Table 1. Clinical Characteristics, Pulmonary Function, and Airway Wall Dimensions of Asthma Patients and Controls at Baseline

Characteristic	Control Subjects (n = 28)	Asthma Patients (n = 45)	*P* Value
	Mean ± SD or Number (%)		
Age (years)	54.4 ± 13.8	48.1 ± 15.9	0.10
Male sex	16 (57)	25 (56)	0.89*
Body surface area (m^2)	1.59 ± 0.16	1.62 ± 0.17	0.43
FEV_1 (% of predicted)	107.7 ± 16.5	78.8 ± 24.4	<0.0001
FVC (% of predicted)	105.0 ± 13.3	96.7 ± 24.3	0.14
FEV_1/FVC (%)	81.1 ± 5.3	68.6 ± 12.0	<0.001
Mid-forced expiratory flow (% of predicted)	87.8 ± 20.3	48.5 ± 27.9	<0.0001
Maximum expiratory flow at 25% FVC (% of predicted)	74.2 ± 25.7	34.2 ± 22.8	<0.0001
Airway wall area (mm^2)	17.6 ± 4.3	28.3 ± 7.9	<0.0001
Airway wall area/body surface area (mm^2/m^2)	11.2 ± 3.0	17.5 ± 4.6	<0.0001
Percent wall area (%)	55.1 ± 6.7	65.1 ± 6.6	<0.0001
Total airway area (mm^2)	32.9 ± 9.9	43.9 ± 13.1	<0.001
Total airway area/body surface area (mm^2/m^2)	20.9 ± 6.8	27.2 ± 7.9	<0.001
Luminal area (mm^2)	15.2 ± 6.1	15.6 ± 6.4	0.79
Luminal area/body surface area (mm^2/m^2)	9.7 ± 4.1	9.7 ± 4.0	1.0

* Chi-squared test.
FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity.

theophylline in 24 patients) and were withdrawn from the trial if they used other medications. On day 85, CT scanning, blood sampling, and pulmonary function tests were repeated. Patients who had respiratory infections or exacerbations of asthma within the previous 8 weeks were excluded. CT scans and pulmonary function tests were done once in the control subjects.

A 12-week treatment period was chosen because pulmonary function and airway inflammation improve and may reach a plateau after 2 to 12 weeks of inhaled corticosteroid therapy (19,20,30,31). Changes in airway wall thickness assessed with serial CT scans during such treatment have not been reported. In preliminary studies, susceptible patients showed a response during 8 to 12 weeks of treatment.

Statistical Analysis

Values are reported as means (± SD) or medians (interquartile range). Differences between groups were assessed with unpaired *t* tests. Paired *t* tests or Wilcoxon signed-rank tests were used to compare variables before and after treatment. Relations among data were assessed with Pearson correlation tests or Spearman rank correlation tests. Analyses were performed with StatView 4.5 (Abacus Concepts, Berkeley, California). Statistical significance was set at $P < 0.05$.

RESULTS

Six of the 51 enrolled patients were excluded: 2 had exacerbations of asthma requiring medication, 2 had respiratory tract infections at follow-up examinations, 1 refused repeated CT scanning, and 1 was lost to follow-up. In the 45 patients who completed the study, the mean duration of asthma was 7.3 ± 10.5 years (median, 3.4 years; interquartile range, 1.0 to 10.0 years). The asthma was mild in 9 patients, moderate in 25, and severe in 11.

There were no differences in age, sex, or body surface area between asthma patients and controls at baseline (Table 1). However, asthma patients had significantly lower FEV_1, FEV_1/FVC, mid-forced expiratory flow, and maximum expiratory flow at 25% FVC than did controls, as well as significantly greater airway wall area, airway wall area/body surface area, and percent wall area, indicating thickened airways. Total airway area and total airway area/body surface area were also greater in these patients. Luminal area and luminal area/body surface area were similar in the two groups.

Effects of Treatment with Inhaled Corticosteroid

FEV_1, FVC, FEV_1/FVC, mid-forced expiratory flow, and maximum expiratory flow at 25% FVC all increased significantly after treatment, but the values (% of predicted) for FEV_1, mid-forced expiratory flow, and maximum expiratory flow at 25% FVC remained lower than in controls (Table 2). The serum eosinophil cationic protein level decreased significantly after treatment.

Airway wall area, airway wall area/body surface area, and percent wall area decreased significantly albeit modestly after treatment (Table 2; Figure 1). For example, airway wall area/body surface area decreased by 1.9 ± 3.5 mm^2/m^2 (10.5% ± 17.9%). However, the post-treatment values were still higher than those in the control subjects. Total wall area and total wall area/body surface area de-

Table 2. Pulmonary Function, Serum Eosinophil Cationic Protein Level, and Airway and Lung Dimensions in Asthma Patients before and after Treatment with Inhaled Corticosteroid

Characteristic	Before Treatment	After Treatment	*P* Value
	Mean ± SD or Median (Interquartile Range)		
FEV_1 (L)	2.3 ± 1.0	2.7 ± 0.9	<0.0001
FEV_1 (% of predicted)	78.8 ± 24.4	95.6 ± 17.7*	<0.0001
FVC (L)	3.2 ± 1.2	3.5 ± 0.9	0.003
FVC (% of predicted)	96.7 ± 24.3	105.4 ± 22.6	0.04
FEV_1/FVC (%)	68.6 ± 12.0	75.3 ± 11.4	<0.0001
Mid-forced expiratory flow (L/s)	1.8 ± 1.2	2.5 ± 1.3	<0.0001
Mid-forced expiratory flow (% of predicted)	48.5 ± 27.9	66.9 ± 27.7*	<0.0001
Maximum expiratory flow at 25% FVC (L/s)	0.8 ± 0.7	1.1 ± 0.8	<0.0001
Maximum expiratory flow at 25% FVC (% of predicted)	34.2 ± 22.8	45.8 ± 25.0*	<0.001
Serum eosinophil cationic protein (μg/L)	19.1 (11.8–35.1)	12.0 (4.4–20.7)	<0.001
Airway wall area (mm^2)	28.3 ± 7.9	25.1 ± 8.2†	<0.001
Airway wall area/body surface area (mm^2/m^2)	17.5 ± 4.6	15.6 ± 5.1†	<0.001
Percent wall area (%)	65.1 ± 6.6	61.2 ± 6.7†	<0.001
Total airway area (mm^2)	43.9 ± 13.1	41.5 ± 13.9†	0.06
Total airway area/body surface area (mm^2/m^2)	27.2 ± 7.9	25.8 ± 8.5†	0.08
Luminal area (mm^2)	15.6 ± 6.4	16.4 ± 7.0	0.30
Luminal area/body surface area (mm^2/m^2)	9.7 ± 4.0	10.2 ± 4.2	0.32
Lung area (cm^2)	116.4 ± 19.9	118.1 ± 19.7	0.34
Lung area/body surface area (cm^2/m^2)	74.0 ± 12.0	75.1 ± 11.8	0.34

* Significantly lower than the values of control subjects in Table 1 ($P = 0.04$ for FEV_1, $P < 0.05$ for mid-forced expiratory flow, and $P < 0.0001$ for maximum expiratory flow at 25% FVC).

† Significantly greater than the values of control subjects in Table 1 ($P < 0.0001$ for airway wall area and airway wall area/body surface area, $P < 0.001$ for percent wall area, $P = 0.005$ for total airway area, and $P = 0.01$ for total airway area/body surface area).

FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity.

creased slightly but insignificantly and were still greater than the control values. Luminal area and luminal area/body surface area were not affected by treatment.

Lung area and lung area/body surface area did not change significantly after treatment. The change in lung area/body surface area was small (0.8% ± 4.4%) and was

Figure 1. Computed tomographic scans of a 52-year-old man with asthma before (left) and after (right) treatment with beclomethasone. Arrows indicate the apical bronchus of the right upper lobe. The airway was assessed at the same level, based on anatomic landmarks such as blood vessels and bronchi.

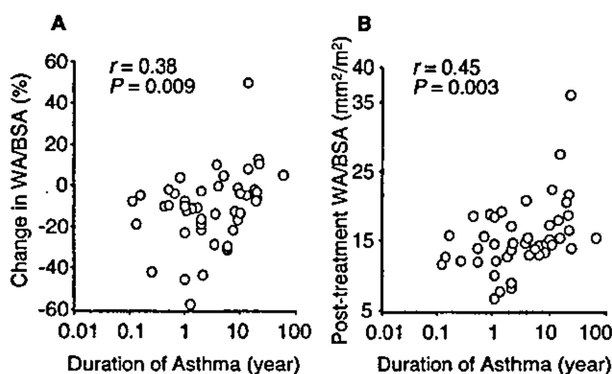

Figure 2. Duration of asthma in relation to the percent change in airway wall area/body surface area (WA/BSA) in response to treatment (A) and post-treatment airway wall area/body surface area (B). In patients with longer-standing asthma, airway wall thickness was reduced less (A) and airway wall thickening after treatment was more prominent (B).

not related to changes in airway wall area/body surface area ($r = -0.19$, $P = 0.20$) or luminal area/body surface area ($r = 0.22$, $P = 0.14$).

Relation of Airway Wall Thickness to Clinical Indexes before and after Treatment

The decrease in eosinophil cationic protein level was associated with a decrease in airway wall area/body surface area ($r = 0.39$, $P = 0.009$) and an increase in FEV_1 ($r = 0.45$, $P = 0.003$). The decrease in airway wall area/body surface area and the increase in FEV_1 were also associated ($r = 0.44$, $P = 0.004$). The duration of asthma at entry was inversely related to the percent decrease in airway wall area/body surface area (Figure 2A) and was directly related to airway wall area/body surface area after (Figure 2B) but not before (Table 3) treatment. Post-treatment FEV_1, FEV_1/FVC, mid-forced expiratory flow, and maximum expiratory flow at 25% FVC correlated negatively with post-treatment airway wall area/body surface area. These correlations were stronger than those observed between the pretreatment values (Table 3).

DISCUSSION

This study shows that treatment with moderate doses of inhaled corticosteroid for 12 weeks reduced airway wall thickness, as assessed by CT scans, modestly but significantly in patients with persistent asthma. However, airway wall thickness remained significantly greater than in controls.

The components of airway wall thickening that responded to treatment may have included inflammatory factors, since the decrease in wall thickness/body surface area correlated with a decrease in serum eosinophil cationic protein level. The water or proteoglycan content of the airways might also have been affected. Inhaled corticosteroid treatment may reduce the thickness of the reticular basement membrane (19–22,31,32), but this component would contribute very little to the decrease in total airway wall thickness because the decrease in reticular basement membrane thickness in response to inhaled corticosteroid therapy is generally less than 5 μm (19,20,22,31,32), and the decrease in absolute wall thickness in our study was 164 ± 232 μm, as calculated from the change in airway wall area (assuming that a cross-section of the airways was a right circular cylinder). Other components, such as increased vascularity (33) or airway mucous, might therefore have been modified by treatment, but such pathological details are difficult to evaluate by CT (12). On the other hand, CT is less invasive than bronchial biopsy and can be used to evaluate a broader range of airway or lung dimensions, such as total airway wall thickness, airway luminal area, and lung area, all of which were assessed in this study.

