Genotype	ID	No. of metaphases	No. of chromosome	Ch11	Ch12	Ch13	Ch19
Lig4$^{Nes\text{-}Cre}$, *p53*$^{-/-}$	#4307	9	39 ± 0.47	0/9	1/9	9/9	6/9
	#4308	10	38.9 ± 0.3	0/10	0/10	10/10	0/10
Xrcc2$^{Nes\text{-}Cre}$, *p53*$^{-/-}$	#695	10	40.1 ± 0.83	2/10	3/10	9/10	7/10
	#865	10	39.9 ± 0.3	2/10	10/10	10/10	0/10
	#1079	22	38.95 ± 1.12	7/22	7/22	18/22	8/22
	#1153	18	39.94 ± 0.97	1/18	7/18	18/18	2/18
Brca2$^{Nes\text{-}Cre}$, *p53*$^{-/-}$	#163	8	38.9 ± 1.3	2/8	8/8	8/8	3/8
	#167	4	38.3 ± 0.4	0/4	1/4	4/4	3/4
	#393	11	39.1 ± 0.6	0/11	2/11	11/11	10/11
	#436	18	40.6 ± 8.1	4/18	14/18	18/18	10/18
	#471	8	39.4 ± 0.7	0/8	1/8	8/8	5/8
	#482	7	36.4 ± 1.9	0/7	3/7	7/7	4/7
Brca2$^{Nes\text{-}Cre}$, *p53*$^{+/-}$	#222	12	36.2 ± 1.1	12/12	2/12	12/12	12/12
	#249	6	39.2 ± 0.9	6/6	4/6	6/6	2/6
	#363	6	48.8 ± 13.3	4/6	5/6	5/6	4/6
	#492	14	39.5 ± 0.7	3/14	5/14	14/14	10/14
	#496	7	41.7 ± 12.4	4/7	7/7	7/7	7/7
	#586	9	35 ± 1.3	1/9	9/9	9/9	9/9

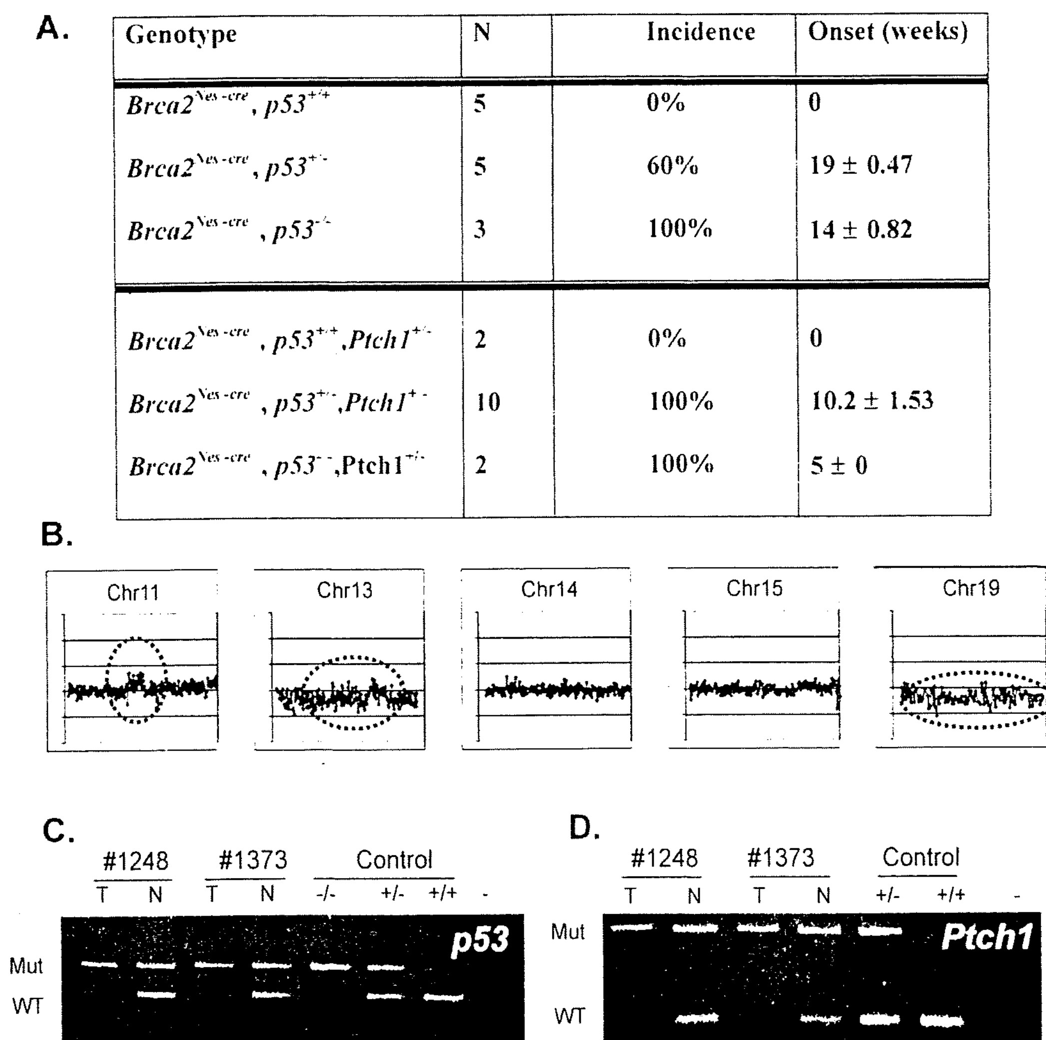
Supplementary Table 2. *Ptch1* mutations in medulloblastomas.

Genotype	tumor ID	Chr13 status	*Ptch1* mutation
$Lig4^{Nes\text{-}Cre};p53^{-/-}$	#236	ND	Δ exon 16-17
	#237	ND	Δ (partial) exon 21
	#246	ND	Δ30nt exon 17
	#247	*Ptch1* locus loss (CGH) Chromosome 13 loss (SKY)	Δ exon 14-15
	#248	*Ptch1* locus loss (CGH) Chromosome 13 loss (SKY)	Δ exon 18
	#249	ND	Δ exon 13
$Xrcc2^{Nes\text{-}Cre};p53^{-/-}$	#865	*Ptch1* genomic region loss	Δ exon 3
	#1004	*Ptch1* locus loss (CGH)	Δ10nt exon 2
	#1079	*Ptch1* locus loss (CGH) Chromosome 13 loss (SKY)	Δ exon 17
	#1153	ND	Δ exon 17
$Lig4/Xrcc2^{Nes\text{-}Cre}, p53^{+/-}$	#950	ND	none detected
	#953	ND	Δ exon 21-exon 23
$Brca2^{Nes\text{-}Cre};p53^{-/-}$	#330	ND	Δ exon14-15
	#848	ND	Δ2nt exon18
	#964	*Ptch1* locus loss (CGH) Chromosome 13 loss (SKY)	Δ24nt exon18
	#1025	*Ptch1* locus loss (CGH) Chromosome 13 loss (SKY)	Δ71nt exon15
$Brca2^{Nes\text{-}Cre};p53^{+/-}$	#162	*Ptch1* genomic region loss	Δ exon8
	#725	*Ptch1* locus loss (CGH)	Δ1nt exon16
	#921	*Ptch1* locus loss (CGH) Chromosome 13 loss (SKY)	Δ143nt exon16
	#1039	ND	Δ5nt exon11-12

Supplementary Table 3. Summary of BAC loss in *Brca2*-null medulloblastoma.

