Apr;7(4):525-532.
25. Skokos D, Le Panse S, Villa I, et al.. Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. J Immunol. 2001;166(2):868-876.
26.Reddy MM, Weissman AM, Mazza DS, et al. Circulating elevated levels of soluble CD23, interleukin-4, and CD20+CD23+ lymphocytes in atopic subjects with elevated serum IgE concentrations.Ann Allergy. 1992;69(2):131-134.

FIGURE LEGENDS

Figure 1

Fig 1: Representative dot plots for FITC-conjugated anti-CD19 and RPE-conjugated anti-CD23 antibodies for the control group (a) and the AR group (b). The proportion of CD23-bearing B cells was measured by flow cytometry as CD19 and CD23 double-positive plots.

Figure 2

Fig 2: CD23 expression on B cells. (a) % positive values for CD23 on B cells from patients with AR were significantly higher than for the control group. (b) A similar tendency was seen in the MFI of CD23 expression on B cells, which was significantly increased for patients with AR, compared with controls. (c) Correlation plot between % positive values for CD 23 on B cells and MFI of CD23 expression on B cells. (d) The % positive values for CD 23 on B cells was significantly correlated with the MFI of CD23 expression on B cells for the control group (closed circles) and the AR group (open circles)

Figure 3

Fig 3: % positive values for chemokine receptor expression on mucosal CD4 T cells. Data for the CXCR3 subtype, assumed to be Th1 cells, are shown in (a), and those for the CCR4 subtype, assumed to be Th2 cells, are shown in (b). There is no significant difference in % positive values for CXCR3 on CD4 cells from patients with AR and controls (a), while the % positive values for CCR4 on mucosal CD4 cells from patients with AR were significantly higher than in controls.

Figure 4

Fig 4: Correlation plot for % positive values for CD23 on mucosal B cells and % positive values for CCR4 on mucosal CD4 cells. There is no significant correlation between these data in the control group (closed circles) or the AR group (open circles)

Figure 5

Fig 5: Correlation plot for % positive values for CD23 on mucosal B cells and the total IgE level. There is no significant correlation between these data.

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Airway Dilatation after Inhalation of Short-acting β-agonist

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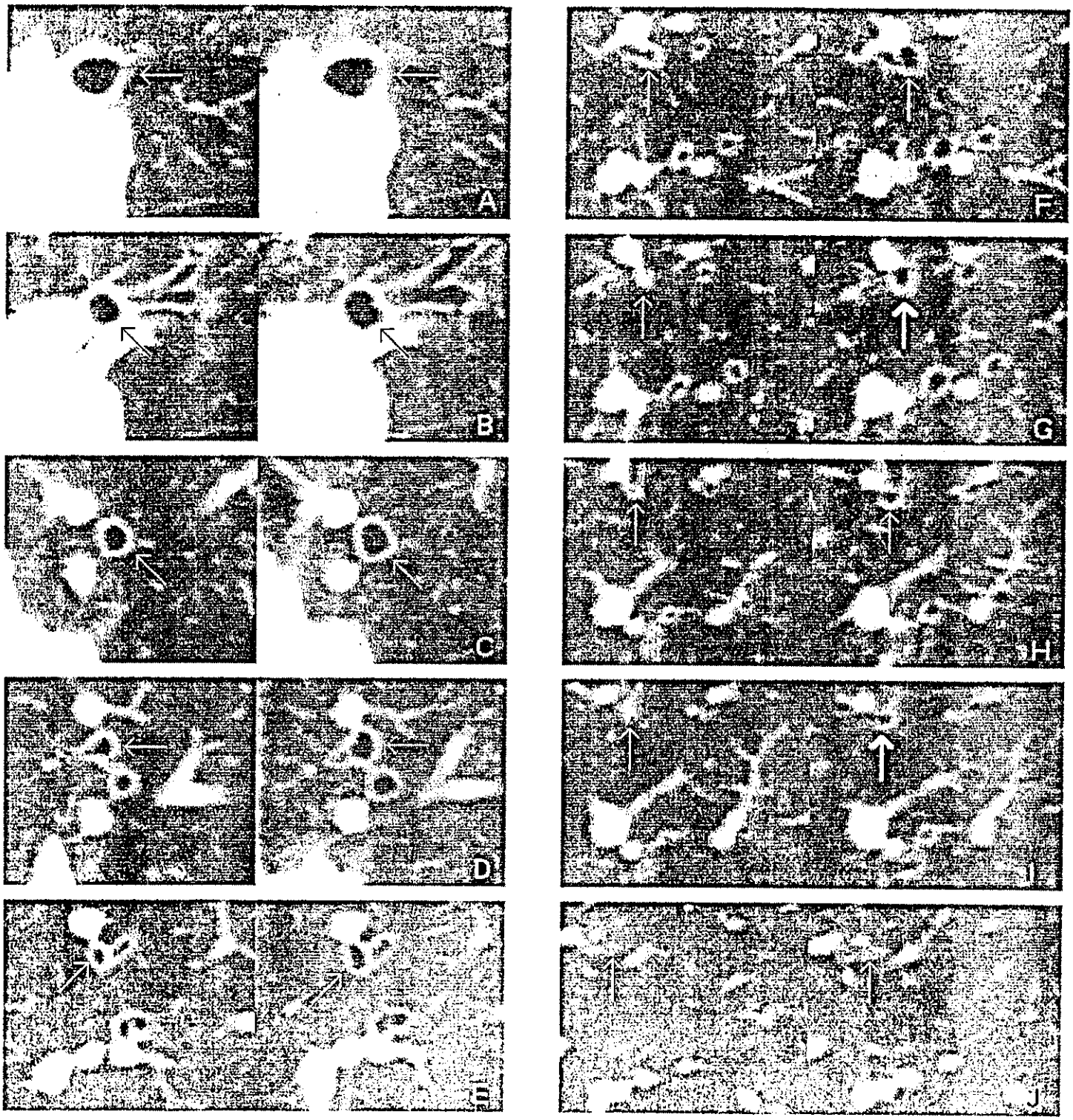
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Legend for the figure:

A 37-year-old female with bronchial asthma of 25-year history underwent high-resolution multi-slice helical computed tomographic scan for the morphological evaluation. A forced expiratory volume in one second of 1.27 liter (49.8 percent of the predicted value) was increased by 0.49 liter after the inhalation of short-acting ß-agonist, salbutamol. Cross-sectional multiplanar reconstruction obtained in the right upper lobe revealed an increase in the dimension of airway calibers. Each panel has two images side-by-side; a view before the inhalation at the left and the corresponding one after the inhalation at the right (Panel A: segmental bronchus or 3rd generation; Panel B: 4th generation at the branching point; Panel C: more periphery of 4th generation with full wall visualization; Panels D, E, F, H, and J are generations from 5th to 9th division, thin arrows). Panels G and I show pre-inhalation airway occlusions (thin arrows) which opened up after the inhalation (thick arrows).

(150 words)

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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 KJ1-26$^+$ 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 (KJ1-26$^+$ 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

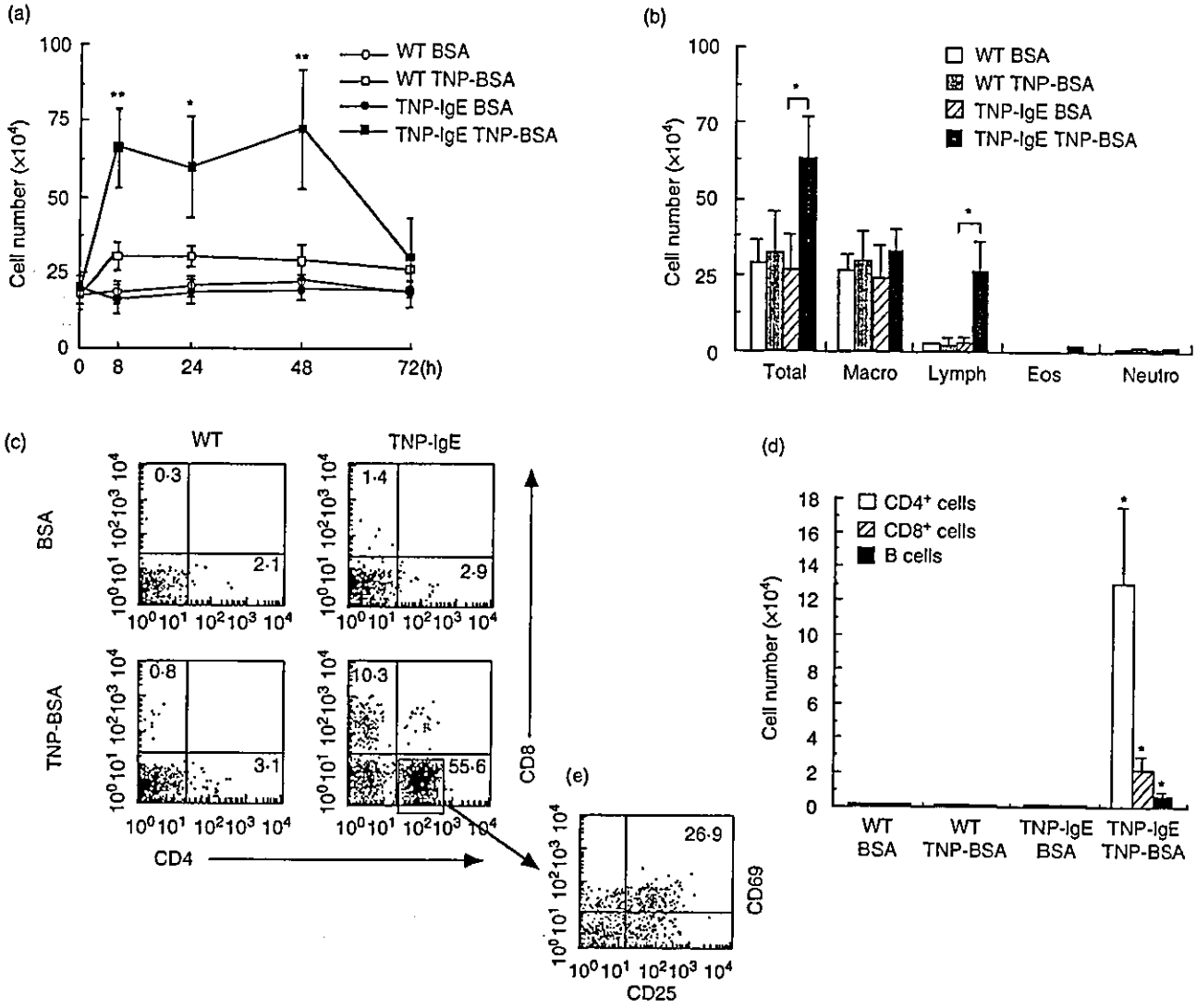

Fig. 1. IgE cross-linking induces CD4⁺ T cell recruitment into the airways. (a) Kinetics of antigen-induced airway inflammation in TNP-IgE mice. TNP-BSA or BSA (as a control) was administered intranasally to TNP-IgE mice and the littermate WT mice. At indicated times after the administration, bronchoalveolar 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