Inhaled corticosteroid had less effect on airway wall thickening in patients with long-standing asthma. Post-treatment airway wall thickness, but not pretreatment thickness, was related to disease duration. These findings indicate that the component of airway wall thickening resistant to inhaled corticosteroid treatment may increase relatively or absolutely in long-standing disease not treated by inhaled corticosteroid. We have demonstrated in this study a possible association between de-

Table 3. Correlation between Airway Wall Area/Body Surface Area and Clinical Indexes before and after Treatment

	Airway Wall Area/Body Surface Area			
	Before Treatment		After Treatment	
Clinical Indexes	*r*	*P* Value	*r*	*P* Value
Duration of asthma at entry (years)	0.23	0.12	0.45	0.003
Serum eosinophil cationic protein (μg/L)	−0.15	0.32	−0.16	0.28
FEV_1 (% of predicted)	−0.13	0.39	−0.32	0.04
FEV_1/FVC (%)	−0.32	0.04	−0.49	<0.001
Mid-forced expiratory flow (% of predicted)	−0.24	0.11	−0.40	0.008
Maximum expiratory flow at 25% FVC (% of predicted)	−0.22	0.15	−0.38	0.01

FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity.

layed introduction of inhaled corticosteroid treatment and a poor morphological response of the airways in patients with asthma. Although not assessable by CT, airway smooth muscle hypertrophy, submucosal gland hyperplasia, increased cartilage, or extracellular matrix deposition, particularly outside the inner airway wall (i.e., around smooth muscle cells or bundles and in the adventitial layer), might contribute to persistent airway wall thickening (1–6,34,35). The relation between airway wall thickness and airflow obstruction was stronger after than before treatment (Table 3). Therefore, thickening that persists after treatment with inhaled corticosteroids may have clinical implications.

The airway luminal area in patients with asthma was similar to that in controls at baseline and did not change after treatment, even though airway wall thickness decreased. Therefore, the differences in FEV_1 between patients and controls at baseline and before and after treatment cannot be explained by differences or changes in airway luminal area. In our previous CT study, the luminal area of the same bronchus evaluated was also similar in asthmatic patients and control subjects and was unrelated to the severity of asthma (18). We speculate that wall thickening of the central airways may correlate with luminal narrowing of more distal airways (18), which may be the primary site of flow limitation in asthma (36,37). Thickening of the adventitial layer of the airway (e.g., from extracellular matrix deposition) may attenuate the transmission of distending force generated by surrounding lung parenchyma to oppose smooth muscle shortening, making the airways more likely to narrow (2–4,8). Such an effect may be more pronounced in distal airways, which have smaller internal diameters and may lack cartilage (37). These airways, however, are too small to be assessed by CT (12). The lack of change in luminal area after inhaled corticosteroid therapy could reflect potential effects of lung volume on airway dimensions (11,13). However, such effects were unlikely because neither lung area nor lung area/body surface area changed after treatment, and the changes in these indexes did not correlate with the change in luminal area or luminal area/body surface area.

Some limitations of the study should be mentioned. The dose of beclomethasone was not individually adjusted and might have been too low in some patients to control inflammation optimally. Persistent airway wall thickening may thus not necessarily be resistant to treatment. Optimizing inhaled corticosteroid dosing is controversial (32,38). We chose the fixed-dose strategy to rule out the effects of different doses on airway inflammation and airway dimensions. Airway wall thickening that remained after treatment might have been decreased by more prolonged therapy or higher doses of inhaled corticosteroid, as suggested by studies of reticular basement membrane thickening (31,32) and airway hyperresponsiveness (39,40), or by using a more potent corticosteroid such as fluticasone or budesonide. In Japan, such agents were not available until recently, and beclomethasone has therefore been widely used in clinical studies (22,41).

Another potential limitation is that airway inflammation was not assessed directly (e.g., by measuring the level of eosinophils or eosinophil cationic protein in induced sputum). However, sputum induction does not always yield samples suitable for examination, and serum levels of eosinophil cationic protein correlate well with the degree of eosinophilic airway inflammation (27–29). Indeed, decreased serum levels of eosinophil cationic protein after treatment were associated with an increase in FEV_1.

In conclusion, airway wall thickening as assessed by CT responds partially to treatment with inhaled corticosteroid. The decrease in airway wall thickness may reflect reduced airway inflammation. Thickening resistant to the effects of inhaled corticosteroid, most likely involving structural changes, may progress in patients with long-standing asthma not treated with inhaled corticosteroid and lead to chronic airflow obstruction. Long-term studies evaluating airway wall thickness by CT may confirm whether early treatment with inhaled corticosteroid prevents airway remodeling in patients with asthma.

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IgE-dependent enhancement of Th2 cell-mediated allergic inflammation in the airways

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SUMMARY

T helper 2 (Th2) cell-derived cytokines, including interleukin (IL)-4, IL-5 and IL-13, play important roles in causing allergic airway inflammation. In contrast to Th2 cells, however, the role of IgE and mast cells in inducing allergic airway inflammation is not understood fully. In the present study, we addressed this point using transgenic mice expressing trinitrophenyl (TNP)-specific IgE (TNP–IgE mice), which enable us to investigate the role of IgE without the influence of antigen-specific T cell activation and other immunoglobulins. When the corresponding antigen, TNP–BSA, was administered intranasally to TNP–IgE mice, a large number of $CD4^+$ T cells were recruited into the airways. In contrast, TNP–BSA administration did not induce eosinophil recruitment into the airways or airway hyperreactivity. Furthermore, when ovalbumin (OVA)-specific Th2 cells were transferred to TNP–IgE mice and the mice were challenged with inhaled OVA, TNP–BSA administration increased OVA-specific T cell recruitment and then enhanced Th2 cell-mediated eosinophil recruitment into the airways. These results indicate that IgE-induced mast cell activation principally induces $CD4^+$ T cell recruitment into the airways and thus plays an important role in enhancing Th2 cell-mediated eosinophilic airway inflammation by recruiting Th2 cells into the site of allergic inflammation.

Keywords eosinophils IgE mast cells transgenic mice

INTRODUCTION

Allergic airway inflammation is a cardinal feature of asthma and is associated with intense eosinophil and $CD4^+$ T cell infiltration in the airways, and the chronic inflammatory process causes epithelial damage and airway hyperreactivity (AHR) [1–3]. It has been shown that T helper 2 (Th2) cells and their cytokines such as interleukin (IL)-4, IL-5 and IL-13 play important roles in inducing allergic airway inflammation [2,4,5]: IL-5 mediates antigen-induced eosinophil recruitment into the airways [6,7] and IL-13 induces goblet cell hyperplasia and AHR [8,9].

In addition to Th2 cell-mediated allergic inflammation, IgE-dependent activation of mast cells is suggested to be involved in the pathogenesis of asthma [10–13]. IgE cross-linking by specific antigens triggers the activation of mast cells, resulting in the synthesis and release of a variety of mediators and cytokines that induce the early phase asthmatic response [12,13]. However, the role of IgE and mast cells in allergic airway inflammation and AHR is not well defined. While it has been demonstrated that features of asthma, including eosinophilic airway inflammation and AHR, can be elicited in the absence of IgE antibodies [14–16] or mast cells [17], it has been shown recently that mast cells play an important role in antigen-induced eosinophil recruitment into the airways and AHR in the situation in which mice are sensitized and challenged with antigens under weak protocols but not under strong protocols [18,19]. The fact that antigen sensitization and challenges induce IgE production, Th2 cell activation and cytokine production and eosinophilic inflammation altogether makes it difficult to evaluate the role of IgE and mast cells in allergic airway inflammation and AHR in asthma [1,20,21]. Thus, the role of IgE-dependent mast cell activation in inducing allergic airway inflammation and AHR still remains to be determined.

To determine whether IgE-dependent mast cell activation induces allergic airway inflammation and AHR, we examined the effect of IgE cross-linking by antigens on airway inflammation using trinitrophenyl (TNP)-specific IgE transgenic mice (TNP–IgE mice) [22], which enables us to investigate the role of IgE

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without the influence of antigen-specific T cell activation and other immunoglobulins. Our results indicate that IgE-dependent mast cell activation induces $CD4^+$ T cell but not eosinophil recruitment into the airways and thus enhances Th2 cell-mediated eosinophilic airway inflammation by recruiting Th2 cells into the site of allergic inflammation.

MATERIALS AND METHODS

Mice

TNP-specific IgE transgenic mice (TNP–IgE mice) [22] with a BALB/c background and littermate wild-type (WT) mice were used in this study. Ovalbumin (OVA)-specific TCR transgenic mice (DO11·10 mice) with a BALB/c background were described previously [23]. All experiments were performed according to the NIH guidelines.

Antigen-induced airway inflammation in TNP–IgE mice

To determine whether IgE cross-linking by a relevant antigen induces airway inflammation, polyvalent TNP–BSA solution in saline (the molar ratio of TNP : BSA = 22 : 1, 6·7 mg/ml, 80 μl/mouse) [22] was administered intranasally to TNP–IgE mice or WT mice. As a control, BSA solution (6·7 mg/ml) was used. At indicated times after TNP–BSA or BSA administration, the number of lymphocytes, eosinophils, neutrophils and macrophages in bronchoalveolar lavage fluid (BALF) was evaluated as described previously [24]. A fraction of cells were subjected to a flow cytometric analysis for surface phenotyping of CD4, CD8 and B220 [24]. The expression of CD25 and CD69 on $CD4^+$ T cells was also evaluated using the corresponding antibodies (BD PharMingen, San Diego, CA, USA).

To determine whether prostaglandins are involved in IgE-dependent airway inflammation, we examined the effect of acetylsalicylic acid, a well-defined cyclooxygenase inhibitor, on antigen-induced airway inflammation in TNP–IgE mice. TNP–IgE mice were injected intraperitoneally with acetylsalicylic acid (3 or 6 mg/mouse in 0·5 ml of saline) at 30 min before the intranasal TNP–BSA administration and the number of lymphocytes, eosinophils, neutrophils and macrophages in BALF was evaluated at 48 h after TNP–BSA administration. We also examined the effect of a cysteinyl leukotriene 1 receptor antagonist pranlukast and anti-tumour necrosis factor (TNF)-α antibody on antigen-induced airway inflammation in TNP–IgE mice. TNP–IgE mice were injected intraperitoneally with pranlukast (75 μg/mouse in 0·2 ml of saline) (Ono Pharmaceutical, Osaka, Japan) or goat antimouse TNF-α antibody [75 μg/mouse in 0·2 ml of phosphate buffered saline (PBS) (Genzyme, Cambridge, MA, USA)] at 30 min and at 12 h, respectively, before the intranasal TNP–BSA administration and the number of inflammatory cells in BALF was evaluated 48 h after TNP–BSA administration.

Cytokine levels in BALF

The amounts of IL-4, IL-5 and IFN-γ in the BALF were determined by the enzyme immunoassay as described previously [24]. The detection limits of these assays were 15 pg/ml of IL-4 and IL-5 and 50 pg/ml of IFN-γ.

Measurement of airway reactivity

Forty-eight hours after intranasal TNP–BSA or BSA administration, airway reactivity to aerosolized methacholine (3·1–50 mg/ml) was measured using whole body plethysmograph (Buxco Electronics, Sharon, CT, USA) as described previously [25]. Briefly, unrestrained conscious mice were placed in whole body plethysmographic chambers and, after 5 min of stabilization, dose–response curves to aerosolized methacholine were generated. Increasing concentrations of methacholine were aerosolized for 3 min each, and mean airway bronchoconstriction readings, as assessed by enhanced respiratory pause (Penh), were obtained over 10-min periods. As controls, BALB/c mice (age 7–8 weeks, Charles River Laboratories, Atsugi, Japan) were immunized intraperitoneally twice with 4 μg of OVA (Sigma Chemical Co., St Louis, MO, USA) in 4 mg of aluminium hydroxide at a 2-week interval. Fourteen days after the second immunization, sensitized mice were challenged with the inhaled OVA (50 mg/ml in saline) or saline for 20 min three times every 24 h. Twenty-four hours after the final OVA challenge, airway reactivity to aerosolized methacholine was measured as described above.

