10 μg/ml of either anti-ST2 mAb or control antibody and then stained with PE-conjugated goat anti-mouse IgG at 10 μg/ml for 60 min at 4°C. For intracellular staining, the cells were fixed with PBS containing 4% paraformaldehyde at 4°C for 30 min followed by permeabilization in PBS containing 0.1% Tween 20 at 4°C for 30 min. The cells were then stained and analyzed using an Experimental

Physics and Industrial Control System, XL System II (Coulter, Miami, FL, USA).

Survival Assay

Highly purified eosinophils were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Differential analysis of apoptotic and live cells was performed using a MEBCYTO apoptosis kit (MBL) and flow cytometry as previously described.[19] Early apoptotic cells were quantitatively determined by their ability to bind annexin V and exclude propidium iodide (PI). Cells stained with PI were considered to be necrotic cells. Cells without binding to annexin V or PI were judged to be alive.

Adhesion Assay

A 96-well culture plate (IWAKI, Tokyo, Japan) was coated with 100 μl of BSA (20 mg/ml), fibronectin (20 μg/ml), ICAM-1 (100 ng/ml) or VCAM-1 (100 ng/ml) dissolved in PBS overnight at 4°C. The coated wells were washed twice with blocking buffer (2% BSA in PBS) and incubated with 100 μl of this buffer for 1 h at 37°C. The wells were washed twice with RPMI 1640 medium containing 0.3% HSA before adding eosinophils.

Approximately 3×10^4 highly purified eosinophils were added to each well containing the stimulating reagent dissolved in RPMI 1640 medium including 0.3% HSA and incubated at 37°C in 5% CO_2 for 45 min. In some experiments, either anti-CD18 mAb, anti-CD29 mAb or control antibody at 10 μg/ml was added to each well. After incubation, the wells were gently washed twice with RPMI 1640 medium to remove nonadherent cells. Eosinophil adhesion was monitored by quantification of the EPO activity released from the adherent eosinophils as previously reported.[16,20] In brief, 200 μl of 50 mM Tris-HCl, pH 8.0, containing 0.1% (v/v) Triton X-100, 0.1 mM *O*-phenylenediamine dihydrochloride (Sigma) and 50 mM hydrogen peroxide was added to each well. The plate was left at room temperature until reaching the desired extinction, and the reaction was terminated with 50 μl of 1 M sulfuric acid solution. The optimal density was read at 490/570 nm using an ELISA plate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). Data were analyzed with the Microplate Manager III program (Bio-Rad Laboratories), and the numbers of the adherent cells were calculated from a calibration curve established with varying known numbers of eosinophils. The adherent cells were expressed as a percentage of the total eosinophils added to each well.

CD11b Expression

CD11b expression experiments were performed as previously described.[21] Briefly, following stimulation, purified cells were incubated with 10 μg/ml of either PE-conjugated anti-CD11b mAb or PE-conjugated control mouse IgG1 and then stained with FITC-conjugated anti-CD16 antibody at 10 μg/ml. Stained cells were analyzed by flow cytometry. Cells that stained negative for CD16 were identified as eosinophils. The median values of fluorescence intensity of eosinophils were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESF), as previously described.[21] Surface expression levels were semi-quantified using the following formula: ΔMESF = (MESF of cells stained with anti-CD11b mAb) − (MESF of cells stained with control IgG).

Enzyme Immunoassay for LTC4 and Eosinophil-Derived Neurotoxin

For enzyme immunoassay (EIA), 5×10^5 cells per ml of highly purified eosinophils were cultured with stimulating reagents in RPMI medium containing 0.3% HSA for 18 h at 37°C, and the supernatant was collected after centrifugation. Cell lysates were obtained by addition of 0.5% nonidet P-40 (Sigma) to the cell pellets. Samples were stored at −80°C until assay.

Immunoreactive LTC4 was measured using an EIA kit for LTC4 (Cayman Chemicals, Ann Arbor, MI, USA; detection range: 10–1000 pg/ml) by following the manufacturer's instructions.