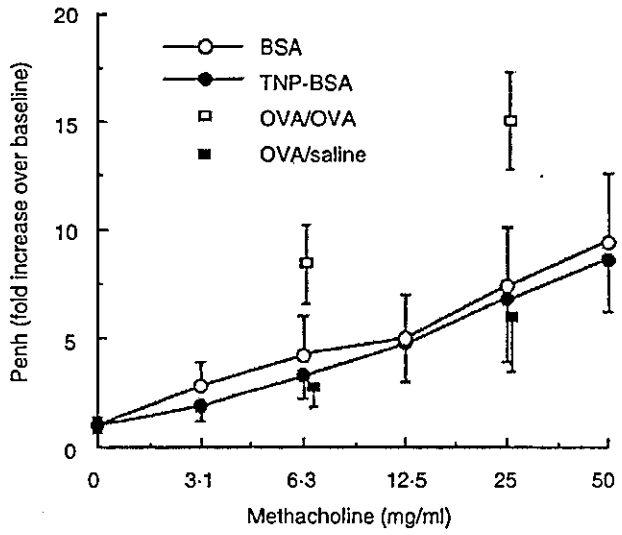

Fig. 2. IgE cross-linking does not induce airway hyperreactivity. TNP–BSA or BSA was administered intranasally to TNP–IgE mice. Forty-eight hours after TNP–BSA or BSA administration, airway reactivity was measured using a Buxco system where mice were exposed to increasing concentrations of aerosolized 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.

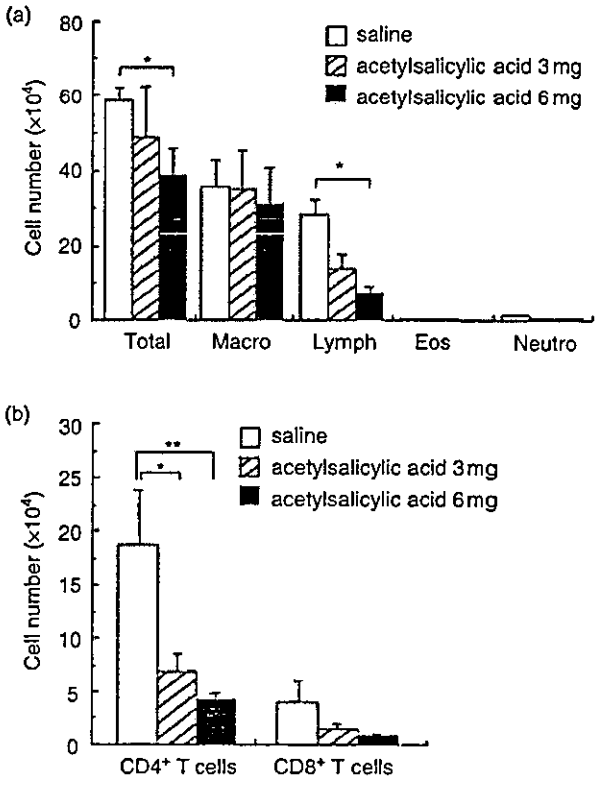

Fig. 3. Acetylsalicylic acid inhibits IgE-induced CD4⁺ T cell recruitment into the airways. TNP–IgE mice were injected intraperitoneally with acetylsalicylic acid (3 mg or 6 mg/mouse) or saline (as a control), and 30 min later TNP–BSA was administered intranasally to the mice. The number of lymphocytes, 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, $*P < 0{\cdot}05$, $**P < 0{\cdot}01$.