Adoptive transfer experiments for antigen-induced eosinophil recruitment into the airways

To determine whether IgE cross-linking enhances Th2 cell-mediated allergic inflammation in the airways, we performed adoptive cell transfer experiments in which OVA-specific Th2 cells from DO11·10 mice were transferred to TNP–IgE mice and the eosinophilic airway inflammation was induced by inhaled OVA challenge in the mice. Briefly, splenocytes from DO11·10 mice were stimulated with OVA323–339 peptide (50 ng/ml) in the presence of recombinant IL-4 (5 ng/ml) at 37°C for 48 h. Cells were then cultured with IL-4 and IL-2 (5 ng/ml) for another 72 h. After dead cells were removed by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ, USA), the recovered cells were injected intravenously to TNP–IgE mice or WT mice ($2{\cdot}0 \times 10^6$ cells/mouse). The frequency of cell populations of transferred cells was 80–90% of OVA-specific $CD4^+$ T cells as $KJ1\text{-}26^+$ $CD4^+$ T cells [23], 2–5% of $CD8^+$ T cells, and 5–10% of $B220^+$ cells. Two days after the cell transfer, these mice were challenged with inhaled OVA (50 mg/ml) or saline (as a control) for 20 min and TNP–BSA or BSA was then administered intranasally to the mice 30 min after the OVA challenge. The number of eosinophils and antigen-specific $CD4^+$ T cells derived from DO11·10 mice ($KJ1\text{-}26^+$ T cells) in BALF was evaluated at 48 h after the intranasal TNP–BSA or BSA administration.

Data analysis

Data are summarized as mean ± s.d. The statistical analysis of the results was performed by unpaired t-test. P-values < 0·05 were considered significant.

RESULTS

IgE cross-linking induces $CD4^+$ T cell recruitment into the airways

We first examined whether IgE cross-linking by a relevant antigen induced airway inflammation using TNP–IgE mice. As shown in Fig. 1a, the intranasal administration of the corresponding antigen, TNP–BSA, significantly increased the number of inflammatory cells in BALF at 8–48 h in TNP–IgE mice but not in WT mice (n = 8 mice at each time-point). As expected, however, intranasal administration of BSA did not induce inflammatory cell recruitment into the airways in TNP–IgE mice or WT mice (Fig. 1a). The analysis of BALF cells showed that the number of lymphocytes was significantly increased after TNP–BSA

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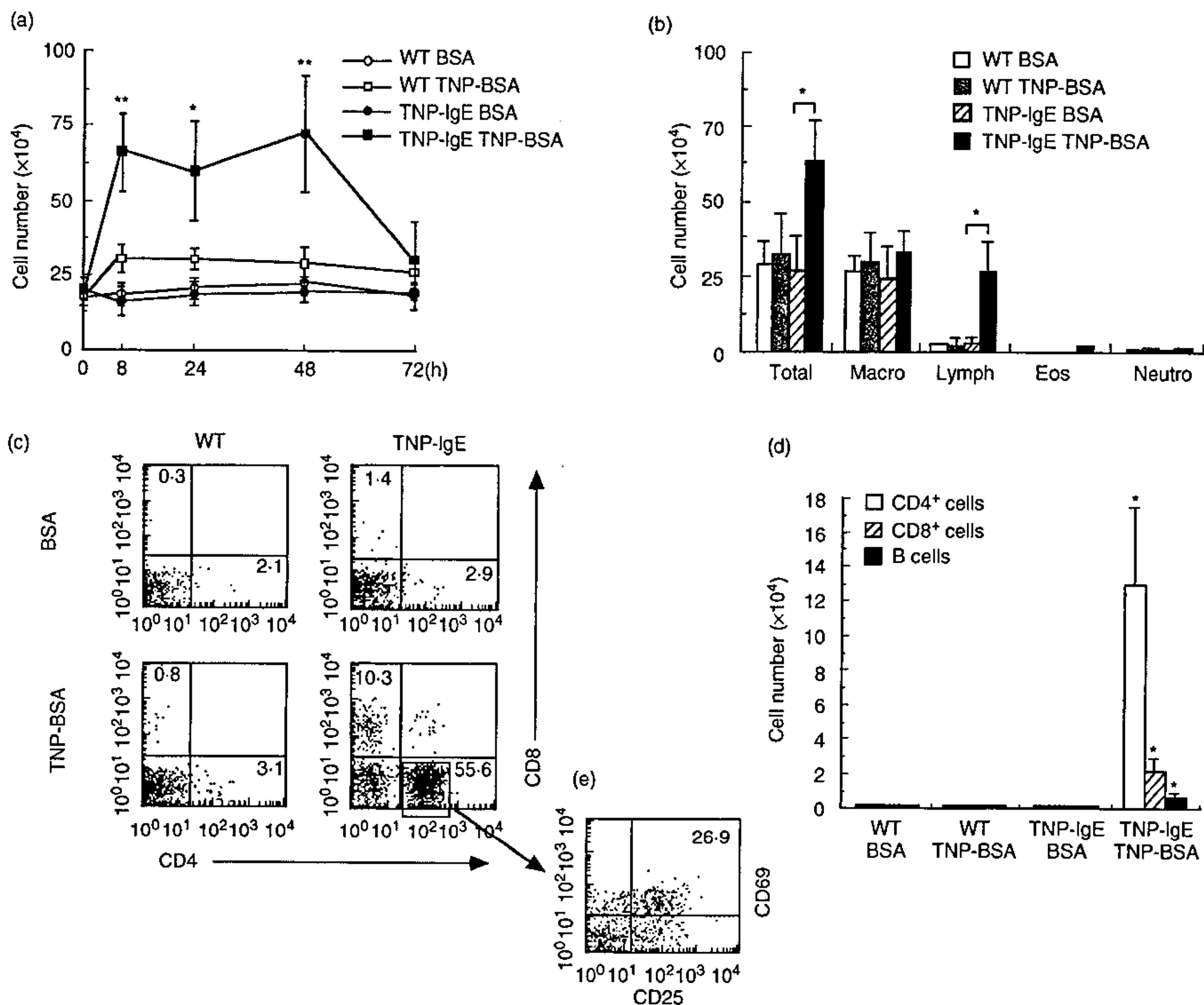

Fig. 1. IgE cross-linking induces CD4⁺ T cell recruitment into the airways. (a) Kinetics of antigen-induced airway inflammation in TNP–IgE mice. TNP–BSA or BSA (as a control) was administered intranasally to TNP–IgE mice and the littermate WT mice. At indicated times after the administration, bronchoalveolar lavage (BAL) was performed and the number of cells in BAL fluid (BALF) was counted. Data are means ± s.d. for eight mice in each group. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, significantly different from the mean value of the corresponding control response. (b) Cellular components in BALF. The number of lymphocytes, eosinophils, neutrophils and macrophages in BALF was evaluated 48 h after TNP–BSA or BSA administration by counting 500 cells stained with Wright–Giemsa solution. $n = 8$ mice in each group, $^{*}P < 0{\cdot}01$. (c) CD4 *versus* CD8 staining of BALF cells. BALF cells were subjected to FACS analysis 48 h after TNP–BSA or BSA administration. Shown is a representative staining of CD4 *versus* CD8 (gating on lymphocyte population) from five independent experiments. (d) The number of CD4⁺ cells, CD8⁺ cells and B cells in BALF 48 h after the challenge. $n = 8$ mice in each group. $^{*}P < 0{\cdot}01$, significantly different from the mean value of the corresponding control response. (e) CD25 *versus* CD69 staining of CD4⁺ T cells. BALF cells were subjected to FACS analysis 48 h after TNP–BSA administration. Shown is a representative CD25 *versus* CD69 staining of CD4⁺ T cells from five independent experiments.

administration in TNP–IgE mice (TNP–BSA $28{\cdot}0 \pm 9{\cdot}8$ *versus* BSA $2{\cdot}1 \pm 1{\cdot}0 \times 10^4$ at 48 h, $n = 8$ mice in each group, $P < 0{\cdot}01$) (Fig. 1b). In contrast, the number of eosinophils, neutrophils or macrophages was not significantly increased in TNP–IgE mice and WT mice at 48 h after TNP-BSA administration (Fig. 1b). FACS analysis revealed that the majority of lymphocytes in BALF of TNP–BSA-administered TNP–IgE mice were CD4⁺ T cells ($60{\cdot}2 \pm 10{\cdot}4\%$, $n = 6$) (Fig. 1c,d). The number of CD8⁺ T cells and B cells was also slightly increased in TNP–BSA-administered TNP–IgE mice (Fig. 1c,d). Although approximately 25% of CD4⁺ T cells exhibited an activated phenotype (CD25⁺ CD69⁺) (Fig. 1e), the levels of IL-4, IL-5 and IFN-γ in the BALF were still undetectable after TNP–BSA administration in TNP–IgE mice (data not shown). Histological analysis showed that the intranasal administration of TNP–BSA also induced lymphocyte recruitment in the perivascular and peribronchial spaces of the lung in TNP–IgE mice (data not shown).

IgE cross-linking does not enhance airway reactivity to methacholine

Next, we examined the effect of IgE cross-linking on airway reactivity to methacholine in TNP–IgE mice. TNP–BSA or BSA was

administered intranasally to TNP–IgE mice and WT mice and, 48 h later, the airway reactivity to aerosolized methacholine was measured by whole body plethysmograph. The intranasal administration of TNP-BSA did not increase airway reactivity to methacholine in TNP–IgE mice as compared with BSA administration ($n = 5$ in each group) (Fig. 2). As anticipated, TNP–BSA did not increase airway reactivity to methacholine in WT mice and the airway reactivity was comparable to that in TNP–BSA-administered TNP–IgE mice (data not shown). These results indicate that IgE cross-linking is not sufficient for the induction of airway hyperreactivity in this system.

Cyclooxygenase inhibitor prevents IgE-induced CD4⁺ T cell recruitment into the airways

We then determined which mediators are involved in IgE-induced $CD4^+$ T cell recruitment into the airways. Because it has been suggested that prostaglandin D_2 (PGD_2) from mast cells is involved in $CD4^+$ T cell recruitment [26], we examined the effect of acetylsalicylic acid on the IgE-induced $CD4^+$ T cell recruitment in TNP–BSA-administered TNP–IgE mice. As shown in Fig. 3, the number of $CD4^+$ T cells in BALF in TNP–BSA-administered TNP–IgE mice was significantly decreased by acetylsalicylic acid (acetylsalicylic acid (6 mg) $3{\cdot}9 \pm 1{\cdot}0$ *versus* saline $18{\cdot}3 \pm 5{\cdot}4 \times 10^4$, $n = 5$, $P < 0{\cdot}01$). The number of $CD8^+$ T cells in BALF tended to be decreased by acetylsalicylic acid but the differences were not statistically significant (Fig. 3b). On the other hand, a cysteinyl leukotriene 1 receptor antagonist pranlukast did not decrease the number of $CD4^+$ T cells and $CD8^+$ T cells in BALF in TNP–BSA-administered TNP–IgE mice (data not shown). In addition, the administration of neutralizing antibody to TNF-α did not decrease the number of $CD4^+$ T cells and $CD8^+$ T cells in the BALF in TNP–BSA-administered TNP–IgE mice (data not shown). Taken together, these results suggest that prostaglandins are involved in IgE-induced $CD4^+$ T cell recruitment into the airways.