Brca2$^{Nes\text{-}Cre}$, p53$^{+/-}$	
BAC loss	**Genes present on the BAC**
RP23-258H23	*990023K05Rik, Dclre1a, Nhlrc2*
RP23-454P10	*Adra2a, Shoc2*
RP23-477C17	ND
RP23-86D6	*Zdhhc6, Acsl5, Tectb, Gucy2g*
RP23-338A15	*Pdcd11, Sh3pxd2a, 2810048617Rik, Neurl, XP_909191.1, XP_986592.1*
RP23-435N12	*Tix1, Lbx1, Btrc*
RP23-467A11	*Gbf1, Pitx3, Nfhb2, Psd, Elovl3*
RP23-445L1	*Trim8, Arl3, Sfxn2, D19Nsu162e, Sufu*
Brca2$^{Nes\text{-}Cre}$, p53$^{-/-}$	
BAC loss	**Genes present on the BAC**
RP23-147A1	*Obfc1, Slk, Col17a1, 6330577E15Rik, D19Erttd652e*
RP23-44K15	*Kcnk18, Pdzd8, Rps12, Slc18a2, 4930442E04Rik*

Supplementary Figure 1.



A.

Genotype	N	Incidence	Onset (weeks)
Brca2$^{Nes-cre}$, *p53*$^{+/+}$	5	0%	0
Brca2$^{Nes-cre}$, *p53*$^{+/-}$	5	60%	19 ± 0.47
Brca2$^{Nes-cre}$, *p53*$^{-/-}$	3	100%	14 ± 0.82
Brca2$^{Nes-cre}$, *p53*$^{+/+}$,*Ptch1*$^{+/-}$	2	0%	0
Brca2$^{Nes-cre}$, *p53*$^{+/-}$,*Ptch1*$^{+/-}$	10	100%	10.2 ± 1.53
Brca2$^{Nes-cre}$, *p53*$^{-/-}$,Ptch1$^{+/-}$	2	100%	5 ± 0

Supplemental Figure 1: *Ptch1* loss accelerates medulloblastoma in *Brca2*^{Nescre} mice. (A) Loss of Ptch1 decreases the latency of medulloblastoma formation in *Brca2*$^{Nes-cre}$*;p53*$^{+/-}$ and *Brca2*$^{Nes-cre}$*;p53*$^{-/-}$ mice. (b) Cytogenetic changes reveal loss of Chr13 containing the WT *Ptch1* allele and in *Brca2*$^{Nes-cre}$*;p53*$^{+/-}$; chr11 containing p53 and chr19 are also altered. (C) PCR analysis shows that *p53* and *Ptch1* are inactivated in the medulloblastoma.

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ORIGINAL ARTICLE

Prognosis of Adult Asthma After Normalization of Bronchial Hyperresponsiveness by Inhaled Corticosteroid Therapy

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Background: Inhaled corticosteroids (ICSs) are the most effective anti-inflammatory drugs for adult asthma and can improve not only clinical symptoms but also bronchial hyperresponsiveness (BHR). However, the prognosis of adult asthma has not been well studied, and it remains to be elucidated precisely how long treatment with ICSs should be continued once clinical remission is achieved. Objectives: We examined whether ICS use could be withdrawn or reduced without exacerbation of disease. Methods: We retrospectively studied 374 adult patients with asthma to determine which factors predicted the elimination or reduction of ICS treatment without exacerbations of disease after the achievement of normalized BHR to acetylcholine. The patients were classified into three groups: Group 1 had symptoms within 6 months of normalization and needed to continue therapy; group 2 received the equivalent of $\geq$400 μg fluticasone propionate until BHR normalization, did not have symptoms in the 6 months after normalization, and then had their doses of ICSs halved; and group 3 received the equivalent of $\leq$200 μg fluticasone propionate at an enrollment, did not have symptoms in the 6 months after normalization, and then had all ICSs withdrawn. The primary outcome measure was the presence of clinical symptoms. We used multiple logistic regression and a Kaplan-Meier analysis to analyze the factors predicting remission. Results: Twenty-nine patients in group 3 remained asymptomatic for more than 30 months (mean 47.1 $\pm$ 12.4 months) after discontinuing ICS therapy. The predictive markers of remission were low levels of eosinophils in the sputum, high %V_{50} at the first hospital visit, and the need for only a low daily dose of ICS to induce normalized BHR. Conversely, patients with severe BHR at the first hospital visit, low %FEV_1 at normalized BHR, and a need for high-dose ICSs to reach normalized BHR could not reduce or discontinue treatments. Conclusion: Some adult patients with asthma whose BHR is normalized by ICS therapy can achieve remission from disease exacerbation after discontinuation of ICSs. However, patients with severe asthma or asthma of long duration may not achieve remission even if their BHR is normalized.

Keywords bronchial asthma, bronchial hyperresponsiveness, prognosis, adult, inhaled corticosteroids

INTRODUCTION

Childhood asthma is generally considered to be a disorder with good prognosis. Almost 50% of all children with asthma will become asymptomatic by the time they reach adulthood (1–5). Conversely, 30% to 80% of those who were asthmatic in childhood will relapse in later life (1, 3, 6, 7). The factors predicting persistence or relapse are childhood sensitization to house dust mites, bronchial hyperresponsiveness (BHR) in childhood, female sex, current smoking, and early age at onset (8). The factors predicting remission of asthma in childhood are high (>90%) percentage forced expiratory volume in 1 second (FEV_1) both at first testing in childhood and after 30 years (5), or absence of BHR to methacholine challenge at 15 years of age (9). Many follow-up studies have shown that BHR in childhood tends to diminish or disappear with age (1, 3, 4).

In contrast to childhood asthma, adult asthma is generally considered incurable, but the prognosis of adult patients with asthma is unknown. However, several studies have shown that growing older and having asthma over a long duration are associated with declining FEV_1 (10, 11) and irreversible airway obstruction (12) in adults with asthma.

BHR is an important risk factor for the development of asthma and is a characteristic feature of the disease. Measurement of BHR is not only a useful adjunct to the diagnosis of asthma (13)—but also an indicator of the severity of the disease (14, 15) and of the prognosis. About 65% of children with asthma in whom all treatments were withdrawn after both clinical symptoms and lung function improved, but who had remaining BHR to histamine, developed asthma exacerbations after withdrawal (16).

Inhaled corticosteroids (ICSs) are successfully used to treat allergic airway inflammation, and their use improves both the clinical symptoms (13, 17) and BHR (18, 19). Once adult asthma has been controlled for at least 3 months, a gradual reduction in maintenance therapy should be attempted to identify the minimum dose required to maintain control of adult asthma. Several reports have suggested that it is difficult to administer a stepwise dose reduction of ICSs in adults

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Abbreviations: FEV_1 = forced expiratory volume in 1 second; ICS = inhaled corticosteroid; BHR = bronchial hyperresponsiveness; ACh = acetylcholine chloride; PC = provocative concentration; NO = nitric oxide; LTRA = leukotriene receptor antagonist; FP = fluticasone propionate; GINA = Global Initiative for Asthma; RIST = radioimmunosorbent test; ELISA = enzyme-linked immunosorbent assay.

(20, 21). Some studies have attempted to identify markers that will predict the success of ICS dose step-down; these markers include the concentration of eosinophils in the sputum (22, 23), the nitric oxide (NO) concentration in the exhaled breath (24), normalized BHR (22), or well-controlled clinical symptoms (25–27). However, there are no guidelines to indicate how long to continue treatment once clinical remission is achieved, and even the Global Initiative for Asthma (GINA) guidelines do not make it clear whether, or when, adult asthmatics can stop ICS therapy (28).