The concentration of eosinophil-derived neurotoxin (EDN) was measured using an EDN ELISA kit (MBL) by following the manufacturer's instructions. The detection limit was 0.62 ng/ml.

Statistics

All data are expressed as the mean ± s.e.m. Differences between values were analyzed by the one-way ANOVA test. When this test indicated a significant difference, Fisher's protected least significant difference test was used to compare individual groups.

RESULTS

Eosinophils Express ST2

First, eosinophil expression of mRNA for ST2 was quantified by real-time PCR in comparison with neutrophils and mast cells. As shown in Figure 1a, ST2 mRNA expression was observed in eosinophils as well as neutrophils and mast cells. Consistent with previous reports,[10] mast cells expressed high levels of mRNA for ST2. The expression level of ST2 by eosinophils was lower than that by mast cells (about 10-fold lower) but much higher than that by neutrophils (about 10-fold higher). Certain cytokines have been previously reported to regulate their respective receptors;[22] thus, we tested the effect of IL-33 on ST2 mRNA expression. However, the ST2 mRNA expression level by eosinophils did not change as a result of 4 h incubation with IL-33 at 100 ng/ml (Figure 1b). By flow cytometric analysis, we found that hardly any ST2 was expressed on the surface of eosinophils (data not shown), but intracellular staining of eosinophils showed a low but detectable level of ST2 (Figure 1c).

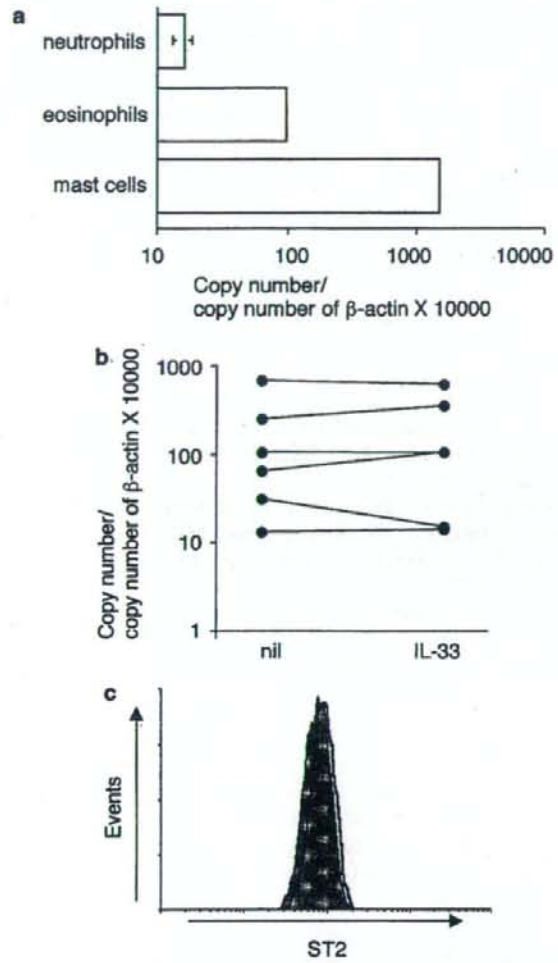

Figure 1 Real-time quantitative PCR analysis and flow cytometric detection for ST2, a receptor for IL-33. (**a**) Quantitative PCR was performed using cDNAs from highly purified eosinophils ($n=10$), neutrophils ($n=9$) and mast cells ($n=4$). The data are calculated as follows: copy number of ST2 gene/copy number of β-actin gene $\times$ 10 000. (**b**) Highly purified eosinophils ($n=6$) were incubated with and without IL-33 at 100 ng/ml for 4 h before extraction of the RNA. The expression levels for the same donor are connected with a solid line in the graph. (**c**) Fixed eosinophils were analyzed for ST2 protein expression by flow cytometry. The cells stained with the control antibody are indicated with a thin line, and the cells stained with anti-ST2 antibody are indicated with a thick line. The data are representative of three separate experiments using cells from different donors and showing similar results.