IgE cross-linking enhances Th2 cell-mediated eosinophil recruitment into the airways

Finally, we studied whether IgE-dependent mast cell activation contributed to Th2 cell-mediated eosinophil recruitment into the airways by the adoptive transfer system of antigen-specific Th2 cells to TNP–IgE mice. OVA-specific Th2 cells prepared from DO11·10 mice were transferred to TNP–IgE mice or WT mice, and 2 days later the mice were challenged with the inhaled OVA or saline (as a control) for 20 min. TNP–BSA or BSA was then administered intranasally to the mice and the number of eosinophils and OVA-specific $CD4^+$ T cells (KJ1-26⁺ $CD4^+$ cells) in BALF was counted at 48 h after TNP–BSA or BSA administration. Without the cell transfer of OVA-specific Th2 cells, the inhaled OVA did not significantly induce eosinophil recruitment into the airways in TNP–IgE mice or WT mice (data not shown). When OVA-specific Th2 cells were transferred to WT mice and TNP–IgE mice, the inhaled OVA similarly induced eosinophil (Fig. 4a) and OVA-specific $CD4^+$ T cell (Fig. 4b) recruitment into

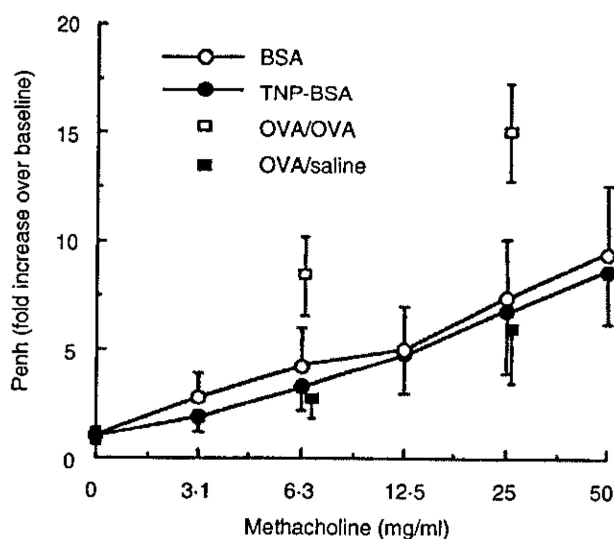

Fig. 2. IgE cross-linking does not induce airway hyperreactivity. TNP–BSA or BSA was administered intranasally to TNP–IgE mice. Forty-eight hours after TNP–BSA or BSA administration, airway reactivity was measured using a Buxco system where mice were exposed to increasing concentrations of aerosolized methacholine (3·1–50 mg/ml). Airway reactivity was expressed as enhanced pause (Penh) values for each concentration of methacholine over baseline response. As controls, OVA-sensitized BALB/c mice were challenged three times with the inhaled OVA or saline, and 24 h later airway reactivity to aerosolized methacholine was measured. Data are means ± s.d. for five mice in each group.

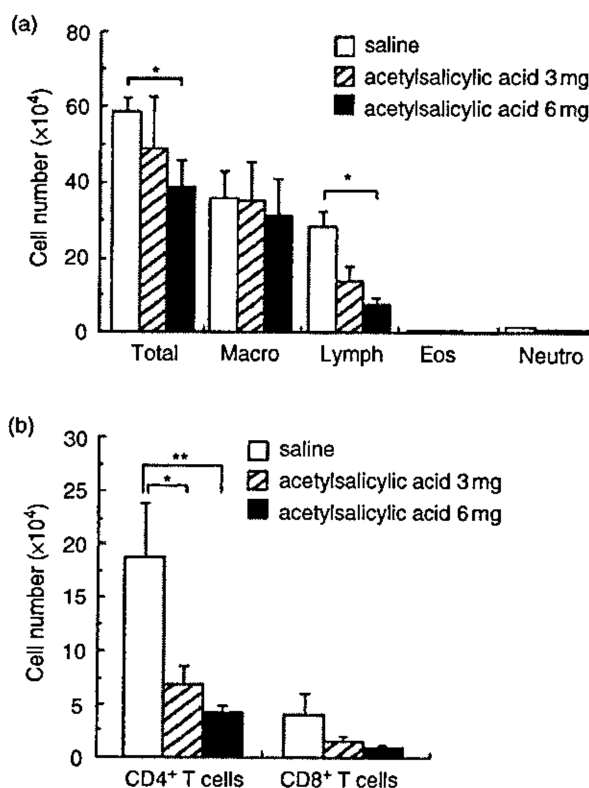

Fig. 3. Acetylsalicylic acid inhibits IgE-induced $CD4^+$ T cell recruitment into the airways. TNP–IgE mice were injected intraperitoneally with acetylsalicylic acid (3 mg or 6 mg/mouse) or saline (as a control), and 30 min later TNP–BSA was administered intranasally to the mice. The number of lymphocytes, eosinophils, neutrophils and macrophages in BALF was counted 48 h after the TNP–BSA administration (a). The number of $CD4^+$ and $CD8^+$ T cells in BALF was also analysed by FACS (b). Data are means ± s.d. for five mice in each group. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

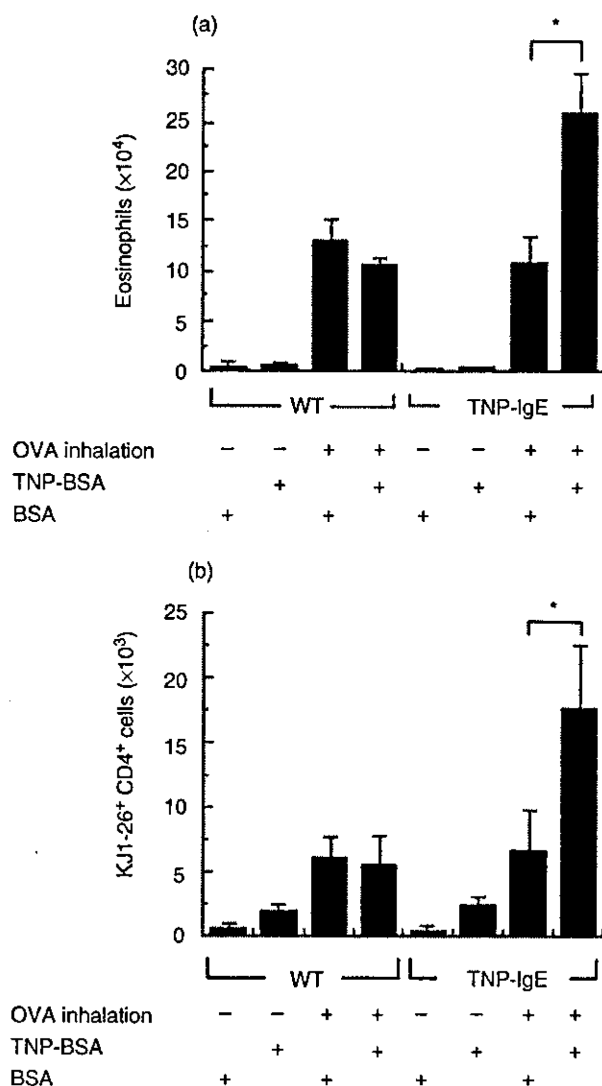

Fig. 4. IgE cross-linking enhances Th2 cell-mediated eosinophil recruitment into the airways. OVA-specific Th2 cells were prepared and transferred to TNP–IgE mice or WT mice as described in the Methods. Two days later, the mice were challenged with the inhaled OVA or saline (as a control) for 20 min. TNP-BSA or BSA was then administered intranasally to the mice, and the number of eosinophils (a) and OVA-specific $CD4^+$ T cells ($CD4^+$ $KJ1\text{-}26^+$) (b) in BALF was counted 48 h after TNP–BSA or BSA administration. Data are means ± s.d. for eight mice in each group, $*P < 0{\cdot}01$.

the airways in both mice. On the other hand, intranasal administration of TNP–BSA alone did not induce eosinophil recruitment into the airways in TNP–IgE mice or WT mice even when Th2 cells were transferred to these mice (Fig. 4a). Interestingly, TNP–BSA administration significantly enhanced OVA-induced eosinophil ($n = 8$, $P < 0{\cdot}01$) (Fig. 4a) and OVA-specific $CD4^+$ T cell ($n = 8$, $P < 0{\cdot}01$) (Fig. 4b) recruitment into the airways in TNP–IgE mice but not in WT mice. FACS analysis revealed that the majority of OVA-specific $CD4^+$ T cells in the BALF exhibited an activated phenotype (data not shown). These results suggest that IgE-dependent mast cell activation enhances Th2 cell-mediated allergic airway inflammation by recruiting Th2 cells into the airways.

DISCUSSION

In this study, we show that using IgE transgenic mice without antigen sensitization, IgE cross-linking by a relevant antigen directly induces $CD4^+$ T cell recruitment into the airways in a prostaglandin-dependent manner (Figs 1 and 3). We also show that, although IgE cross-linking alone does not induce eosinophil recruitment into the airways, IgE cross-linking significantly enhances Th2 cell-mediated eosinophil recruitment into the airways (Fig. 4). Therefore, these results indicate that IgE-dependent mast cell activation enhances Th2 cell-mediated allergic airway inflammation by recruiting Th2 cells into the airways.

In a previous study [22], we showed that mast cells in ear skin of TNP–IgE mice were heavily loaded with TNP-specific IgE as detected by immunohistochemical staining. In contrast, such IgE-loaded mast cells were undetectable in WT mice even though the comparable numbers of mast cells existed in ear skin of TNP–IgE mice and WT mice. We also found that the epicutaneous application of picryl chloride carrying a TNP group induced an immediate cutaneous reaction in TNP–IgE mice but not in WT mice. Moreover, using peritoneal mast cells, we found that IgE bound to FcεRI on c-kit^+ mast cells in TNP–IgE mice. Therefore, it is suggested that intranasal administration of TNP–BSA induces mast cell activation through the cross-linking of FcεRI in TNP–IgE mice. However, it is still possible that FcεRI on basophils and eosinophils [27,28] as well as other IgE receptors including CD23 and Fcγ receptors [29] may be involved in TNP–BSA-induced $CD4^+$ T cell recruitment in TNP–IgE mice.

We show that IgE cross-linking principally induces $CD4^+$ T cell recruitment into the airways and thus enhances Th2 cell-mediated eosinophilic airway inflammation by recruiting Th2 cells into the airways. This implies that both antigen-specific IgE antibody on mast cells and antigen-specific Th2 cells cooperate synergistically to induce antigen-induced eosinophilic airway inflammation in asthma. Our findings are consistent with the previous observations that using mast cell-deficient mice, the role of mast cells in antigen-induced eosinophil recruitment into the airways can be detected only in the situation in which mice were weakly sensitized and challenged with antigens and thereby subsequent Th2 cell-mediated eosinophil recruitment was modest [19].

We demonstrate that, however, IgE-dependent mast cell activation alone is not sufficient for the induction of eosinophil recruitment into the airways (Fig. 1) or the induction of AHR (Fig. 2). In contrast to the convincing function of IgE and mast cells in the early phase reaction [30], the roles of IgE in allergic airway inflammation and AHR in the late phase are still controversial. In the previous study using IgE-deficient mice, it was demonstrated that the features of asthma, including eosinophil infiltration into the airways and AHR in the late phase, can be elicited in the absence of IgE [14], suggesting that IgE is not essential for the induction of allergic airway inflammation. On the other hand, in a previous study with the mice sensitized passively with antigen-specific IgE followed by the corresponding antigen challenge, it has been reported that antigen-induced mast cell activation induces eosinophil recruitment into the airways and induces AHR [31]. Interestingly, in their study it was reported that the repeated antigen challenges are required for the induction of eosinophilic airway inflammation and AHR in the passively sensitized mice [31]. Therefore, it is possible that antigen-specific T cells may be activated during the period of antigen

challenges and that these activated T cells may contribute to the induction of eosinophilic airway inflammation and AHR. This notion is in agreement with our finding that IgE cross-linking by antigens significantly induces eosinophilic airway inflammation only when antigen-specific Th2 cells are activated simultaneously by antigens (Fig. 4).

We have also found that IgE-induced $CD4^+$ T cell recruitment into the airways is significantly decreased by a cyclooxygenase inhibitor acetylsalicylic acid (Fig. 3) but not by a cysteinyl leukotriene 1 receptor antagonist pranlukast (data not shown), suggesting that prostaglandins are involved in IgE-induced $CD4^+$ T cell accumulation in the airways. Moreover, our findings that $CD4^+$ T cells are accumulated preferentially into the airways (Fig. 1d) suggest that the IgE-induced $CD4^+$ T cell recruitment is not due simply to an increase of vascular permeability. In this regard, it has been shown that PGD_2 is the major cyclooxygenase metabolite produced by mast cells in response to antigen challenge [32]. In addition, the importance of PGD_2 in allergic airway inflammation has recently been demonstrated by using mice deficient in PGD_2 receptor, DP [33]. More recently, it has been shown that PGD_2 also induces chemotaxis of Th2 cells through a novel PGD_2 receptor, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) [26]. Therefore, it is suggested that PGD_2 may be involved in IgE-induced $CD4^+$ T cell recruitment into the airways.