We retrospectively studied a total of 374 adult patients with asthma who showed both improvement in their symptoms and normalization of BHR to ACh to determine whether ICS use could be withdrawn or reduced without exacerbation of disease. In addition, we examined which markers were predictive of asthma remission in adult patients.

MATERIALS AND METHODS

Patients

Male and female adult outpatients with asthma who made their first visit to the Clinical Research Centre for Allergy and Rheumatology at the National Hospital Organization, Sagamihara, between 1973 and 2006 were eligible for this study. The patients were diagnosed according to the criteria of the American Thoracic Society (29), and the severity of asthma was classified as defined by the current GINA guidelines (28).

Study Design

In a retrospective study, all data would be obtained from patients or perhaps interviews with patients in the clinical examination. The study was approved by the ethics committee of the hospital, and informed consent was obtained from each patient. All patients received ICS therapy. After ICS therapy, all patients had not only long clinical remissions (for at least 6 months) but also normalized BHR to inhaled ACh (defined as PC_{20}-ACh >20 mg/mL).

At the first hospital visit after enrollment, we measured lung function (%FEV_1, FEV_1/VC [%], maximum expiratory flow at 50% of vital capacity [%V50], %V25), concentration of IgE in the serum, and eosinophil score in non-inducible sputum and peripheral blood according to the classification of Hansel (30). Intracutaneous skin testing and tests for BHR to ACh were performed within 1 month after the first visit and after treatment with ICSs had achieved normalized BHR to ACh.

Several years after treatment, BHR to ACh was examined to determine whether the ICS dose could be reduced or discontinued. The patients were classified into two groups according to whether they demonstrated symptoms of asthma within the 6 months after BHR normalization. The patients with symptoms within 6 months of BHR normalization required continued therapy (group 1: 58 patients). Of the 316 patients without symptoms for more than 6 months, 33 patients were excluded because they did not want to reduce or discontinue ICS therapy. We classified the remaining 283 patients who all wished to reduce or discontinue ICS therapy into two groups. Patients in the reducing group (group 2: 134 patients) who had received more than 400 μg of an ICS (FP equivalent) until they showed an improved BHR to ACh had their ICS dosages reduced by half. Patients in the withdrawing group (group 3: 149 patients) who had received less than 200 μg FP equivalent at an enrollment had all ICSs discontinued (Figure 1). The withdrawing group was further classified into three groups: the nonremission group (62 patients), who had exacerbation of symptoms within 30 months; the remission group (29 patients), who showed no symptoms for more than 30 months; and the unknown prognosis group (58 patients), for whom we did not have clear information about the exacerbation of symptoms after 30 months of the discontinuation of ICS therapy. Thirty-two of the patients in group 1 received only ICS therapy after BHR normalization, and the 26 other patients also received theophylline, long-acting β-agonists, oral β-agonists, inhaled anticholinergic agents, and/or leukotriene receptor antagonists (LTRAs). Patients in groups 2 and 3 received only ICSs at study entry. The primary outcome measure was clinical symptoms. Asthma was defined as being exacerbated when wheeze and nocturnal cough occurred more than once a week or the patient needed to use inhaled short-acting β-agonists more than twice a month.

The exclusion criteria included pulmonary disease other than asthma, exacerbation of asthma within 6 months before enrollment, and any significant abnormality on physical examination, or laboratory evaluation.

Measurement of Bronchial Hyperresponsiveness to ACh

Inhalation testing was performed by a modification of the method described by Chai *et al.* (31). Acetylcholine chloride (ACh; Ovisot, Daiichi Pharmaceutical Co, Ltd, Tokyo, Japan) at a concentration of 0.157, 0.316, 0.625, 1.25, 2.5, 5, 10, or 20 mg/mL was prepared by dilution in buffered saline solution (pH 7.4). The FEV_1 was measured with a spirometer (Auto Spiro AS-303; Minato Medical Science Co, Ltd, Osaka, Japan) after each inhalation. All anti-asthma medications were withheld for at least 12 hours before the provocation test measurements. The subjects inhaled ACh aerosol from a nebulizer (DeVilbiss 646; DeVilbiss, Somerset, PA) by tidal breathing for 2 minutes. The operating airflow rate of this device was 5 L/min. Isotonic saline was inhaled first as a control. Patients with an FEV_1 of less than 1.00 L or more than 10% below the FEV_1 measured with the saline control before the inhalation of ACh were not tested further. Increasing concentrations of ACh were inhaled by the remaining patients until the FEV_1 decreased by more than 20% of its post-saline value or until the maximum concentration of ACh was reached. The percentage decrease in the FEV_1 from the post-saline value was plotted against the log concentration of inhaled ACh. Bronchial sensitivity was expressed as PC_{20} and was defined as the provocative concentration (PC) of the agonist in the inhaled aerosol leading to a decrease in the FEV_1 of 20%. At the end of the test, any decrease in the FEV_1 was reversed by the inhalation of salbutamol (0.5 mL of 5 mg/mL salbutamol solution). Subjects with a PC_{20}-ACh of less than 20 mg/mL were defined as having a positive BHR. The provocation testing with ACh was performed within 1 month after the first hospital visit.

Intracutaneous Skin Testing and Measurement of Serum IgE

Atopy was defined as a positive intracutaneous skin reaction to at least one antigen. Intracutaneous skin testing