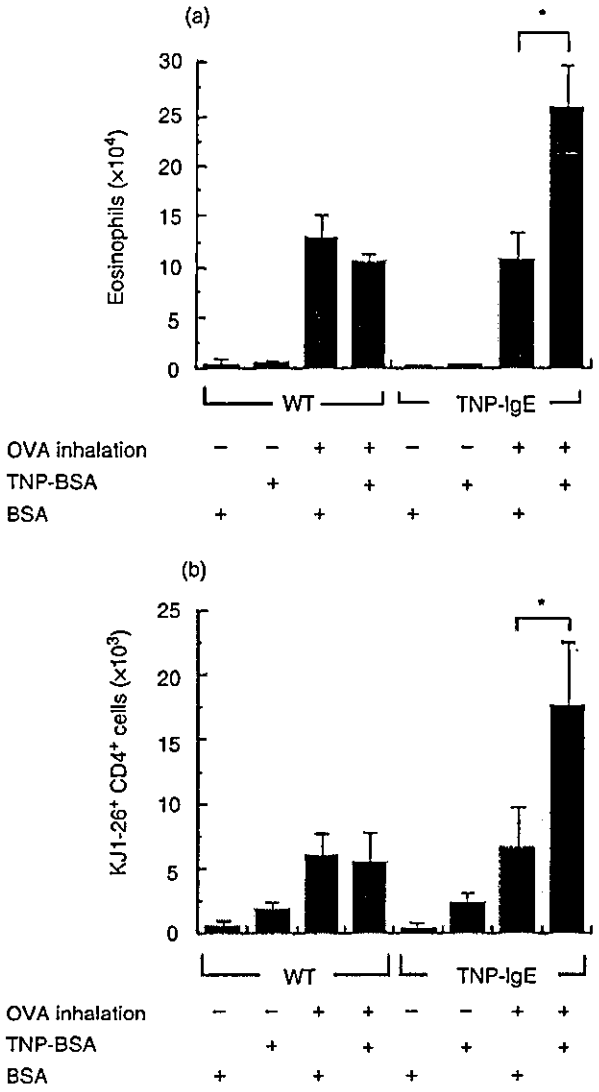

Fig. 4. IgE cross-linking enhances Th2 cell-mediated eosinophil recruitment into the airways. OVA-specific Th2 cells were prepared and transferred to TNP–IgE mice or WT mice as described in the Methods. Two days later, the mice were challenged with the inhaled OVA or saline (as a control) for 20 min. TNP-BSA or BSA was then administered intranasally to the mice, and the number of eosinophils (a) and OVA-specific CD4⁺ T cells (CD4⁺ KJ1-26⁺) (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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REFERENCES

1 Gleich GJ. The eosinophil and bronchial asthma: current understanding. J Allergy Clin Immunol 1990; 85:422–36.
2 Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. Annu Rev Immunol 1999; 17:255–81.
3 Busse WW, Lemanske RF Jr. Asthma. N Engl J Med 2001; 344:350–62.
4 Foster PS, Mould AW, Yang M *et al.* Elemental signals regulating eosinophil accumulation in the lung. Immunol Rev 2001; 179:173–81.
5 Hamelmann E, Gelfand EW. IL-5-induced airway eosinophilia – the key to asthma? Immunol Rev 2001; 179:182–91.
6 Nakajima H, Iwamoto I, Tomoe S *et al.* CD4$^+$ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. Am Rev Respir Dis 1992; 146:374–7.
7 Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med 1996; 183:195–201.
8 Wills-Karp M, Luyimbazi J, Xu X *et al.* Interleukin-13: central mediator of allergic asthma. Science 1998; 282:2258–61.
9 Grunig G, Warnock M, Wakil AE *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998; 282:2261–3.
10 Oettgen HC, Geha RS. IgE in asthma and atopy: cellular and molecular connections. J Clin Invest 1999; 104:829–35.
11 Platts-Mills TA. The role of immunoglobulin E in allergy and asthma. Am J Respir Crit Care Med 2001; 164:S1–5.
12 Galli SJ, Costa JJ. Mast cell–leukocyte cytokine cascades in allergic inflammation. Allergy 1995; 50:851–62.
13 Metcalfe DD, Baram D, Mekori YA. Mast cells. Physiol Rev 1997; 77:1033–79.
14 Mehlhop PD, van de Rijn M, Goldberg AB *et al.* Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. Proc Natl Acad Sci USA 1997; 94:1344–9.
15 Korsgren M, Erjefalt JS, Korsgren O, Sundler F, Persson CG. Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. J Exp Med 1997; 185:885–92.
16 MacLean JA, Sauty A, Luster AD, Drazen JM, De Sanctis GT. Antigen-induced airway hyperresponsiveness, pulmonary eosinophilia, and chemokine expression in B cell-deficient mice. Am J Respir Cell Mol Biol 1999; 20:379–87.
17 Takeda K, Hamelmann E, Joetham A *et al.* Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. J Exp Med 1997; 186:449–54.
18 Kobayashi T, Miura T, Haba T *et al.* An essential role of mast cells in the development of airway hyperresponsiveness in a murine asthma model. J Immunol 2000; 164:3855–61.
19 Williams CM, Galli SJ. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. J Exp Med 2000; 192:455–62.
20 Holt PG, Macaubas C, Stumbles PA, Sly PD. The role of allergy in the development of asthma. Nature 1999; 402:B12–7.
21 Humbert M, Menz G, Ying S *et al.* The immunopathology of extrinsic (atopic) and intrinsic (non-atopic) asthma: more similarities than differences. Immunol Today 1999; 20:528–33.