On the other hand, because it has also been shown that thromboxanes are involved in the accumulation of lymphocytes in the airways of a guinea pig asthma model [34], other prostanoids such as thromboxanes might be involved in IgE-induced $CD4^+$ T cell recruitment in TNP–IgE mice. It is also possible that acetylsalicylic acid may directly decrease the $CD4^+$ T cell recruitment by inhibiting adhesion of T cells to the endothelium [35].

Although it has been shown that mast cell mediators induce short-term AHR [36], our results suggest that IgE cross-linking alone does not significantly induce persistent AHR. A previous study also showed that anti-IgE antibody treatment of sensitized mice prevented systemic anaphylactic reactions, but failed to affect the development of persistent AHR associated with airway inflammation [37]. On the other hand, some studies revealed that IgE and mast cells were necessary for AHR associated with airway inflammation 24 h after antigen challenge [18,19]. The differences in the role of mast cells in the development of AHR may be explained by the differences in the relative contribution to AHR of activated T cells and their cytokines such as IL-13 [2] and eosinophils [7,37]. In addition, in the cell transfer experiments, we found that WT and TNP–IgE mice that had received OVA-specific Th2 cells and subsequent inhaled OVA challenge showed no significant increase in airway reactivity to methacholine even after TNP–BSA administration. It is consistent with the previous findings that AHR associated with mild airway eosinophilia induced by passive sensitization with IgE or exclusive airway sensitization and challenges with antigens could be detected only by *in vitro* airway smooth muscle contraction to electrical field stimulation but not by *in vivo* hyperresponsiveness to inhaled methacholine [31,38].

In summary, we have shown that IgE cross-linking by antigens of mast cells induces $CD4^+$ T cell recruitment into the airways and consequently enhances Th2 cell-mediated eosinophil recruitment into the airways. Although the molecular mechanisms underlying this phenomenon remains to be determined, our results show a novel relationship between IgE-dependent mast cell activation and Th2 cell-mediated allergic inflammation in the late-phase allergic airway responses.

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Indispensable Role of Stat5a in Stat6-Independent Th2 Cell Differentiation and Allergic Airway Inflammation[1]

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It is well-recognized that Stat6 plays a critical role in Th2 cell differentiation and the induction of allergic inflammation. We have previously shown that Stat5a is also required for Th2 cell differentiation and allergic airway inflammation. However, it is the relative importance and redundancy of Stat6 and Stat5a in Th2 cell differentiation and allergic airway inflammation are unknown. In this study we addressed these issues by comparing Stat5a-deficient (Stat5a$^{-/-}$) mice, Stat6$^{-/-}$ mice, and Stat5a- and Stat6 double-deficient (Stat5a$^{-/-}$ Stat6$^{-/-}$) mice on the same genetic background. Th2 cell differentiation was severely decreased in Stat6$^{-/-}$CD4^{+} T cells, but Stat6-independent Th2 cell differentiation was still significantly observed in Stat6$^{-/-}$CD4^{+} T cells. However, even in the Th2-polarizing condition (IL-4 plus anti-IFN-γ mAb), no Th2 cells developed in Stat5a$^{-/-}$Stat6$^{-/-}$ CD4^{+} T cells. Moreover, Ag-induced eosinophil and lymphocyte recruitment in the airways was severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with that in Stat6$^{-/-}$ mice. These results indicate that Stat5a plays an indispensable role in Stat6-independent Th2 cell differentiation and subsequent Th2 cell-mediated allergic airway inflammation. *The Journal of Immunology,* **2005, 174: 3734–3740.**

Newly activated CD4^{+} T cells differentiate into at least two functionally distinct subsets, Th1 and Th2 cells, as defined by their patterns of cytokine production (1, 2). Th1 cells produce IFN-γ and lymphotoxin and are responsible for delayed-type hypersensitivity reactions, promoting control of intracellular pathogens (1, 2). Th2 cells produce IL-4, IL-5, and IL-13 and provide an excellent helper function for Ab production, particularly of IgE (1, 2). Th2 cells are essential for promoting host defense against helminths, but uncontrolled Th2 cell activation to noninvasive Ags (allergen) causes atopic disorders, including asthma (3, 4).

Over the last several years, significant progress has been made in the molecular mechanisms for Th2 cell differentiation (5–7). Although early studies have indicated that Stat6 (8–10), a cytosolic latent transcription factor that is rapidly activated after cellular exposure to IL-4 and IL-13, is essential for Th2 cell differentiation through the induction of GATA3 (5–7), recent studies have revealed that Stat6-deficient (Stat6$^{-/-}$) CD4^{+} T cells make a considerable amount of IL-4 upon stimulation with TCR (11). In addition, it has been demonstrated that Th2 cell-mediated allergic airway inflammation is still observed in Stat6$^{-/-}$ mice (12–15). Therefore, in addition to the Stat6-dependent pathway, the Stat6-independent pathway participates in in vitro Th2 cell differentiation as well as in vivo Th2 cell-mediated immune responses.

In contrast, we have shown that Ag-induced IL-5 production and eosinophil recruitment in the airways are decreased in Stat5a$^{-/-}$ mice (16). In addition, we have shown that Th cell differentiation in Stat5a$^{-/-}$ mice is biased toward the Th1 type at single cell levels and that retrovirus-mediated expression of Stat5a restores the impaired Th2 cell differentiation of Stat5a$^{-/-}$CD4^{+} T cells (17). Consistent with these findings, it has recently been shown that the enforced expression of a constitutively active form of Stat5a induces IL-4 production in CD4^{+} T cells by enhancing the accessibility of the IL-4 gene (18). These findings suggest that the intrinsic expression of Stat5a in CD4^{+} T cells plays an important role in Th2 cell differentiation and the induction of allergic airway inflammation. However, the relative importance and redundancy of Stat5a-mediated Th2 cell differentiation and Stat6-mediated Th2 cell differentiation are still unclear.

In the present study we addressed these issues by comparing Th2 cell differentiation in Stat5a$^{-/-}$ mice, Stat6$^{-/-}$ mice, and Stat5a and Stat6 double-deficient (Stat5a$^{-/-}$Stat6$^{-/-}$) mice in the same genetic background. We also examined allergic airway inflammation in these mice as a model of in vivo Th2 cell-mediated immune responses. We found that Th2 cell differentiation and allergic airway inflammation were severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with those in Stat5a$^{-/-}$ mice or Stat6$^{-/-}$ mice. Our results suggest that Stat5a is essential for Th2 cell differentiation in the absence of Stat6 activation and vice versa.

Materials and Methods

Mice

Stat5a-deficient (Stat5a$^{-/-}$) mice (19) and Stat6-deficient (Stat6$^{-/-}$) mice (8) were backcrossed to BALB/c mice (Charles River Laboratories) for eight generations. Stat5a$^{+/-}$Stat6$^{+/-}$ male mice were mated with Stat5a$^{+/-}$Stat6$^{+/-}$ female mice to obtain Stat5a$^{+/+}$Stat6$^{+/+}$ mice (wild-type (WT)[3] mice), Stat5a$^{-/-}$Stat6$^{+/+}$ mice (Stat5a$^{-/-}$ mice), Stat5a$^{+/+}$Stat6$^{-/-}$ mice (Stat6$^{-/-}$ mice), and Stat5a$^{-/-}$Stat6$^{-/-}$ mice within the litter. All experiments were performed according to the guidelines of Chiba University.

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[3] Abbreviations used in this paper: WT, wild type; BALF, bronchoalveolar lavage fluid; PAS, periodic acid-Schiff.

Flow cytometric analysis

Cells were stained and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software. The following Abs were purchased from BD Pharmingen: anti-CD4-FITC, -PE, -allophycocyanin, and -PerCP (H129.19); anti-CD8-FITC and -PE (53-6.7); anti-B220-allophycocyanin (RA3-6B2); anti-IgM-FITC (R6-60.2); anti-CD69-FITC (H1.3F3); anti-CD62L-FITC (MEL-14); anti-TCR Vβ8.1,2-FITC (MR5-2); and anti-pan-NK-PE (DX5). Before staining, FcRs were blocked with anti-CD16/32 Ab (2.4G2; BD Pharmingen). Negative controls consisted of isotype-matched, directly conjugated, nonspecific Abs (BD Pharmingen).

Cell culture

Splenocytes (2×10^6 cells/ml) from WT mice, $Stat5a^{-/-}$ mice, $Stat6^{-/-}$ mice, and $Stat5a^{-/-}Stat6^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb (mAb) (5 μg/ml; clone 145-2C11; BD Pharmingen) in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM L-glutamine, and antibiotics in a 24-well microtiter plate at 37°C for 48 h. Where indicated, IL-12 (15 ng/ml; PeproTech EC) was added to polarize toward Th1 cells (Th1 condition), and IL-4 (15 ng/ml; PeproTech EC) and anti-IFN-γ mAb (15 μg/ml; clone XMG1.2; BD Pharmingen) were added to polarize toward Th2 cells (Th2 condition) (17). Cells were washed with PBS, then cultured for another 3 days in Th0 (no exogenous cytokines), Th1, or Th2 conditions in the presence of IL-2 (20 U/ml; PeproTech).

Intracellular cytokine analysis

Intracellular cytokine staining for IL-4 vs IFN-γ was performed as described previously (17). In brief, cultured splenocytes were washed with PBS and restimulated with plate-bound anti-CD3 mAb at 37°C for 6 h, with monensin (2 μM) (Sigma-Aldrich) added for the final 4 h. After being stained with anti-CD4-PerCP, cells were fixed with IC FIX (BioSource International), permeabilized with IC PERM (BioSource International), and stained with anti-IL-4-PE (BVD4-1D11; BD Pharmingen) and anti-IFN-γ-allophycocyanin (XMG1.2; BD Pharmingen) for 30 min at 4°C. The cytokine profile (IL-4 vs IFN-γ) of $CD4^+$ cells was analyzed on a FACSCalibur using CellQuest software.

Ag-induced allergic inflammation in the airways

Allergic airway inflammation was induced by the inhalation of OVA (Sigma-Aldrich) in sensitized mice as described previously (20). Briefly, mice (aged 7–8 wk) were immunized i.p. twice with 4 μg of OVA in 4 mg of aluminum hydroxide at a 2-wk interval. Twelve to 14 days after the second immunization, the sensitized mice were given aerosolized OVA (50 mg/ml) dissolved in 0.9% saline by a DeVilbiss 646 nebulizer three times, for 20 min each time, at 24-h intervals. As a control, 0.9% saline alone was administered by the nebulizer. Forty-eight hours after the last inhalation, trachea and lung were excised, fixed in 10% buffered-formalin, and embedded in paraffin. The specimens (3 μm thick) of the trachea were stained with Luna and H&E solutions. The number of eosinophils in the submucosal tissue of trachea was counted in Luna-stained sections and expressed as the number of eosinophils per length of the basement membrane of trachea, which was measured with a digital curvimeter.

Lung sections were stained with H&E and periodic acid-Schiff (PAS) according to standard protocols. The magnitude of inflammatory cell infiltration in the perivascular and peribronchial spaces on H&E-stained lung sections was evaluated by a semiquantitative scoring system as described previously (21): +5 signified a large (more than three cells deep) widespread infiltrate around the majority of vessels and bronchioles, and +1 signified a small number of inflammatory foci. The H&E-stained sections were coded and then examined by two observers in a blind manner, the sum of the scores from each lung was divided by the number of airways examined for the score, and the average of the two determinations for each section was used for subsequent calculations. PAS-stained lung sections were also categorized according to the abundance of PAS^+ goblet cells and assigned numerical scores as described previously (22): 0, <5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%.

The numbers of eosinophils, lymphocytes, and macrophages recovered in the bronchoalveolar lavage fluid (BALF) were evaluated as described previously (16). In short, after bronchoalveolar lavage was performed with 2 ml of PBS, BALF was centrifuged at 400 × g for 5 min at 4°C, and differential cell counts were performed on cytospin cell preparations stained with Wright-Giemsa solution.