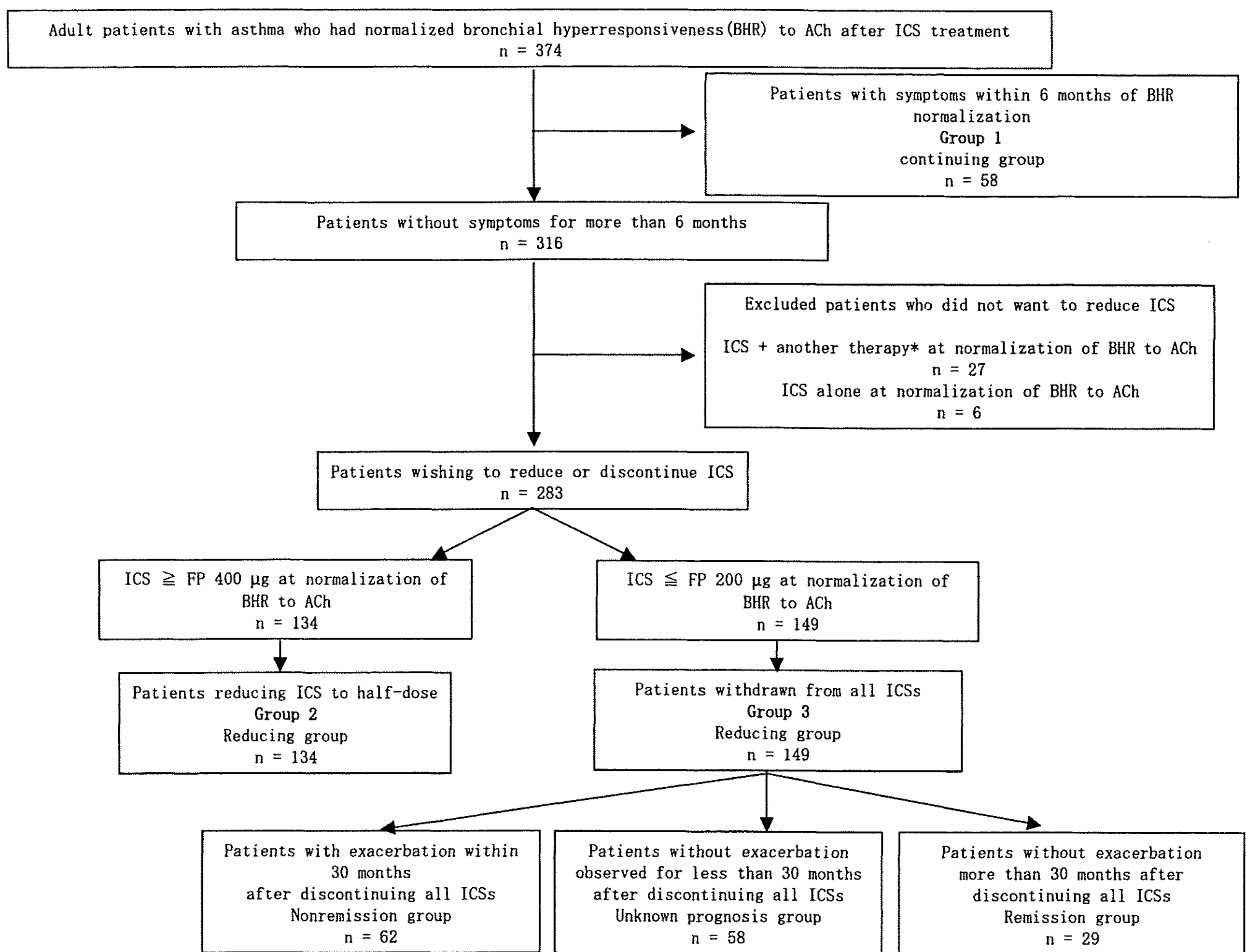

FIGURE 1.—Protocol and follow-up of patients. *Theophylline, long-acting β-agonists, oral β-agonists, inhaled anti-cholinergic agents and/or LTRAs.

was performed within 1 month after the first hospital visit, using a modification of the method described by Solley *et al.* (32). One solution containing all 6 allergens, each containing 0.05 mL of an allergen—house dust mite, cat, dog, *Alternaria tenuis*, *Aspergillus fumigatus*, or ragweed—were injected intracutaneously and the result was judged 15 minutes after injection. After a prick test to confirm the reaction to histamine, histamine (0.4 μg) was then injected intradermally and used as a positive control. Skin reactions were judged positive if they were larger than the histamine reaction. IgE in serum (IU/mL) was measured with a radioimmunosorbent test (RIST) by an enzyme-linked immunosorbent assay (ELISA) using the nephrometry method (BN II; Dade Behring Inc, Deerfield, IL) (33).

Score of Eosinophils in Non-Induced Sputum

Sputum was collected and analyzed by the classification of Hansel (30). The level of eosinophilia was scored as 0: none, 1: few, 2: slight, 3: mild, 4: moderate, 5: severe. Scores of 0 and 1 were obtained by examining the entire smear; scores of 2 to 5 were measured in single 400× microscopic fields.

Analysis of data

Statistical analysis. All values are expressed as means ± SD unless otherwise specified. Statistical comparisons between groups were performed with a twoway analysis of variance (ANOVA) with repeated measures, followed by a *post hoc* comparison by the Newman-Keuls test. Two mean values were compared by the Mann-Whitney U test. A multiple logistic regression analysis was used to calculate the odds ratios, which were adjusted for the effects of other risk factors in the model. Values of $p < 0.05$ were considered statistically significant. The statistical analysis was performed with the StatView 5.0 (SAS Institute; Cary, NC) statistical program.

RESULTS

The mean age of entry of the patients to the study was 51.3 ± 15.1 years (range 17 to 84 years), and the mean age at onset of asthma was 38.8 ± 18.2 years (0 to 79 years). Of the 374 patients included in the study, 12.1% were current smokers. For all 374 patients, BHR to ACh had been normalized (PC_{20}-ACh >20 mg/mL). The patients in group 1 maintained treatment because they had symptoms even after achieving a normalized BHR to ACh. The patients in groups 2 and 3 had

TABLE 1.—Patient characteristics.*

	Group 1 Continuing group N = 58	Group 2 Reducing group N = 134	Group 3 Withdrawing group N = 149	p value
Current age (years)	60.7 ± 15.1 (24–88)	51.6 ± 15.0 (21–82)	52.3 ± 14.4 (20–85)	$p < 0.01$†
Age at entry of study (years)	57.2 ± 15.5 (21–84)	48.9 ± 14.6 (19–77)	49.8 ± 14.5 (17–83)	$p < 0.01$†
Sex: M/F	34/24	55/79	56/93	$p = 0.02$
Age at onset of asthma (years)	38.0 ± 18.7 (0–79)	35.6 ± 16.8 (1–71)	40.4 ± 18.3 (1–75)	N.S.
Age at first hospital visit (years)	46.2 ± 15.1 (18–80)	42.7 ± 14.7 (17–72)	45.1 ± 15.0 (16–75)	N.S.
Duration of asthma (from age at onset to study entry) (years)	19.4 ± 14.6	13.7 ± 11.1	9.4 ± 10.5	$p < 0.01$††
Type: atopy/ nonatopy	35/23	72/62	79/70	N.S
At first hospital visit				
Log IgE RIST in serum	2.30 ± 0.67	2.37 ± 0.63	2.12 ± 0.68	N.S.
Proportion of eosinophils (%) among WBCs	7.4 ± 5.0	7.1 ± 6.4	5.9 ± 6.4	N.S.
Score of eosinophils in sputum** (score ≤2/≥3)	14/17	35/39	47/47	N.S.
FEV1/VC (%)	65.6 ± 14.6	71.8 ± 13.2	75.0 ± 11.5	$p < 0.01$†††
FEV1 (% of predicted)	76.0 ± 18.1	86.1 ± 18.0	92.8 ± 21.3	$p < 0.01$††
V50 (% of predicted)	48.1 ± 25.2	64.0 ± 28.6	73.4 ± 29.5	$p <0.01$††
V25 (% of predicted)	42.3 ± 23.2	52.6 ± 26.6	60.3 ± 27.7	$p < 0.01$††††
Log ACh PC_{20}(mg/mL)	0.55 ± 0.60	0.70 ± 0.55	0.95 ± 0.53	$p < 0.01$††††††
At normalization of BHR to ACh after ICS therapy				
FEV1 (% of predicted)	84.8 ± 16.3	92.4 ± 16.6	99.9 ± 16.6	$p < 0.01$††
V50 (% of predicted)	58.1 ± 27.5	69.1 ± 25.0	77.7 ± 26.6	$p < 0.01$††††
V25 (% of predicted)	50.9 ± 24.9	60.5 ± 34.4	62.7 ± 23.4	$p < 0.01$†††††
ICS therapy until ACh PC_{20}>20 mg/mL				
Duration from onset of asthma to initiation of ICS therapy (years)	9.4 ± 12.6	8.1 ± 9.7	4.7 ± 9.2	$p < 0.01$††††††
Daily dose of ICS (μg; converted to FP equivalent)	768.4 ± 331.2	713.5 ± 361.7	410.4 ± 207.5	$p < 0.01$††††††
Duration of ICS therapy (years)	5.7 ± 3.9	4.4 ± 3.8	3.4 ± 3.1	$p < 0.01$††††

Group 1 patients had symptoms and continued on the same dose of ICS after stabilization of BHR; group 2 patients were without symptoms and reduced their ICS to half-dose; and group 3 patients were without symptoms and discontinued all ICS therapy.

*Data are presented as the mean ± SD. NS = not significant.

** According to the Hansel classification(30).

†: Group 1 vs. group 2: $p < 0.01$, group 2 vs. group 3: N.S., group 1 vs. group 3: $p < 0.01$.

††: Group 1 vs. group 2: $p < 0.01$, group 2 vs. group 3: $p < 0.01$, group 1 vs. group 3: $p < 0.01$.