22 Matsuoka K, Taya C, Kubo S *et al.* Establishment of antigen-specific IgE transgenic mice to study pathological and immunobiological roles of IgE *in vivo*. Int Immunol 1999; 11:987–94.
23 Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4$^+$ CD8$^+$ TCR^{lo} thymocytes *in vivo*. Science 1990; 250:1720–3.
24 Kagami S, Nakajima H, Kumano K *et al.* Both Stat5a and Stat5b are required for antigen-induced eosinophil and T cell recruitment into the tissue. Blood 2000; 95:1370–7.
25 Hamelmann E, Schwarze J, Takeda K *et al.* Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am J Respir Crit Care Med 1997; 156:766–75.
26 Hirai H, Tanaka K, Yoshie O *et al.* Prostaglandin D_2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven transmembrane receptor CRTH2. J Exp Med 2001; 193:255–61.
27 Metzger H. The receptor with high affinity for IgE. Immunol Rev 1992; 125:37–48.
28 Gounni AS, Lamkhioued B, Ochiai K *et al.* High-affinity IgE receptor on eosinophils is involved in defence against parasites. Nature 1994; 367:183–6.
29 Corry DB, Kheradmand F. Induction and regulation of the IgE response. Nature 1999; 402:B18–23.
30 Martin TR, Galli SJ, Katona IM, Drazen JM. Role of mast cells in anaphylaxis. Evidence for the importance of mast cells in the cardiopulmonary alterations and death induced by anti-IgE in mice. J Clin Invest 1989; 83:1375–83.
31 Oshiba A, Hamelmann E, Takeda K *et al.* Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. J Clin Invest 1996; 97:1398–408.
32 Lewis RA, Soter NA, Diamond PT, Austen KF, Oates JA, Roberts LJ II. Prostaglandin D_2 generation after activation of rat and human mast cells with anti-IgE. J Immunol 1982; 129:1627–31.
33 Matsuoka T, Hirata M, Tanaka H *et al.* Prostaglandin D_2 as a mediator of allergic asthma. Science 2000; 287:2013–7.
34 Takami M, Tsukada W. Effects of DP-1904a thromboxane synthetase inhibitor, on the antigen-induced airway hyperresponsiveness and infiltration of inflammatory cells in guinea-pigs. Prostaglandins Leukot Essent Fatty Acids 1998; 59:407–14.
35 Gerli R, Paolucci C, Gresele P *et al.* Salicylates inhibit adhesion and transmigration of T lymphocytes by preventing integrin activation induced by contact with endothelial cells. Blood 1998; 92:2389–98.
36 Martin TR, Takeishi T, Katz HR, Austen KF, Drazen JM, Galli SJ. Mast cell activation enhances airway responsiveness to methacholine in the mouse. J Clin Invest 1993; 91:1176–82.
37 Hamelmann E, Cieslewicz G, Schwarze J *et al.* Anti-interleukin 5 but not anti-IgE prevents airway inflammation and airway hyperresponsiveness. Am J Respir Crit Care Med 1999; 160:934–41.
38 Hamelmann E, Takeda K, Oshiba A, Gelfand EW. Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness – a murine model. Allergy 1999; 54:297–305.

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Tyk2 Is Essential for IFN-α-Induced Gene Expression in Mast Cells

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Key Words
Mast cells · Tyk2 · IFN-α · Stat1 · Immunity, innate

Abstract
Mast cells are recognized not only as the major effector cells of type I hypersensitivity reactions but also as an important player of innate immune response against bacterial infection. Type I IFNs are also involved in the response against bacterial infection. However, the role of type I IFNs and their associated Janus kinase Tyk2 in mast cell functions remains to be determined. In this study, we addressed this issue using Tyk2-deficient ($Tyk2^{-/-}$) bone marrow-derived mast cells (BMMCs). When BMMCs from wild-type (WT) mice were stimulated with IFN-α, they expressed mRNA for IFN-γ-inducible protein 10 (IP-10) and monocyte chemoattractant protein-5 (MCP-5). Interestingly, IFN-α-induced expression of IP-10 and MCP-5 was severely decreased in $Tyk2^{-/-}$ BMMCs. In addition, IFN-α-induced Stat1 phosphorylation was decreased in $Tyk2^{-/-}$ BMMCs. On the other hand, IFN-α-induced Stat1 phosphorylation and IP-10 and MCP-5 expression were normal in $Tyk2^{-/-}$ fibroblasts. These results indicate that IFN-α induces the expression of TNF-α and the chemokines IP-10 and MCP-5 in mast cells and thatTyk2 plays a nonredundant role in IFN-α signaling in mast cells.

Introduction

Mast cells are recognized as the major effector cells of type I hypersensitivity reactions by virtue of possessing the high affinity receptors for IgE and are known to play a critical role in allergic diseases such as atopic rhinitis, asthma, and atopic dermatitis [1, 2]. Recently, a number of studies have revealed that mast cells also play important roles in innate immune responses especially against gram-negative bacteria by recruiting neutrophils and monocytes into the inflammatory site through the production of proinflammatory cytokines such as TNF-α [3, 4]. Some of the bacterial components, including lipopolysaccharide (LPS), directly activate mast cells through their surface receptors [5, 6]. However, the precise mechanisms of mast cell activation in innate immune responses are still largely unknown.

Type I IFNs (IFN-α/β), key immunoregulatory cytokines produced by macrophages and plasmacytoid dendritic cells after the exposure to pathogens, modulate innate and adaptive immune responses [7]. Although the function of type I IFNs is principally associated with the protection against viral infections, recent studies have revealed that type I IFNs are also involved in the immune response against other pathogens [8, 9]. In this regard, it has been shown that preceding IFN-α treatment sensitizes the mice for an enhanced production of TNF-α upon LPS stimulation [10]. It has also been shown that type I IFNs

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are rapidly produced by macrophages upon LPS stimulation [11]. These findings suggest that type I IFNs may participate in immune responses against bacterial infection through the production of TNF-α.

Two Janus kinases (JAKs), Tyk2 and Jak1, are associated with IFN-α/β receptor components IFNAR1 and IFNAR2, respectively [12, 13]. Upon ligand binding, Jak1 and Tyk2 are activated and the activated JAKs phosphorylate Stat1 and Stat2 [12, 13]. Subsequently, these activated STATs associate to form either Stat1 homodimers or Stat1/Stat2 heterodimers, which then translocates to the nucleus to induce gene expression [14, 15]. Recently, the physiological function of Jak1 and Tyk2 in type I IFN signaling has been determined using mice lacking Jak1 or Tyk2 [16–19]. These studies have revealed that, whereas Jak1 is essential for responding to type I IFNs in most cell types [16], the requirement of Tyk2 in type I IFN signaling differs depending on cell types [17–19]. It has been demonstrated that Tyk2 is essential for IFN-α signaling in IL-7-dependent B cells [19] but not in fibroblasts [17, 18]. However, the role of Tyk2 in IFN-α signaling in mast cells is unknown.