ELISA

Cultured splenocytes were washed with PBS and restimulated with plate-bound anti-CD3 mAb at 37°C for 12 h. The amounts of IL-4, IL-5, IL-10, and IFN-γ in the culture supernatant were measured by enzyme immunoassay using murine IL-4, IL-5, IL-10, and IFN-γ ELISA kits (BD Pharmingen). The amount of IL-13 in the culture supernatant was measured using an ELISA kit from R&D Systems. The assays were performed in duplicate according to the manufacturer's instructions. The minimum significant values of these assays were 15 pg/ml IL-4 and IL-5 and 30 pg/ml IFN-γ, IL-10, and IL-13.

Data analysis

Data are summarized as the mean ± SD. The statistical analysis of the results was performed by unpaired *t* test. A value of $p < 0.05$ was considered significant.

Results

Normal $CD4^+$ T cell development in $Stat5a^{-/-}Stat6^{-/-}$ mice

It has been shown that not only Stat6 (8–10), but also Stat5a (16–18), play critical roles in Th2 cell differentiation. To investigate the relative importance of Stat5a- and Stat6-mediated signaling in Th2 cell differentiation in detail, we generated $Stat5a^{-/-}$ mice, $Stat6^{-/-}$ mice, and $Stat5a^{-/-}Stat6^{-/-}$ mice on the same genetic background and compared the development and differentiation of $CD4^+$ T cells among these mice. Consistent with the previous reports (16, 23), the number of splenocytes in $Stat5a^{-/-}$ mice was modestly, but significantly, decreased compared with that in WT mice (Fig. 1*A*). The number of splenocytes in $Stat5a^{-/-}Stat6^{-/-}$ mice was also decreased compared with that in $Stat6^{-/-}$ mice (Fig. 1*A*). However, FACS analysis revealed that the frequencies of $CD4^+$ T cells and $CD8^+$ T cells were similar among WT, $Stat5a^{-/-}$, $Stat6^{-/-}$, and $Stat5a^{-/-}Stat6^{-/-}$ mice (Fig. 1*B*). The expression of CD69 and CD62L on $CD4^+$ T cells was also similar among these mice (data not shown). Based on B220 vs IgM staining, B cells in the spleen exhibited normal maturation in these mice (Fig. 1*B*). These results indicate that T and B cells can develop even in the absence of Stat5a and Stat6.

Stat6-independent Th2 cell differentiation depends on Stat5a

We then examined cytokine production from WT, $Stat5a^{-/-}$, $Stat6^{-/-}$, and $Stat5a^{-/-}Stat6^{-/-}$ T cells. Splenocytes were stimulated with plate-bound anti-CD3 mAb in Th0 (no exogenous cytokines), Th1 (in the presence of IL-12), or Th2 (in the presence of

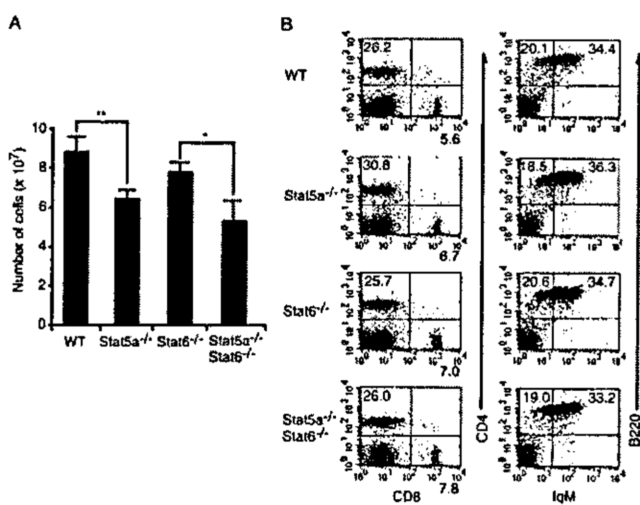

FIGURE 1. Normal T cell and B cell development in $Stat5a^{-/-}Stat6^{-/-}$ mice. *A*, Number of splenocytes in WT, $Stat5a^{-/-}$, $Stat6^{-/-}$, and $Stat5a^{-/-}$-$Stat6^{-/-}$ mice. Data are the mean ± SD from eight mice for each genotype. *, $p < 0.05$; **, $p < 0.01$. *B*, Flow cytometric analysis of splenocytes from 6-wk-old mice. Cells were stained with anti-CD4-PE vs anti-CD8-FITC or anti-B220-allophycocyanin vs anti-IgM-FITC. Shown are representative FACS profiles from five mice in each group.

IL-4 and anti-IFN-γ mAb) conditions for 2 days, then cultured for another 3 days in Th0, Th1, or Th2 conditions in the presence of IL-2. After washing, cells were restimulated with plate-bound anti-CD3 mAb for 12 h, and the amounts of IL-4, IL-5, IL-10, IL-13, and IFN-γ in the culture supernatant were determined. In the Th0 condition, IL-4 and IL-5 production was significantly decreased in Stat5a$^{-/-}$ splenocytes compared with that in WT splenocytes (Fig. 2), consistent with our previous report (17). IL-4 and IL-5 production was more severely decreased in Stat6$^{-/-}$ splenocytes (Fig. 2). However, significant IL-4 and IL-5 production was still detected in Stat6$^{-/-}$ splenocytes (Fig. 2). In contrast, almost no IL-4 or IL-5 was detected in Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes in the Th0 condition (Fig. 2). Furthermore, even when Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes were stimulated with anti-CD3 Ab in Th2 condition, they did not significantly produce IL-4 and IL-5 ($n = 5$; $p < 0.01$; Fig. 2). Similarly, IL-10 and IL-13 production was significantly decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes compared with that in Stat5a$^{-/-}$ or Stat6$^{-/-}$ splenocytes in the Th2 condition (Fig. 2). By contrast, IFN-γ production did not change in Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes in the Th0 condition and, instead, was increased in the Th1 condition compared with that in WT splenocytes or Stat6$^{-/-}$ splenocytes ($n = 5$; $p < 0.01$; Fig. 2). In contrast, no significant differences were observed in the proliferative responses of T cells among these mice in Th0, Th1, and Th2 conditions (data not shown), suggesting that the impaired Th2 cytokine production in Stat5a$^{-/-}$Stat6$^{-/-}$ splenocytes does not result from possible defects in cell proliferation.

Next, we examined Th1/Th2 cell differentiation at single-cell levels (Fig. 3). Splenocytes were stimulated with plate-bound anti-CD3 mAb in Th0, Th1, or Th2 conditions, and the cytokine profile (IL-4 vs IFN-γ) of CD4$^+$ T cells was evaluated by intracellular cytokine analysis. In the Th0 condition, CD4$^+$ T cells that produced IL-4, but not IFN-γ, were significantly decreased in Stat5a$^{-/-}$ mice compared with those in WT mice (Fig. 3, *a* vs *b*). IL-4-producing CD4$^+$ cells were more severely decreased in Stat6$^{-/-}$ mice but IL-4-producing CD4$^+$ cells still developed in

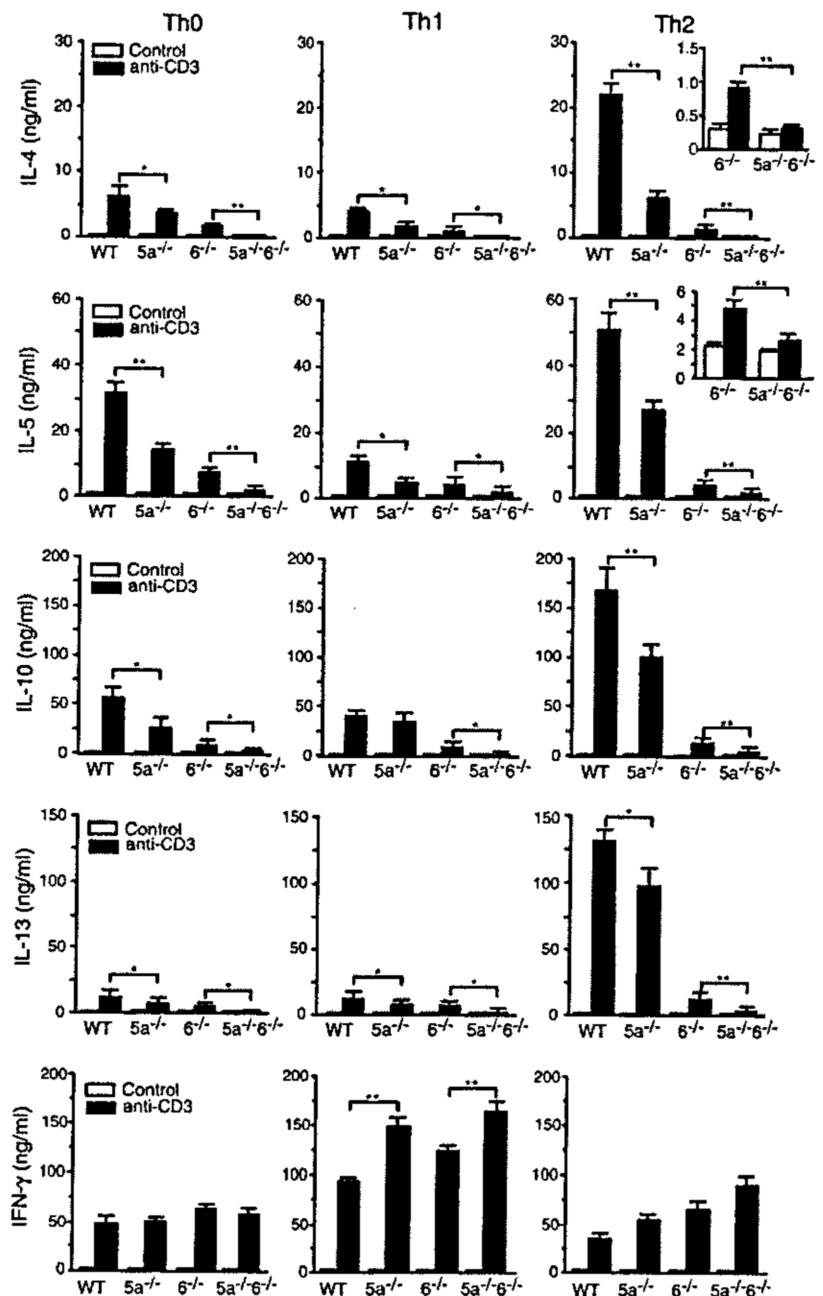

FIGURE 2. Th2 cytokine production is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. Splenocytes from WT, Stat5a$^{-/-}$ (5a$^{-/-}$), Stat6$^{-/-}$ (6$^{-/-}$), or Stat5a$^{-/-}$Stat6$^{-/-}$ (5a$^{-/-}$ 6$^{-/-}$) mice were stimulated with plate-bound anti-CD3 mAb in the nonpolarizing Th0 condition (no exogenous cytokines), the Th1 condition (in the presence of IL-12), or the Th2 condition (in the presence of IL-4 and anti-IFN-γ mAb) for 48 h, then cultured for another 72 h in Th0, Th1, or Th2 conditions in the presence of IL-2. After washing, cells (1×10^6/ml) were restimulated with plate-bound anti-CD3 mAb for 12 h in the absence of exogenous cytokines. The amounts of IL-4, IL-5, IL-10, IL-13, and IFN-γ in the culture supernatant were determined by ELISA. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$.