IL-33 Upregulates Adhesiveness of Eosinophils

As *in vivo* administration of exogenous IL-33 in murine models resulted in local accumulation of eosinophils, we performed a migration assay on eosinophils with IL-33. However, IL-33 added to the lower chambers failed to attract eosinophils. In addition, IL-33 added to the upper chambers did not show enhancement of eosinophil migration toward eotaxin (data not shown). Therefore, next, eosinophil adhesion was quantified by measuring EPO released from lysed adherent cells after 45-min incubation. As shown in Figure 2a, eosinophil adhesion to albumin-, fibronectin-, ICAM-1- and VCAM-1-coated wells was significantly upregulated by IL-33. This effect was apparent at a concentration of 1 ng/ml and increased up to 100 ng/ml. Notably, the effect of IL-33 at 100 ng/ml on eosinophil adhesion was significantly greater than that of IL-5 at 300 pM ($P<0.01$), the most potent known eosinophil-activating cytokine. Two other cytokines of the IL-1 family, ie, IL-1β and IL-18 at 100 ng/ml, did not show any effect on eosinophil adhesion. As shown in Figure 2b, adhesion of eosinophils to albumin-, fibronectin- and ICAM-1-coated wells in the presence of IL-33 was almost completely blocked by anti-CD18 neutralizing antibody, indicating that mainly $\beta 2$ integrin on IL-33-treated eosinophils is involved in the adhesion process to albumin, fibronectin and ICAM-1. On the other hand, adhesion to VCAM-1-coated wells was strongly diminished by the combination of anti-CD18 plus anti-CD29 antibodies, suggesting that eosinophil $\beta 1$ integrin is also involved in adhesion to VCAM-1.

IL-33 Augments CD11b Expression on Eosinophils

Eosinophils have been reported to express β_2 (CD11a, CD11b and CD18) integrins on their surface, and the levels of CD11b on eosinophils are enhanced by eosinophil-activating cytokines such as IL-5. In this study, expression of CD11b on eosinophils was analyzed by flow cytometry. As shown in Figure 3, IL-33 at 1–100 ng/ml significantly upregulated the expression of CD11b on eosinophils dose-dependently, and the effect of IL-33 at 100 ng/ml was stronger than that of IL-5 at 300 pM ($P<0.001$). On the other hand, two other IL-1 family cytokines, IL-1β and IL-18, did not show any effect on eosinophil CD11b expression.

To elucidate the role of ST2, neutralizing antibody for ST2 was added together with IL-33. When eosinophils were pretreated with anti-ST2 neutralizing antibody, the effect of IL-33 on CD11b expression was diminished, as shown in Figure 4a and b, indicating that IL-33 affected eosinophils by binding to and signaling through ST2. When IL-33 at 1 or 10 ng/ml was used to stimulate eosinophils, the effect declined significantly upon addition of anti-ST2 antibody. These results suggest that eosinophil CD11b expression is regulated by IL-33 and its receptor, ST2.

IL-33 Enhances Survival of Eosinophils

Next, we used highly purified eosinophils and analyzed the effect of IL-33 on their viability. As shown in Figure 5a and b, IL-33 at 10–100 ng/ml significantly enhanced the survival of eosinophils dose-dependently although the effect was weaker than that of IL-5 at 300 pM. IL-33 at 100 ng/ml increased the number of live eosinophils by approximately