In this study, we determined whether Tyk2 is essential for IFN-α signaling in mast cells. Our findings have clearly demonstrated that using Tyk2-deficient mice, Tyk2 is required for IFN-α-induced Stat1 phosphorylation and subsequent gene induction in mast cells.

Methods

Mice and Cytokines

Tyk2-deficient ($Tyk2^{-/-}$) mice [17] were backcrossed for more than four generations onto BALB/c mice (Japan SLC, Shizuoka, Japan). The mice were genotyped by PCR as described previously [17] and littermate wild-type (WT) mice were used as controls. Mice were housed in microisolator cages under pathogen-free conditions. All experiments were performed according to the guidelines of the Chiba University. Recombinant murine IFN-α and IL-12 were purchased from R&D systems (Minneapolis, Minn., USA).

Culture of Bone Marrow-Derived Mast Cells

Primary culture of IL-3-dependent bone marrow-derived mast cells (BMMCs) was prepared from 8- to 12-week-old WT or $Tyk2^{-/-}$ mice and maintained as described previously [20]. BMMCs obtained after 4 weeks of culture were >99% mast cells.

Culture of Fibroblasts

Skin fibroblasts were prepared from WT mice or $Tyk2^{-/-}$ mice and maintained as described previously [21].

Flow-Cytometric Analysis

BMMCs were stained and analyzed on FACScalibur (Becton Dickinson, San Jose, Calif., USA) using CELLQuest software. Anti-CD117 (c-kit) antibody (2B8) was purchased from BD PharMingen (San Diego, Calif., USA). Prior to staining, Fc receptors were blocked with anti-CD16/32 antibody (2.4G2, BD PharMingen).

RT-PCR Analysis for IP-10, MCP-5, IFNAR1, IFNAR2, and Jak1

BMMCs or fibroblasts from WT mice or $Tyk2^{-/-}$ mice were stimulated with IFN-α (1,000 U/ml) at 37°C for 3 h. Total cellular RNA was prepared using Isogen solution (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. The first-strand complementary DNA (cDNA) was synthesized from total RNA using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers (Pharmacia Biotech, Buckinghamshire, UK). cDNAs encoding IFN-γ-inducible protein 10 (IP-10), monocyte chemoattractant protein-5 (MCP-5), IFNAR1, IFNAR2, and Jak1 were amplified by PCR using the following primer pairs:

IP-10 5′-GAGATCATTGCCACGATGAA-3′ and 5′-CACTGGGTAAAGGGGAGT-3′, MCP-5 5′-AATCACAAGCAGCCAGTG-3′ and 5′-GGGAACTTCAGGGGGAAATA-3′, IFNAR1 5′-CCTGCTGAATAAGACCAGCAACTTC-3′ and 5′-GTGCTTTACTTCTACAGCGACCGTG-3′, IFNAR2 5′-CAAGCCTCTGCAACAAACCTCTGAC-3′ and 5′-GATTTCTCAGATGACCCATCTTCAG-3′, Jak1 5′-CTGCTAGCATGATGAGACAGGTTTC-3′ and 5′-TTGGAGTCTTCAACACACTCAGGAG-3′. β-Actin was used to normalize the cDNA amount to be used.

Immunoblotting

Preparation of whole cell extracts and immunoblottings were performed as described previously [20]. The following antisera were used: anti-phospho-Stat1 (New England Biolabs, Beverly, Mass., USA) and anti-Stat1 (Upstate Biotechnology, Lake Placid, N.Y., USA).

Data Analysis

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

Results and Discussion

Development of IL-3-Dependent BMMCs Is Normal in $Tyk2^{-/-}$ Mice

To determine the role of Tyk2 in mast cell development, bone marrow cells from WT mice or $Tyk2^{-/-}$ mice were cultured in the presence of IL-3 and the number of mast cells were evaluated every 7 days. As shown in figure 1a, the number of mast cells recovered from the culture was indistinguishable between WT and $Tyk2^{-/-}$ mice. Over 99% of cells obtained after 4 weeks of culture were morphologically mast cells in $Tyk2^{-/-}$ mice as well as in WT mice (data not shown). In addition, $Tyk2^{-/-}$ BMMCs expressed comparable levels of c-kit to WT BMMCs (fig. 1b). These results suggest that Tyk2 is not required for the development of IL-3-dependent mast cells.