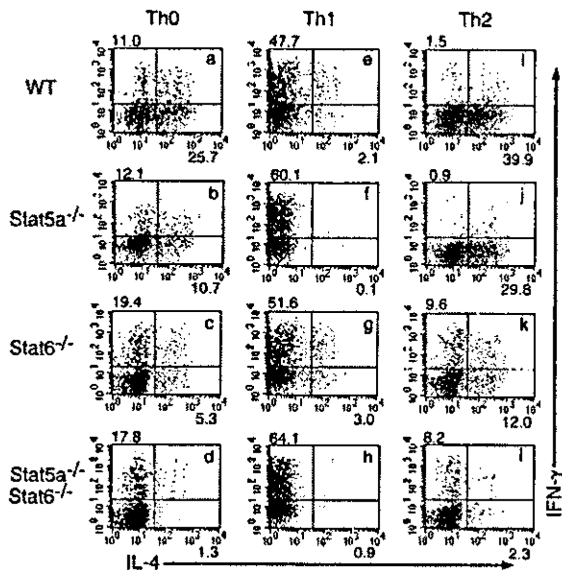

FIGURE 3. Th2 cell differentiation is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. Splenocytes from WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, or Stat5a$^{-/-}$Stat6$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th0, Th1, or Th2 conditions and cultured for another 72 h in Th0, Th1, or Th2 conditions in the presence of IL-2. Cells were washed and restimulated with plate-bound anti-CD3 mAb for 6 h. Intracellular cytokine profiles for IL-4 vs IFN-γ were determined on CD4^{+} T cells. Shown are representative FACS profiles from five mice in each group.

Stat6$^{-/-}$ mice (Fig. 3*c*). Consistent with a previous report (11), IL-4-producing CD4^{+} cells in Stat6$^{-/-}$ mice lacked the expression of DX5, and the frequency of TCR Vβ8^{+} cells was not significantly increased in these cells (data not shown), suggesting that the majority of IL-4-producing CD4^{+} cells in Stat6$^{-/-}$ mice were conventional Th2 cells, but not NK T cells. Importantly, Th2 cells were hardly detected in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 3*d*). The frequency of Th2 cells in the Th0 condition was as follows: WT mice, 24.7 ± 3.4%; Stat5a$^{-/-}$ mice, 10.2 ± 2.6%; Stat6$^{-/-}$ mice, 5.5 ± 1.1%; and Stat5a$^{-/-}$Stat6$^{-/-}$ mice, 1.2 ± 0.3% (mean ± SD; n = 5 experiments in each group).

When splenocytes were cultured in Th2-polarizing conditions, the frequency of Th2 cells increased in Stat5a$^{-/-}$ mice and Stat6$^{-/-}$ mice, although the frequency of Th2 cells was still significantly lower in Stat5a$^{-/-}$ and Stat6$^{-/-}$ mice than that in WT mice (Fig. 3). However, even in the Th2 condition, the frequency of Th2 cells did not significantly increase in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 3*l*). These results indicate that Stat5a is essential for Stat6-independent Th2 cell differentiation and vice versa.

In contrast, in the Th1 condition, CD4^{+} T cells that produced IFN-γ, but not IL-4 (Th1 cells), were significantly increased in Stat5a$^{-/-}$ and Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with those in WT and Stat6$^{-/-}$ mice, respectively (WT mice, 44.9 ± 8.2%; Stat5a$^{-/-}$ mice, 62.3 ± 11.9% ($p < 0.05$); Stat6$^{-/-}$ mice, 50.8 ± 12.9%; Stat5a$^{-/-}$Stat6$^{-/-}$ mice, 66.4 ± 12.3% ($p < 0.05$); $n = 5$; Fig. 3). In contrast, in the Th0 or Th2 condition, Th1 cells were significantly increased in Stat6$^{-/-}$ and Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with those in WT mice and Stat5a$^{-/-}$ mice, respectively (Fig. 3). These results suggest that Stat5a and Stat6 are differently involved in the suppression of Th1 cell differentiation, depending on the cytokine environment.

Interestingly, CD4^{+} T cells that produced both IFN-γ and IL-4 tended to be increased in Stat6$^{-/-}$ mice, but not in Stat5a$^{-/-}$ mice (Fig. 3). These results suggest that Stat6 may also play a role in the suppression of IFN-γ production in developing Th2 cells; this idea is consistent with the previous finding that Stat6 induces the expression of GATA3 (24), a master regulator of Th2 cells that induces Th2 cytokine production and inhibits IFN-γ production in T cells (5–7).

Stat5a-dependent, Stat6-independent Th2 cell differentiation participates in Ag-induced eosinophil and lymphocyte recruitment into the airways

To clarify the in vivo role of Stat5a-dependent, Stat6-independent Th2 cell differentiation, we examined Ag-induced airway inflammation as a model of Th2 cell-mediated in vivo immune responses. Stat5a$^{-/-}$, Stat6$^{-/-}$, Stat5a$^{-/-}$Stat6$^{-/-}$, and control WT mice were immunized twice with OVA; 2 wk later, these mice were challenged with aerosolized OVA three times at 24-h intervals. Forty-eight hours after the last Ag challenge, airway inflammation

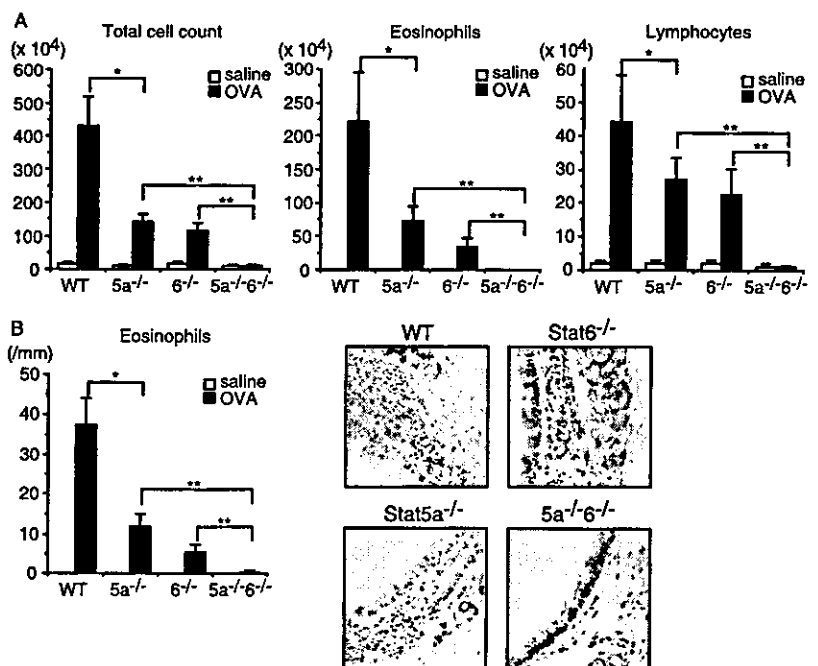

FIGURE 4. Ag-induced eosinophil and lymphocyte recruitment into the airways is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. *A*, OVA-sensitized Stat5a$^{-/-}$, Stat6$^{-/-}$, Stat5a$^{-/-}$Stat6$^{-/-}$, and littermate WT mice were challenged with the inhalation of OVA or saline (as a control) three times at 24-h intervals. The numbers of total cells, eosinophils, and lymphocytes in BALF were evaluated 48 h after the last inhalation. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$. *B*, Similar to *A*, OVA-sensitized mice were challenged with inhaled OVA or saline, and the number of eosinophils infiltrating the submucosal tissue of trachea was evaluated 48 h after the last inhalation. Data are the mean ± SD for five mice in each group. *, $p < 0.05$; **, $p < 0.01$. Representative photomicrographs of trachea sections stained with Luna solution are also shown (×100).

was evaluated (Fig. 4). Consistent with the previous studies (12–16), the number of eosinophils recovered in BALF 48 h after the last Ag challenge was significantly diminished in Stat5a$^{-/-}$ mice as well as in Stat6$^{-/-}$ mice compared with that in WT mice (Fig. 4*A*). However, the eosinophil recruitment in BALF was still observed to a considerable extent in both Stat5a$^{-/-}$ and Stat6$^{-/-}$ mice (Fig. 4*A*). In contrast, Ag inhalation induced no significant eosinophil recruitment in BALF in sensitized Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 4*A*). The number of eosinophils in BALF 48 h after the last Ag inhalation was as follows: WT mice, 222.2 ± 75.6; Stat5a$^{-/-}$ mice, 71.2 ± 22.7; Stat6$^{-/-}$ mice, 34.8 ± 13.1; and Stat5a$^{-/-}$Stat6$^{-/-}$ mice, 0.2 ± 0.2 × 10^4/mice (n = 5 mice in each group; Fig. 4*A*). Ag-induced eosinophil recruitment in BALF was not observed in Stat5a$^{-/-}$Stat6$^{-/-}$ mice even 96 h after the last Ag inhalation (data not shown). The number of eosinophils infiltrating the submucosal tissue of the trachea 48 h after Ag inhalation was also severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with that in Stat5a$^{-/-}$ or Stat6$^{-/-}$ mice ($n = 5$; $p < 0.01$; Fig. 4*B*).

Ag-induced lymphocyte recruitment in BALF was also significantly decreased in Stat5a$^{-/-}$ and Stat6$^{-/-}$ mice ($n = 5$; $p < 0.05$; Fig. 4*A*). Furthermore, virtually no Ag-induced lymphocyte recruitment in BALF was observed in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (n = 5; $p < 0.01$; Fig. 4*A*). Consistent with these data obtained from BALF analysis (Fig. 4*A*), histological analysis showed that inflammatory cell infiltration in the lung after Ag inhalation was significantly decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with Stat5a$^{-/-}$ or Stat6$^{-/-}$ mice ($n = 5$; $p < 0.01$; Fig. 5*A*). In contrast, Ag-induced epithelial goblet cell hyperplasia was severely decreased not only in Stat5a$^{-/-}$Stat6$^{-/-}$ mice; but also in Stat6$^{-/-}$ mice, indicating that Stat6 is absolutely required for Ag-induced epithelial goblet cell hyperplasia (n = 5; Fig. 5, *B* and *C*). Taken together, these results suggest that the Stat5a-dependent, Stat6-independent pathway is involved in in vivo Th2 cell differentiation and subsequent allergic airway inflammation, but not in the induction of epithelial goblet cell hyperplasia.

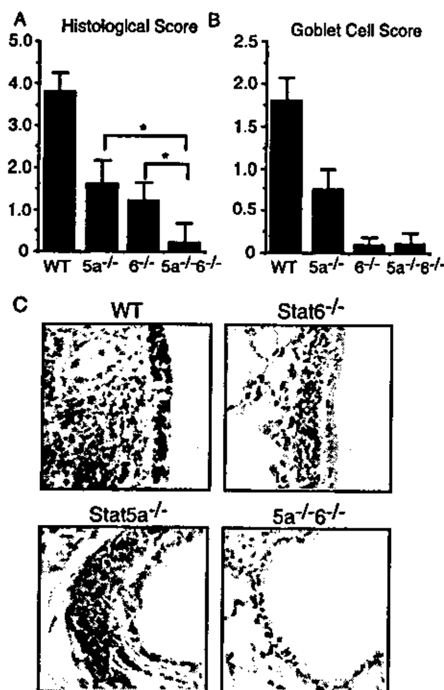

FIGURE 5. The Stat5a-dependent, Stat6-independent pathway induces airway inflammation, but not epithelial goblet cell hyperplasia. OVA-sensitized WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice were challenged with inhaled OVA three times at 24-h intervals. *A*, Forty-eight hours after the last OVA inhalation, lung was removed, and inflammatory cell infiltration into the perivascular and peribronchial spaces was scored as described previously (21). *B*, The degree of goblet cell hyperplasia was scored on PAS-stained sections as described previously (22). Data are the mean ± SD for five mice in each group. *, $p < 0.01$. *C*, Representative photomicrographs of PAS-stained lung sections from these mice are also shown (×100).

Discussion

In this study we show that Stat5a plays an indispensable role in Stat6-independent Th2 cell differentiation and subsequent allergic airway inflammation. We found that Th2 cell differentiation was severely decreased in Stat6$^{-/-}$ CD4$^+$ T cells, but that Stat6-independent Th2 cell differentiation was still observed in Stat6$^{-/-}$ CD4$^+$ T cells (Figs. 2 and 3). However, even in the Th2-polarizing condition, Th2 cells did not significantly develop in Stat5a$^{-/-}$Stat6$^{-/-}$ CD4$^+$ T cells (Figs. 2 and 3), suggesting that the residual Th2 cell differentiation in Stat6$^{-/-}$ CD4$^+$ T cells depends on Stat5a. We also found that Ag-induced eosinophil and lymphocyte recruitment in the airways was severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice compared with that in Stat6$^{-/-}$ mice (Fig. 4). Taken together, our results suggest that the Stat5a-dependent, Stat6-independent pathway participates not only in in vitro Th2 cell differentiation, but also in in vivo Th2 cell-mediated allergic airway inflammation.