†††: Group 1 vs. group 2: $p < 0.01$, group 2 vs. group 3: $p < 0.05$, group 1 vs. group 3: $p < 0.01$.

††††: Group 1 vs. group 2: $p < 0.05$, group 2 vs. group 3: $p < 0.05$, group 1 vs. group 3: $p < 0.01$.

†††††: Group 1 vs. group 2: $p < 0.05$, group 2 vs. group 3: N.S., group 1 vs. group 3: $p < 0.05$

††††††: Group 1 vs. group 2: N.S, group 2 vs. group 3: $p < 0.01$, group 1 vs. group 3: $p < 0.01$.

been disease-free for at least 6 months and had received only ICSs after they had achieved a normalized BHR to ACh.

There were no significant differences among the three groups in the age at onset of asthma or at the first hospital visit, but the age at the entry into the study and the duration of asthma were greater in group 1 than in the other two groups ($p < 0.01$; Table 1). There were more males in group 1 than in the other groups (vs. group 2 and group 3, $p = 0.02$). There were no significant differences among the three groups in terms of IgE in serum or the percentage of eosinophils in white blood cells or sputum at the first hospital visit. At the first hospital visit, FEV_1/VC (%), %FEV_1, %V50, and %V25 were lower in group 1 than in group 3 ($p < 0.01$) and in group 2 (FEV_1/VC [%], %FEV_1, and %V50: $p < 0.01$, %V25: $p < 0.05$). Patients in group 3 had significantly lower BHR to ACh at the first hospital visit than the other two groups (Table 1). When the patients had achieved normalized BHR to ACh after ICS treatment, FEV_1/VC (%), %FEV_1, %V50, and %V25 were lower in group 1 than in groups 2 and 3. The duration from the onset of asthma to the initiation of ICS therapy was shorter in group 3 than in group 1 and group 2 ($p < 0.01$). The daily dose of ICS (converted into the FP equivalent) and the duration of ICS therapy until normalization of BHR to ACh were significantly smaller in group 3 than in the other two groups. Patients in group 1 had significantly more severe, higher doses of ICS and longer duration of asthma than in the other two groups at the first hospital visit.

We used a multivariate logistic regression model to determine the factors predicting the prognosis of patients with asthma in groups 1 and 3 (Table 2). The presence of slight

TABLE 2.—Logistic regression modeling of baseline factors predictive of prognosis in patients with asthma in the continuing group (group 1) and the withdrawing group (group 3).

Variable	Odds ratio	95% confidence interval	Pvalue
Duration of asthma	0.965	0.914–1.019	0.198
At first hospital visit			
%FEV1	1.001	0.951–1.054	0.972
%V50	1.021	0.967–1.078	0.457
%V25	0.981	0.945–1.019	0.322
Log ACh PC_{20}	4.417	1.212–16.091	0.017
At normalization of BHR to ACh after ICS therapy			
% FEV1	1.079	1.079–1.142	0.005
%V50	0.983	0.937–1.007	0.504
%V25	0.966	0.926–1.007	0.937
Therapy			
Daily dose of ICS	0.997	0.995–0.998	0.001
Duration of ICS therapy	1.052	0.806–1.374	0.707

BHR to ACh at the first hospital visit, a high $\%FEV_1$ at normalized BHR, and a low daily dose of ICS until normalization of BHR increased the likelihood that all ICSs could be withdrawn. In contrast, the presence of a low %FEV1 at the time of normalization of BHR to ACh and the need for a high dose of ICSs to achieve a normalized BHR were associated with inability to reduce or discontinue the treatment. These patients needed high doses of ICS, which indicated severe asthma at the first hospital visit. Even if the patients with severe asthma had achieved normalized BHR of ACh, they could not reduce the treatment of asthma.

We used a Kaplan-Meier analysis to assess the prognosis of patients with adult asthma after withdrawing all ICSs (Figure 2). During the observation period (maximum 96 months), 62 patients within group 3 (the nonremission group) experienced exacerbations at a mean of 9.2 ± 7.4 months after stopping all ICSs, and 29 patients within Group 3 (the remission group) were disease-free for more than 30 months (mean 47.1 ± 12.4 months). All patients who had not experienced a disease exacerbation for 30 months after the normalization of the BHR had still not relapsed at the end of the observation period (Figure 2). In another 58 patients within group 3 (the unknown prognosis group), it was not clear whether exacerbation of asthma had occurred after the discontinuation of ICS therapy because their observation periods remained within 30 months (Figure 1). We then examined which background factors were shared by the patients in the remission group who did not experience exacerbations for at least 30 months and which were associated with those in the nonremission group who did have exacerbations within 30 months. The duration of asthma was longer in the nonremission group than in the remission group. The presence of a low eosinophil count in the sputum, high lung function ($\%FEV_1$, %V50), slight BHR to ACh at the first hospital visit, and high %V50 at normalization of BHR were associated with remission of adult asthma (Table 3). As was found in the combined analysis of groups 1 and 3

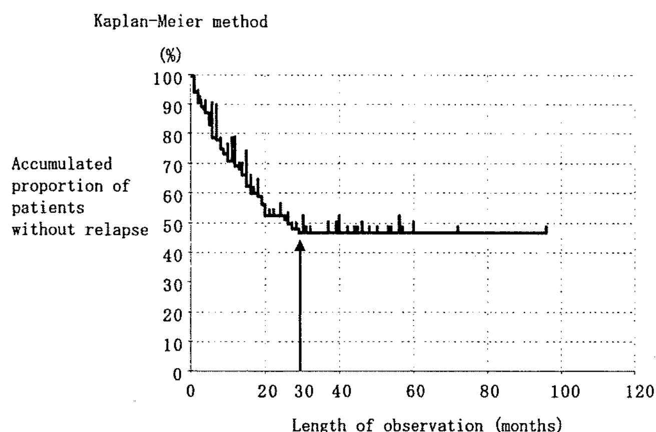

FIGURE 2.—Clinical course of patients withdrawing from all ICS therapy after normalization of BHR to ACh.
The proportion of patients without relapse among those discontinuing all ICS therapy was analyzed by Kaplan-Meier curves after normalization of BHR to ACh. Sixty-two of 149 patients (41.6%) experienced exacerbations during a mean 9.2 ± 7.4 months after discontinuing all ICS therapy. Twenty-nine patients were without disease exacerbation for more than 30 months after stopping all therapy. Patients in the remission group were observed for 47.1 ± 12.4 months.

(Table 2), adult patients with asthma who experienced remission needed only low doses of ICS and a short duration of treatment to normalize BHR to ACh. A multivariate logistic regression model was used to determine the predictive factors of clinical remission of asthma in the nonremission group and the remission group. The presence of few eosinophils in the sputum, high %V50 at the first hospital visit, and the need for only a low daily dose of ICS to induce a normalized BHR to ACh were associated with the remission of asthma (Table 4). The $\%FEV_1$ after patients had reached a normalized BHR to ACh after ICS therapy was inversely correlated with the

TABLE 3.—Background factors in patients with exacerbation and without exacerbation within 30 months in group 3.*