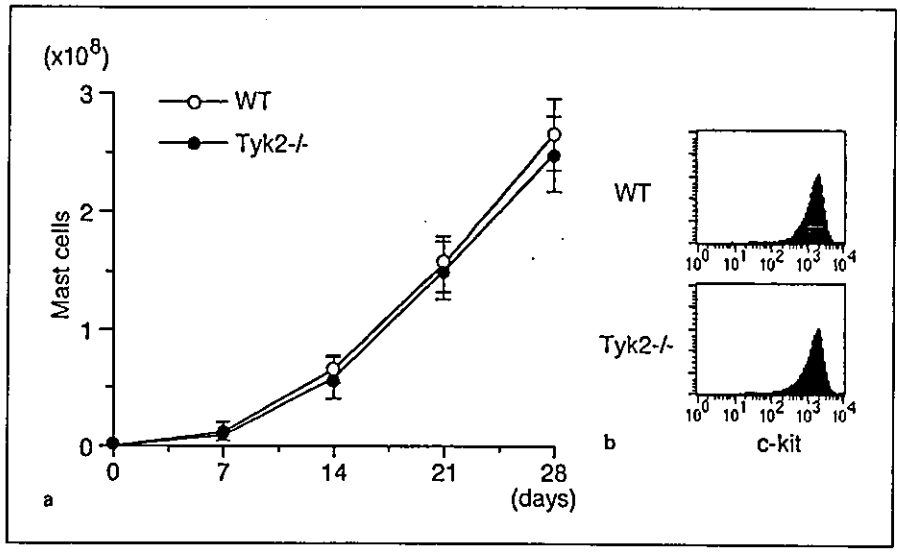

Fig. 1. Development of IL-3-dependent BMMCs is normal in Tyk2$^{-/-}$ mice. **a** Bone marrow cells from WT mice or Tyk2$^{-/-}$ mice were cultured in the presence of IL-3. Indicated days later, the number of BMMCs was evaluated. Data are means ± SD of 5 experiments for each genotype. **b** BMMCs from WT mice or Tyk2$^{-/-}$ mice were stained with anti-c-kit APC and analyzed on FACS. Representative FACS profiles for c-kit staining from five independent experiments are shown.

IFN-α-Induced Expression of IP-10 and MCP-5 Is Diminished in Tyk2$^{-/-}$ BMMCs

Jak1 and Tyk2 are associated with receptors for type I IFNs [12–15]. Using Jak1-deficient mice, it has been shown that Jak1 is essential for biological responses in IFN-α/β signaling [16]. On the other hand, it has been demonstrated that the requirement of Tyk2 in IFN-α/β signaling differs depending on cell types [17–19]. To examine the role of Tyk2 in IFN-α-mediated functions in mast cells, BMMCs from WT mice or Tyk2$^{-/-}$ mice were stimulated with IFN-α and the expression of IFN-responsive genes was analyzed at mRNA levels. As shown in figure 2, IFN-α-induced expression of IP-10 and MCP-5, which play important roles in the host defense to pathogens [22, 23], was severely decreased in Tyk2$^{-/-}$ BMMCs as compared with that in WT BMMCs. On the other hand, Tyk2$^{-/-}$ fibroblasts expressed mRNA for IP-10 and MCP-5 at a level comparable to that in WT fibroblasts (fig. 2). These results suggest that Tyk2 is essential for IFN-α-mediated gene expression in mast cells but not in fibroblasts. On the other hand, IL-12, another cytokine that utilizes Tyk2 as a signaling molecule [24] and augments innate immune responses [25], exhibited no significant effects even on WT BMMCs because of the absence of functional IL-12R (data not shown).

IFN-α-Induced Phosphorylation of Stat1 Is Diminished in Tyk2$^{-/-}$ BMMCs

IFN-α-mediated gene induction was diminished in Tyk2$^{-/-}$ BMMCs (fig. 2). Because most IFN-α-induced responses depend on Stat1 activation [26, 27], we next

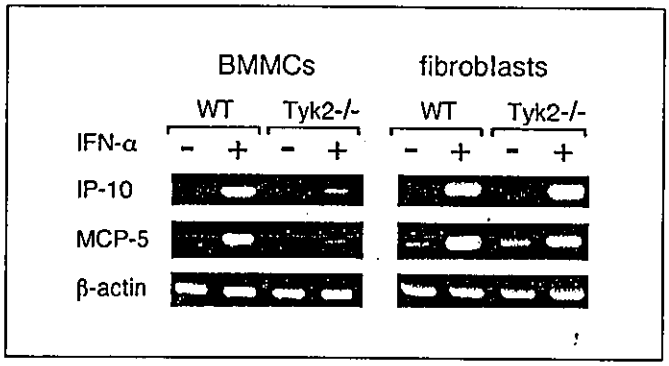

Fig. 2. IFN-α-induced gene expression is diminished in Tyk2$^{-/-}$ BMMCs. BMMCs or fibroblasts from WT mice or Tyk2$^{-/-}$ mice were stimulated with IFN-α (1,000 U/ml) for 3 h. The expression of IP-10 and MCP-5 mRNA was determined by RT-PCR. Representative data from 5 independent experiments are shown.

examined IFN-α-induced phosphorylation of Stat1 in Tyk2$^{-/-}$ BMMCs. As shown in figure 3, IFN-α-induced Stat1 phosphorylation was severely decreased in Tyk2$^{-/-}$ BMMCs as compared with that in WT BMMCs (fig. 3). In contrast, consistent with previous reports [17, 18], IFN-α-induced Stat1 phosphorylation was similarly observed in Tyk2$^{-/-}$ fibroblasts and WT fibroblasts (fig. 3). These results suggest that Tyk2 is essential for IFN-α-induced Stat1 phosphorylation and then for Stat1-dependent gene expression in mast cells.