We show that Stat6 is not necessarily required for Stat5a-mediated Th2 cell differentiation. We found that the impairment of Th2 cell differentiation was more severe in Stat5a$^{-/-}$Stat6$^{-/-}$ CD4$^+$ T cells than that in Stat6$^{-/-}$ CD4$^+$ T cells (Fig. 3), indicating that Stat5a can induce Th2 cell differentiation even in the absence of Stat6 activation. This observation is consistent with a recent finding by Zhu et al. (18) demonstrating that the enforced expression of a constitutively active form of Stat5a induces IL-4 production even in Stat6$^{-/-}$ CD4$^+$ T cells. Because the induction of IL-4R α-chain expression requires IL-4/Stat6-mediated signaling (8–10, 25), it is possible that the Stat5a-dependent pathway plays a role in the initiation of Th2 cell differentiation before developing Th2 cells begin to up-regulate IL-4R α-chain to increase the sensitivity to IL-4/Stat6-mediated signaling. It is also possible that the Stat5a-dependent pathway may function as an amplifier of IL-4/Stat6-mediated Th2 cell differentiation.

Regarding the molecular mechanisms of Stat5a-mediated Th2 cell differentiation, it has recently been shown that activated Stat5a directly interacts with HSII and HSIII sites of the IL-4 gene and then up-regulates the accessibility of the IL-4 gene (18). These results suggest that Stat5a functions as a direct inducer of IL-4 production. In contrast, we found that the enhanced Th1 cell differentiation was responsible in part for the impaired Th2 cell differentiation in Stat5a$^{-/-}$ CD4$^+$ T cells.[4] We also found that the expression pattern of SOCS family proteins was different between WT CD4$^+$ T cells and Stat5a$^{-/-}$ CD4$^+$ T cells (see Footnote 4). Because accumulating evidence suggests that some of SOCS family proteins are involved in cross-regulation of the cytokine network and then regulate Th1 and Th2 cell differentiation (26, 27), the different expression of SOCS family proteins in Stat5a$^{-/-}$ CD4$^+$ T cells may also be involved in the regulation of Th1/Th2 balance.

[4] H. Takatori, H. Nakajima, S. Kagami, K. Hirose, A. Suto, K. Suzuki, M. Kubo, A. Yoshimura, Y. Saito, and I. Iwamoto. Stat5a inhibits IL-12-induced Th1 cell differentiation through the induction of SOCS3 expression. *Submitted for publication.*

We also demonstrate that Stat5a, independently of Stat6, contributes to the induction of Th2 cell-mediated allergic airway inflammation. It has been shown that Ag-induced eosinophil and lymphocyte recruitment in the airways is mediated by Th2 cells secreting IL-5 (20, 28) and IL-4 (29, 30), respectively. Although it is apparent that Stat6 plays an important role in causing allergic airway inflammation (31), it has been demonstrated that in vivo Th2 cell differentiation and allergic airway inflammation are still substantial in Stat6$^{-/-}$ mice (12–15), suggesting that a Stat6-independent mechanism is involved in the development of allergic airway inflammation. In the present study we found that the residual Th2 cell-mediated allergic airway inflammation in Stat6$^{-/-}$ mice was abrogated by the additional deletion of the Stat5a gene (Fig. 4). Therefore, in addition to the Stat6-dependent pathway, the Stat5a-dependent, Stat6-independent pathway participates in in vivo Th2 cell-mediated immune responses such as allergic airway inflammation.

It is still uncertain which cytokine is upstream of Stat5a-mediated Th cell differentiation. A number of immunologically important cytokines, including IL-2, IL-7, and IL-15, have been shown to activate Stat5a in many cell types (32). IL-4 has also been reported to activate Stat5 in some circumstances (33, 34), but we have previously shown that IL-4 does not phosphorylate Stat5a in CD4$^+$ T cells (17). Therefore, it is unlikely that IL-4 is an upstream cytokine for Stat5a-mediated Th2 cell differentiation. In contrast, it has recently been shown that developing Th2 cells express higher levels of IL-2R α-chain and exhibit stronger Stat5 activation than developing Th1 cells (35). This is consistent with a previous finding that Stat5a functions as an enhancer of IL-2 signaling by inducing the expression of IL-2R α-chain (23). Moreover, it has been demonstrated that Th2 cell differentiation is decreased by the neutralization of IL-2 or the blocking of IL-2R (18, 35, 36). Furthermore, it has been demonstrated that IL-2, but not IL-4, IL-9, IL-15, or IL-21, induces Stat5 phosphorylation and IL-4 production in activated CD4$^+$ T cells (37). Therefore, IL-2 is likely to be a cytokine responsible for Stat5a activation during Th2 cell differentiation.

Given that Stat5b is highly homologous to Stat5a (32) and that Stat5a/Stat5b double-deficient mice exhibit a severe defect in T cell responses compared with Stat5a$^{-/-}$ or Stat5b$^{-/-}$ mice (38), it is apparent that Stat5a and Stat5b have overlapping functions. However, the different phenotypes of Stat5a$^{-/-}$ and Stat5b$^{-/-}$ mice underscore the distinctive roles of Stat5a and Stat5b (17, 23, 39). For example, it has been demonstrated that although Stat5a$^{-/-}$ T cells exhibit no detectable defect in anti-CD3-induced proliferation, Stat5b$^{-/-}$ T cells are defective in anti-CD3-induced proliferation (17, 23, 39). These observations suggest that Stat5b is likely to play a role in the proliferation and/or survival of activated T cells, and that this function of Stat5b may not be shared with Stat5a.

Regarding Th cell differentiation, we have previously shown that both Th1 and Th2 cells are decreased in Stat5b$^{-/-}$ mice, whereas Th2, but not Th1, cells are decreased in Stat5a$^{-/-}$ mice (16). Nevertheless, because the number of CD4$^+$ T cells recovered from the culture was significantly lower in Stat5b$^{-/-}$ mice than in Stat5a$^{-/-}$ or WT mice (17), these data on Th cell differentiation in Stat5b$^{-/-}$ mice might be inconclusive. However, our finding that Th2 cells cannot develop in Stat5a$^{-/-}$Stat6$^{-/-}$ mice (Fig. 3) suggests that Stat5b cannot compensate for the role of Stat5a in Stat6-independent Th2 cell differentiation, because Stat5b can be normally expressed and activated in response to IL-2 even in the absence of Stat5a (23, 39).

In conclusion, we have shown that Stat5a activation is required for proper Th2 cell differentiation, and that Stat5a plays an indispensable role in Th2 cell differentiation in the absence of Stat6 activation. Although additional studies are required for complete understanding of the molecular mechanisms of Stat5a-mediated Th2 cell differentiation, our findings provide new insight into the mechanism of Stat6-independent Th2 cell differentiation and allergic airway inflammation.

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Disclosures

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Stat5a Inhibits IL-12-Induced Th1 Cell Differentiation through the Induction of Suppressor of Cytokine Signaling 3 Expression[1]

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In previous studies, we have shown that Th2 cell differentiation is diminished but Th1 cell differentiation is increased in Stat5a-deficient (Stat5a$^{-/-}$) CD4^{+} T cells. In the present study, we clarified the molecular mechanisms of Stat5a-mediated Th cell differentiation. We found that enhanced Th1 cell differentiation and the resultant IFN-γ production played a dominant inhibitory role in the down-regulation of IL-4-induced Th2 cell differentiation of Stat5a$^{-/-}$ CD4^{+} T cells. We also found that IL-12-induced Stat4 phosphorylation and Th1 cell differentiation were augmented in Stat5a$^{-/-}$ CD4^{+} T cells. Importantly, the expression of suppressor of cytokine signaling (SOCS)3, a potent inhibitor of IL-12-induced Stat4 activation, was decreased in Stat5a$^{-/-}$ CD4^{+} T cells. Moreover, a reporter assay showed that a constitutively active form of Stat5a but not Stat6 activated the SOCS3 promoter. Furthermore, chromatin immunoprecipitation assays revealed that Stat5a binds to the SOCS3 promoter in CD4^{+} T cells. Finally, the retrovirus-mediated expression of SOCS3 restored the impaired Th cell differentiation of Stat5a$^{-/-}$ CD4^{+} T cells. These results suggest that Stat5a forces the Th1/Th2 balance toward a Th2-type by preventing IL-12-induced Th1 cell differentiation through the induction of SOCS3. *The Journal of Immunology,* **2005, 174: 4105–4112.**

Over the last several years, significant progress has been made in the regulatory mechanisms of the transition of naive CD4^{+} T cells into mature Th2 cells (1–3). Whereas early studies have demonstrated that Th2 cell differentiation is essentially a Stat6-dependent process (4–6), recent studies have revealed that Stat6-independent pathways also participate not only in in vitro Th2 cell differentiation (7) but also in in vivo Th2 cell-mediated allergic airway inflammation (8, 9). Because the presence of IL-4-producing cells during T cell activation induces subsequent Stat6-dependent Th2 cell differentiation (1–3), it is inferred that Stat6-independent IL-4 production enhances the Stat6-dependent process of Th2 cell differentiation.

Regarding the Stat6-independent pathway, recent studies including ours indicate that Stat5a is involved in Th2 cell differentiation. We have previously shown that Ag-induced Th2 cytokine production and subsequent allergic airway inflammation are decreased in Stat5a-deficient (Stat5a$^{-/-}$) mice (10). We have also shown that Th cell differentiation is biased toward a Th1-type at single cell levels in Stat5a$^{-/-}$ CD4^{+} T cells (11) and that the retrovirus-mediated expression of Stat5a restores the impaired Th2 cell differentiation of Stat5a$^{-/-}$ CD4^{+} T cells (11). Moreover, it has recently been demonstrated that the enforced expression of a constitutively active form of Stat5a induces IL-4 production in CD4^{+} T cells by regulating the accessibility of the IL-4 gene (12). These results suggest that the intrinsic expression of Stat5a within CD4^{+} T cells plays a critical role in Th2 cell differentiation and in the induction of allergic airway inflammation and that Stat5a may function as a direct inducer of IL-4 production. In addition, we have found that, by comparing Stat6$^{-/-}$ mice to Stat5a- and Stat6-double deficient mice, Stat5a is indispensable in Stat6-independent Th2 cell differentiation of Stat6$^{-/-}$ CD4^{+} T cells (44). However, the molecular mechanisms underlying Stat5a-mediated Th cell differentiation are still largely unknown.

In the present study, we determined the molecular mechanisms underlying Stat5a-mediated Th cell differentiation. First, we found that IL-12-induced Stat4 phosphorylation and Th1 cell differentiation were enhanced in Stat5a$^{-/-}$ CD4^{+} T cells. Second, we found that the expression of suppressor of cytokine signaling (SOCS)[4] 3, a potent inhibitor of IL-12/Stat4 signaling (13, 14), was decreased in Stat5a$^{-/-}$ CD4^{+} T cells. Third, we found that Stat5a bound to SOCS3 promoter in CD4^{+} T cells and directly induced SOCS3 expression. Finally, we found that the retrovirus-mediated expression of SOCS3 restored the Th1/Th2 balance of Stat5a$^{-/-}$ CD4^{+} T cells. Taken together, our results indicate that Stat5a induces SOCS3 expression in CD4^{+} T cells and thus inhibits IL-12-induced Th1 cell differentiation, forcing the Th1/Th2 balance toward a Th2-type.

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[4] Abbreviations used in this paper: SOCS, suppressor of cytokine signaling; WT, wild type; ChIP, chromatin immunoprecipitation; LUC, luciferase; MGF, mammary gland factor.

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