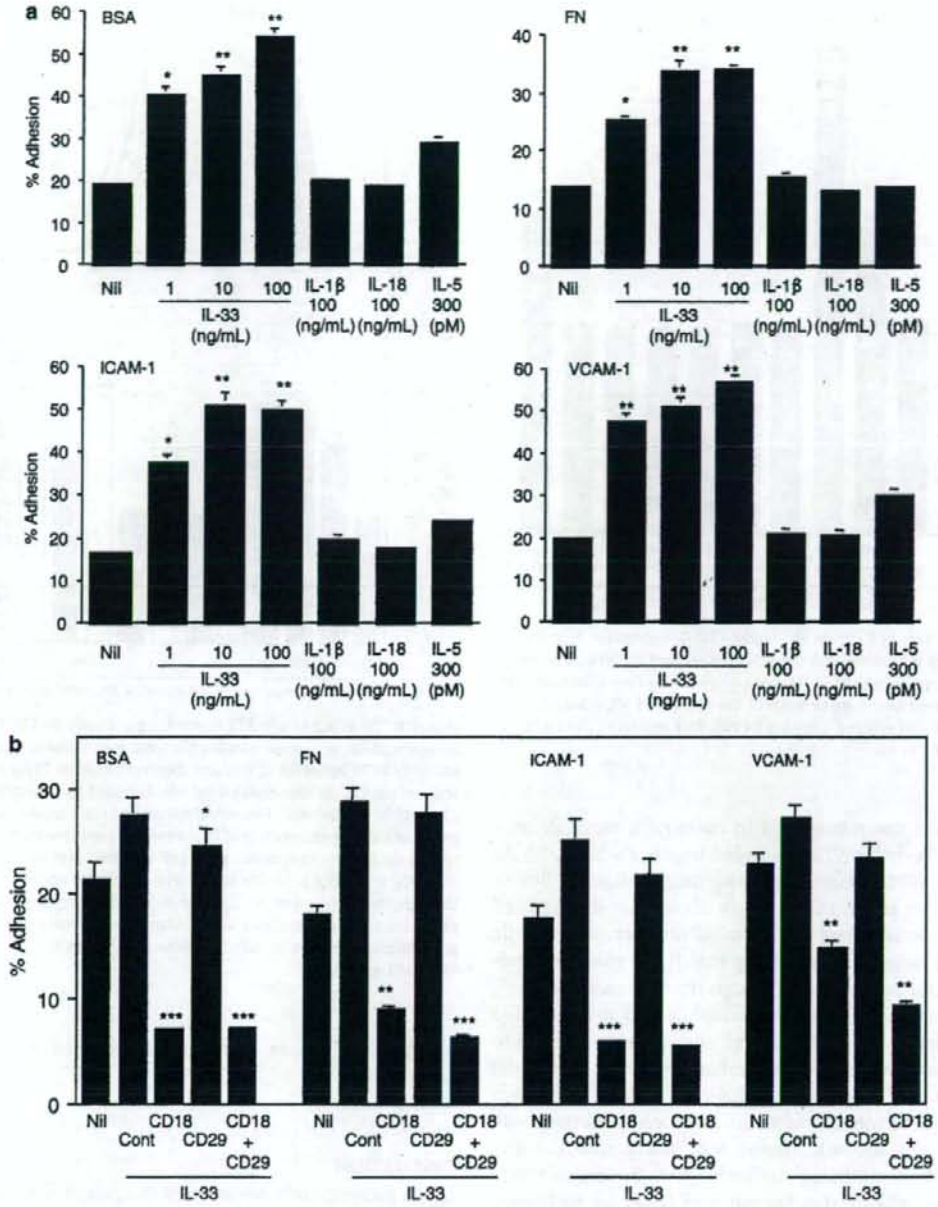

Figure 2 IL-33 enhances adhesion of eosinophils. (**a**) Human eosinophils were used to analyze adhesion to BSA-, fibronectin-, ICAM-1- or VCAM-1-coated culture plates. The cells were incubated with the indicated concentrations of IL-33 or IL-1β at 100 ng/ml, IL-18 at 100 ng/ml or IL-5 at 300 pM for 45 min. The number of adherent cells is expressed as a percentage of the total number of cells placed in each well. Bars represent the s.e.m. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* medium alone (nil). (**b**) Human eosinophils were preincubated with either neutralizing antibody or control antibody at 10 μg/ml plus IL-33 at 100 ng/ml, for 45 min. The number of adherent cells is expressed as the percentage of the total number of cells placed in each well. Bars represent the s.e.m. ($n = 4$–5). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* control antibody.

20% after 24 h. Apoptotic cells, ie, positive for annexin V staining and negative for PI staining, were significantly decreased by addition of IL-33 (Figure 5c). IL-1β and IL-18, at 100 ng/ml, did not enhance the survival of eosinophils, indicating that this effect is specific for IL-33 among these cytokines.