	Remission group (without exacerbation) 29 patients	Nonremission group (with exacerbation) 62 patients	*P* value
Current age (years)	56.6 ± 14.5	53.3 ± 13.8	N.S
Age at entry of study (years)	53.5 ± 14.2	51.5 ± 13.7	N.S
Sex: M/F	8/21	20/42	N.S
Age at onset of asthma (years)	46.2 ± 17.5	41.7 ± 17.0	N.S
Age at first hospital visit (years)	50.2 ± 14.0	45.4 ± 14.9	N.S
Duration of asthma (from age at onset to study entry) (years)	6.9 ± 9.1	10.9 ± 9.5	$P < 0.01$
Type: atopy/nonatopy	11/18	34/28	N.S
At first hospital visit			
Log IgE RIST in serum at first hospital visit	2.02 ± 0.75	2.18 ± 0.58	N.S
Number of eosinophils (%) in WBC	4.5 ± 3.5	6.1 ± 4.5	N.S
Score of eosinophils in sputum (score≤2/≥3)	16/8	15/25	$P = 0.024$
FEV1 (% of predicted)	98.4 ± 15.5	88.4 ± 19.8	$P = 0.029$
V50 (% of predicted)	91.4 ± 26.8	70.0 ± 31.1	$P < 0.01$
V25 (% of predicted)	81.0 ± 31.2	64.4 ± 36.9	$P = 0.058$
Log ACh PC_{20} (μg/mL) within 1 month after the first hospital visit	4.13 ± 0.36	3.88 ± 0.56	$P = 0.031$
At normalization of BHR to Ach after ICS therapy			
FEV1 (% of predicted)	103.5 ± 14.9	98.7 ± 17.8	N.S
V50 (% of predicted)	88.1 ± 29.7	73.2 ± 22.4	$P = 0.021$
V25 (% of predicted)	69.3 ± 29.8	58.5 ± 21.3	N.S
ICS therapy until ACh PC_{20}> 20mg/mL			
Duration from onset of asthma to therapy with ICS (years)	2.1 ± 4.1	4.7 ± 7.1	$P = 0.051$
Daily dose of ICS (μg) (converted to FP equivalent)	311.1 ± 182.6	427.4 ± 196.9	$P < 0.01$
Duration of ICS therapy (years)	2.1 ± 2.1	4.0 ± 3.3	$P < 0.01$

*Data are presented as means ± SD.
N.S., not significant; WBCs, white blood cells.

TABLE 4.—Logistic regression model of baseline factors predictive of remission of asthma.

Variables	Odds ratio	95% confidence interval	p value
At first hospital visit			
Score of eosinophils in sputum	0.013	0.004–0.498	0.038
At first hospital visit			
$\%V_{50}$	1.053	1.005–1.103	0.007
Log ACh PC_{20}	0.704	0.039–12.772	0.814
Therapy			
Daily dose of ICS	0.996	0.993–0.999	0.007
Duration of ICS therapy	0.63	0.342–1.161	0.089

duration of asthma in all patients ($r = -0.34$, $p < 0.01$, data not shown). This result suggests that asthma of long duration causes irreversible airway obstruction.

Discussion

There have been few prospective studies of the prognosis of adult patients with asthma. ICSs are the most effective agents for controlling persistent asthma (13, 17), and they improve not only clinical symptoms but also BHR (18, 19). However, it is not known whether ICSs can induce disease remission in adult patients with asthma. Our unpublished data showed that 89.4% of 39 adult patients with asthma who did not achieve normalized BHR to ACh (mean PC_{20}-ACh of 1.59 mg/mL) had deteriorated within a median of 7.63 ± 0.9 months after discontinuing all ICSs (data not shown). This demonstrates the importance of BHR improvement to the remission of asthma (2, 9, 34) and to the success of stepwise reduction of ICS therapy (23).

In the current study, 374 adult patients with asthma all achieved normalization of BHR to ACh after ICS therapy, in addition to improvements in their symptoms. In children with asthma, the predictors of recurrence after discontinuation of ICSs are deterioration in lung function and an increase in urinary excretion of eosinophil protein X (35). However, there is no evidence that can be used to determine how long to continue treatment in adults once clinical remission is achieved, and even the GINA guidelines do not make it clear whether, or when, adult asthmatics can stop ICS therapy (20).

Step-down dosing is a commonly used strategy for patients receiving high- or low-dose ICSs (20, 24–28), but there is insufficient evidence in adult asthma for discontinuation of ICSs. Twenty-nine of our patients did not experience asthma exacerbation for more than 30 months after withdrawing all ICSs, up to a maximum observation period of 96 months. These results show that ICS therapy can induce remission or cure in some adult patients with asthma. The factors predictive of remission were the presence of few eosinophils in the airway, high %V50 at the first hospital visit, and the need for only a low daily dose of ICS to induce normalized BHR to ACh; this is consistent with the findings of Leuppi *et al.* (22). Thus, most of the patients with remission had mild asthma of short duration. This contrasts with the important predictive factors for children with asthma who develop clinical remission, which are a higher FEV_1 at the first hospital visit and at remission, rather than the absence of BHR to histamine at remission (2, 5, 36).

The patients in group 1 continued therapy with the same doses of ICSs and/or other medications, such as theophylline, long-acting β-agonists, and LTRAs, even after normalization of BHR to ACh. The characteristics of these patients were a long duration of asthma, late induction of ICS therapy, severe BHR to ACh at the first hospital visit, and low lung function at the first hospital visit and when BHR to ACh was normalized after ICS therapy. This study demonstrated that the $\%FEV_1$ at normalized BHR to ACh was inversely correlated with the duration of asthma. Several previous reports have suggested that a long duration of asthma is associated with a decline in FEV_1 in adult asthma (10–12). Ulrik *et al.* (12) conducted a 10-year follow-up study in 92 nonsmoking adult asthmatics and found that a higher degree of bronchodilator reversibility at enrollment and long-term treatment with oral corticosteroids were associated with irreversible airway obstruction at follow-up. Vonk *et al.* (10) suggested that patients diagnosed with severe asthma had a higher risk of developing irreversible airflow limitation 26 years later. These findings suggest that a long duration of asthma may lead to airway remodelling (37–39). This explains why patients with severe obstructive impairment cannot reduce their ICS doses and may not be able to achieve remission despite the normalization of BHR to ACh following ICS therapy. These results are consistent with the conclusion that it is difficult to taper ICSs in patients with severe asthma (22).

After the normalization of BHR to ACh by ICS treatment, there are therefore two opposing prognoses: future remission or cure and persistent exacerbation perhaps due to airway remodeling. Can adult patients with severe asthma and an irreversible airflow limitation improve their airway remodelling? Early ICS intervention in adult asthmatics reduces the area of airway smooth muscle with hypertrophy and hyperplasia (40) and decreases basement membrane thickness (41). Furthermore, there might be major improvements in the basic protein density and reticular basement membrane thickness in the subepithelium of adult patients with clinical remission of asthma (42).

More females than males were enrolled in this study. This could reflect the greater prevalence of asthma in females than in males or could have occurred because more female than male patients had regular medical examinations after withdrawing from all therapy. Future randomized prospective studies are therefore necessary.

This study demonstrated that some adult patients with asthma may achieve either remission or cure after obtaining a normalized BHR to ACh by induction ICS therapy. The predictive factors for remission in adult asthma were mild asthma needing low-dose ICSs, early intervention with ICS therapy, mild eosinophilia in the airways, and high lung function at the first hospital visit.

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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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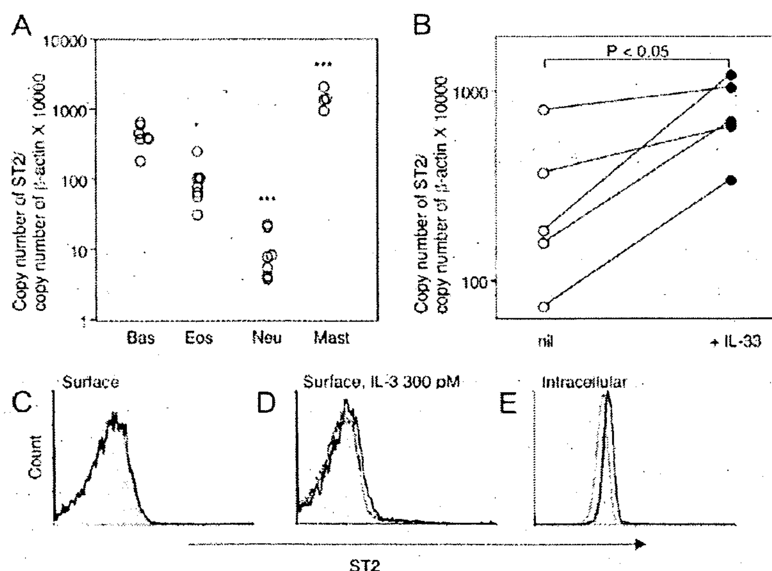

FIGURE 1. Real-time quantitative PCR and flow cytometric analysis for ST2. *A*, cDNAs from highly purified basophils ($n = 7$), eosinophils ($n = 7$), and neutrophils ($n = 7$) were tested. The data are expressed as the following ratio: copy number of ST2 gene/copy number of β-actin gene × 10,000. *, $p < 0.05$ 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 FcεRI 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 ~2.4 × 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).

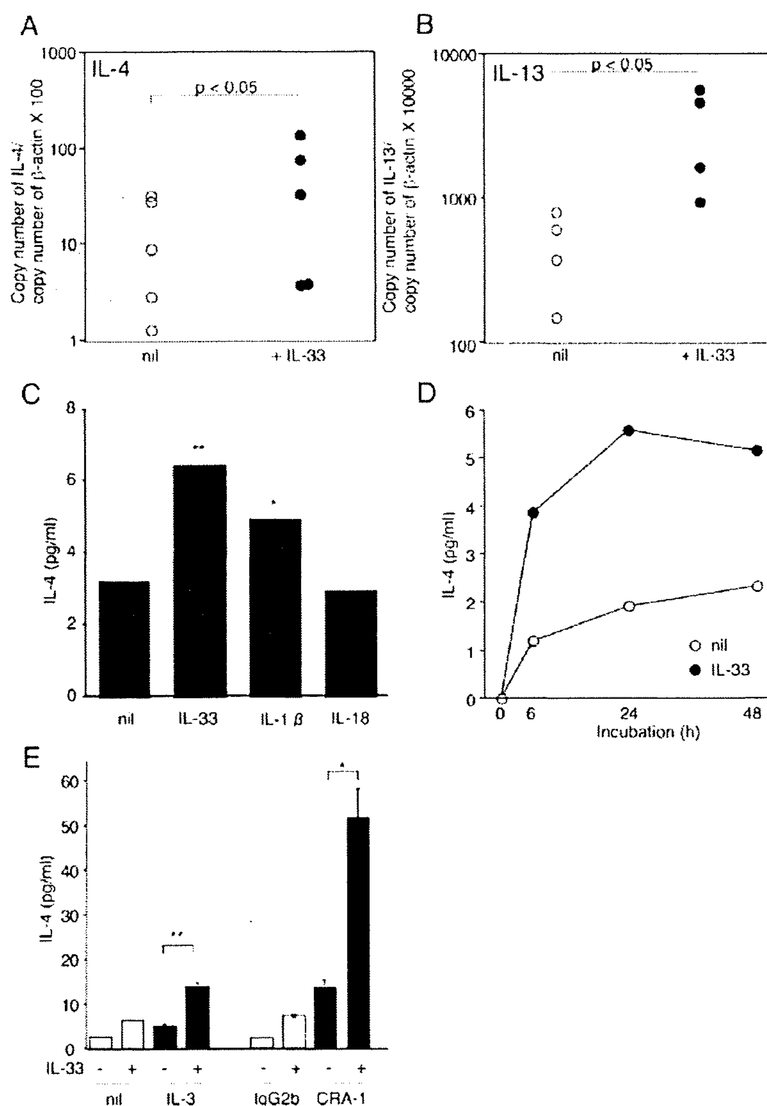

FIGURE 2. *A* and *B*, IL-33 up-regulates IL-4 and IL-13 production by human basophils. Highly purified basophils were incubated with (●) and without (○) IL-33 at 100 ng/ml for 4 h, and then IL-4 (*A*) and IL-13 (*B*) mRNA expression was analyzed by real-time PCR. Data are expressed as the ratio to the 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

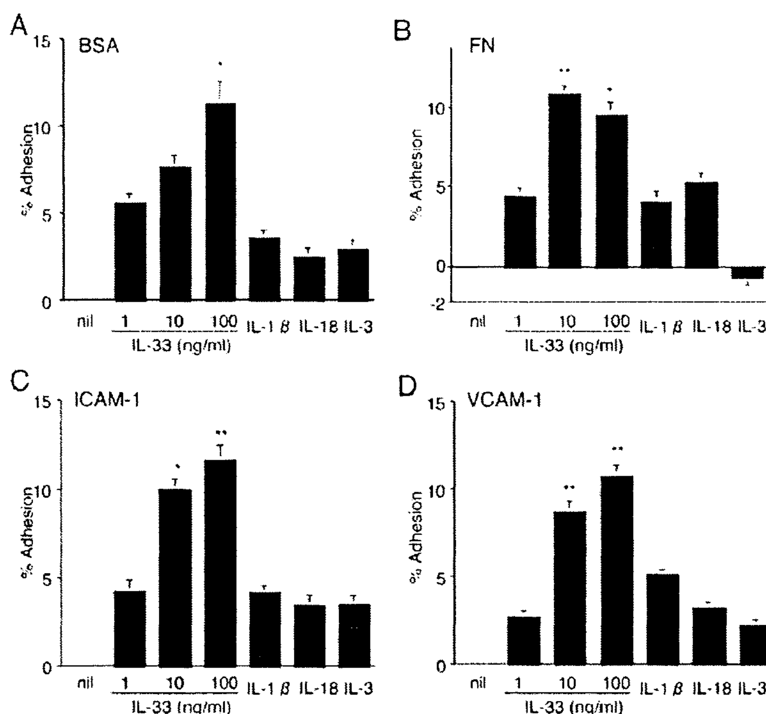

FIGURE 3. IL-33 enhances adhesiveness of human basophils. Percoll-separated basophils were preincubated with and without IL-33 (1, 10, 100 ng/ml), IL-1β (100 ng/ml), IL-18 (100 ng/ml), or IL-3 (300 pM) for 45 min and then dispensed into BSA- (*A*), fibronectin- (*B*), ICAM-1- (*C*) or VCAM-1- (*D*) coated 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

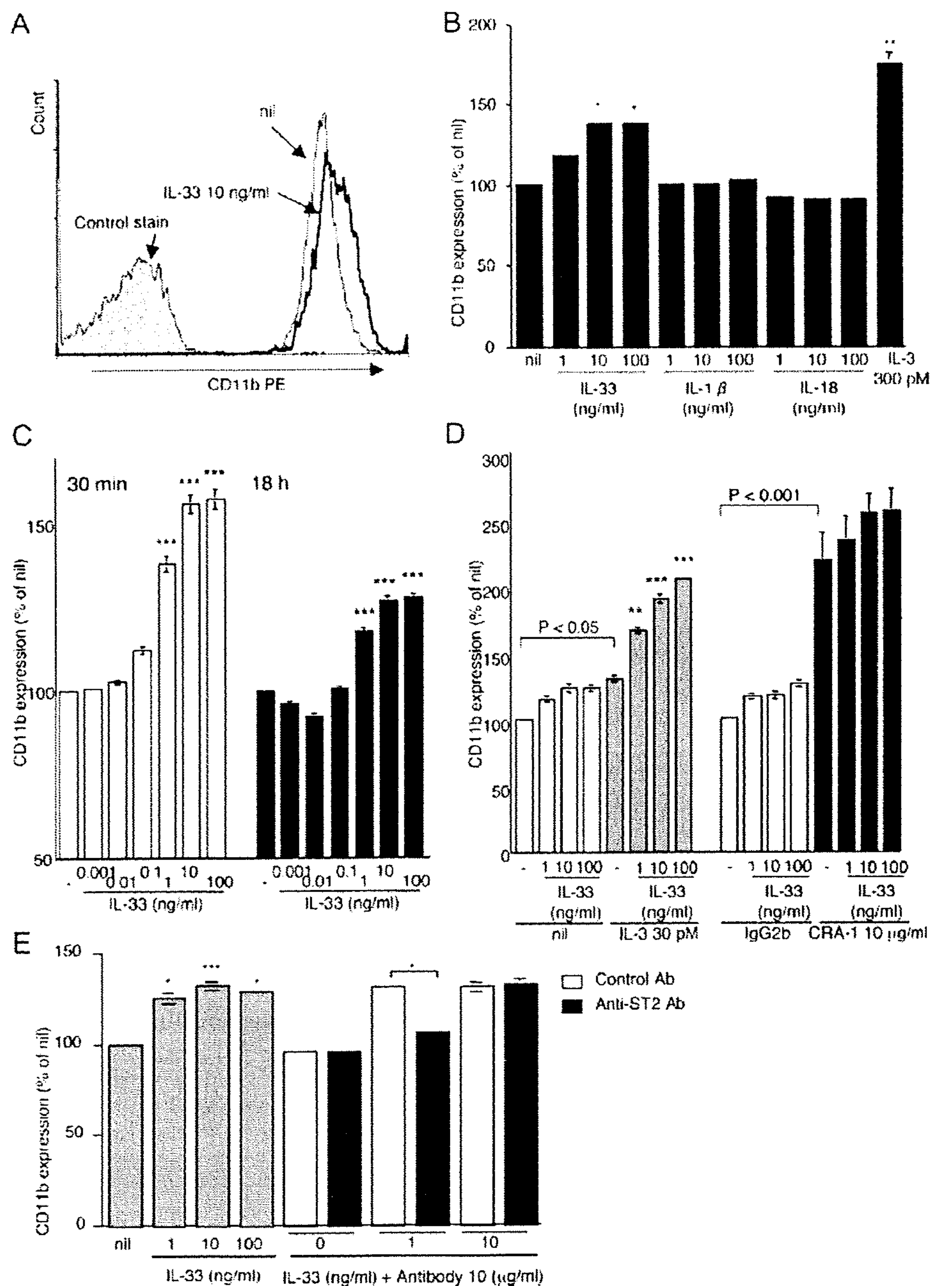

FIGURE 4. IL-33 enhances basophil CD11b expression through their surface ST2 receptor. *A*, Percoll-separated basophils were incubated with medium alone (thin line) or IL-33 at 10 ng/ml (thick line) for 30 min. Then the surface CD11b expression level was assessed by flow cytometry. Data are representative 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. 2D, 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. 2E, 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. 3A, 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. 4C). 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. 4C). 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. 4D, 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. 4E. 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. 4E).

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. 5A). However, when added to the upper chamber with the cells, IL-33

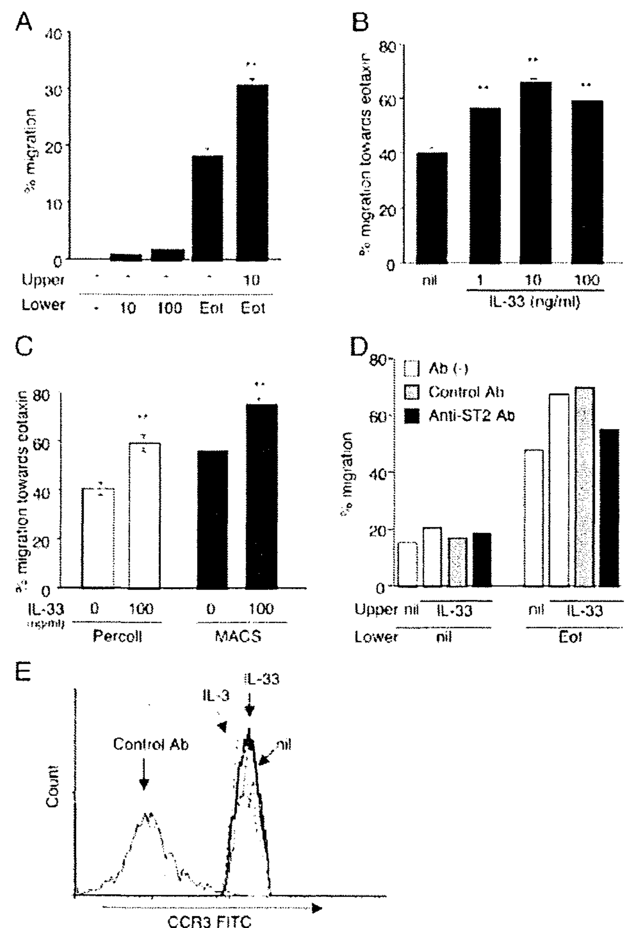

FIGURE 5. IL-33 enhances human basophil migration toward eotaxin. *A*, Two × 10^4 Percoll-separated basophils were added to the upper chamber. IL-33 at 10 or 100 ng/ml or eotaxin (Eot) at 10 nM was added to the lower chamber. Cells mixed with IL-33 at 10 ng/ml were also tested for migration toward eotaxin. 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.

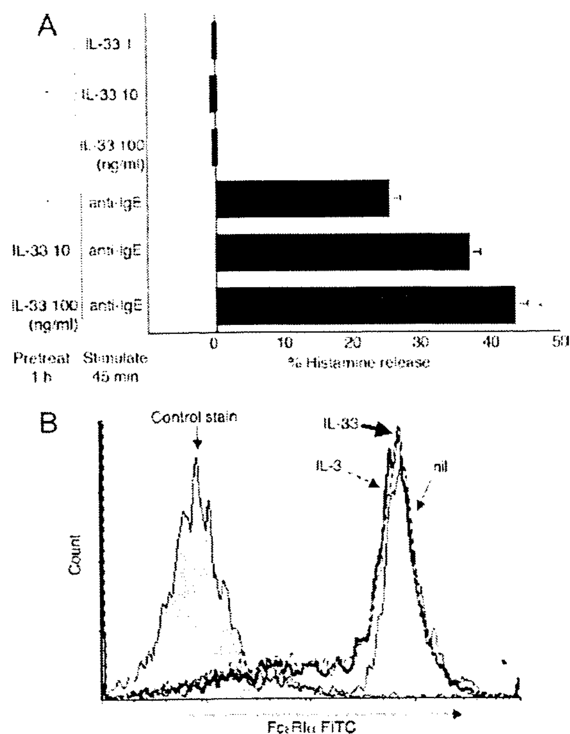

FIGURE 6. IL-33 enhances basophil degranulation following IgE cross-linkage. *A*, Percoll-separated basophils were incubated with and without IL-33 at the indicated concentrations for 1 h and then stimulated with either IL-33 at the indicated concentrations or anti-IgE Ab at 14 μg/ml. The percentage 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 $\beta1$ and $\beta2$ integrins on their surface (14), and in earlier studies we demonstrated that $\beta2$ 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 $\beta2$ 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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