Because IFN-α-induced Stat1 phosphorylation was diminished in Tyk2$^{-/-}$ BMMCs but not in Tyk2$^{-/-}$ fibro-

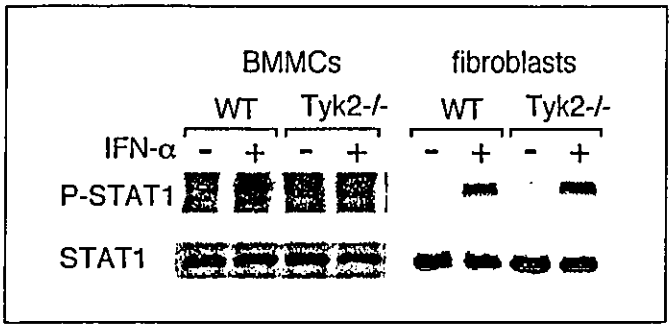

Fig. 3. Tyk2 is essential for IFN-α-induced Stat1 phosphorylation in BMMCs but not in fibroblasts. WT BMMCs, Tyk2$^{-/-}$ BMMCs, WT fibroblasts, or Tyk2$^{-/-}$ fibroblasts were stimulated with or without IFN-α (1,000 U/ml) for 30 min. Whole cell extracts were subjected to Western blotting with anti-phospho-Stat1 antibody or anti-Stat1 antibody. Representative data from 5 independent experiments are shown.

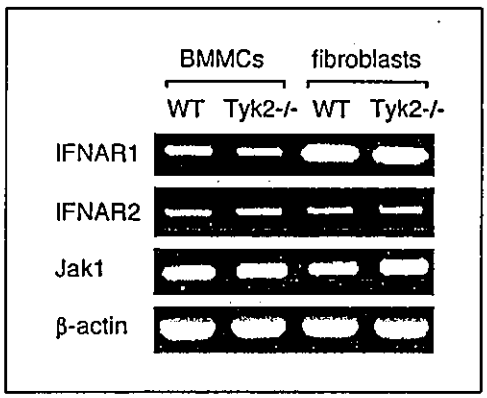

Fig. 4. Expression of IFNAR1 is lower in BMMCs than that in fibroblasts. Total cellular RNA was prepared from WT BMMCs, Tyk2$^{-/-}$ BMMCs, WT fibroblasts, or Tyk2$^{-/-}$ fibroblasts. Expression of IFNAR1, IFNAR2, and Jak1 transcripts was examined by RT-PCR analysis. Representative data from 5 independent experiments are shown.

blasts, we next compared the expression of IFN-α receptor components (IFNAR1 and IFNAR2) and Jak1 in BMMCs and fibroblasts. As shown in figure 4, regardless of the presence or absence of Tyk2, the expression of IFNAR1 was significantly lower in BMMCs than that in fibroblasts. On the other hand, the expression of IFNAR2 and Jak1 was comparable between BMMCs and fibroblasts (fig. 4). These results suggest that the limited expression of IFNAR1 in mast cells may account for the requirement of Tyk2 in IFN-α signaling.

Concluding Remarks

In the present study, we show that IFN-α induces the expression of the chemokines IP-10 and MCP-5 in mast cells and that Tyk2 is essential for IFN-α-induced gene expression in mast cells but not in fibroblasts. We found that IFN-α induced mRNA expression of IP-10 and MCP-5, important chemokines for innate immune responses [22, 23], in WT BMMCs, but the chemokine expression was diminished in Tyk2$^{-/-}$ BMMCs but not in Tyk2$^{-/-}$ fibroblasts. In addition, we found that IFN-α-induced Stat1 phosphorylation was decreased in Tyk2$^{-/-}$ BMMCs but not in Tyk2$^{-/-}$ fibroblasts. These results suggest that Tyk2 is required for IFN-α-induced Stat1 phosphorylation and subsequent gene expression in mast cells but not in fibroblasts.

Recent studies using Tyk2$^{-/-}$ mice have revealed that Tyk2 regulates both acquired and innate immune responses. It has been shown that Tyk2 is essential for IL-12-mediated T cell function, including IFN-γ production and Th1 cell differentiation [17, 18]. Tyk2 is also required for the downregulation of Th2 cell-mediated allergic inflammation in murine models of allergic asthma [28]. In addition, it has been demonstrated that Tyk2 plays an important role in endotoxin shock as a component of type I IFN signaling [29]. These findings suggest that Tyk2 is involved not only in acquired immune responses but also in innate immune responses. Our findings that Tyk2 is required for the IFN-α-induced expression of IP-10 and MCP-5 in mast cells also suggest the important roles of Tyk2 in innate immune responses.

We have shown here that Tyk2 is essential for IFN-α signaling in mast cells but not in fibroblasts. Although further studies are required, our data suggest that the expression levels of IFNAR1 may account for the different requirement for Tyk2 in IFN-α signaling between mast cells and fibroblasts.

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References

1 Galli SJ, Hammel I: Mast cell and basophil development. Curr Opin Hematol 1994;1:33–39.

2 Metcalfe DD, Baram D, Mekori YA: Mast cells. Physiol Rev 1997;77:1033–1079.

3 Echtenacher B, Mannel DN, Hultner L: Critical protective role of mast cells in a model of acute septic peritonitis. Nature 1996;381:75–77.

4 Malaviya R, Ikeda T, Ross E, Abraham SN: Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α. Nature 1996;381:77–80.

5 Malaviya R, Gao Z, Thankavel K, Merwe PA, Abraham SN: The mast cell tumor necrosis factor α response to FimH-expressing *Escherichia coli* is mediated by the glycosylphosphatidylinositol-anchored molecule CD48. Proc Natl Acad Sci USA 1999;96:8110–8115.

6 McCurdy JD, Lin TJ, Marshall JS: Toll-like receptor 4-mediated activation of murine mast cells. J Leukoc Biol 2001;70:977–984.

7 Pestka S, Langer JA, Zoon KC, Samuel CE: Interferons and their actions. Annu Rev Biochem 1987;56:727–777.

8 Decker T, Stockinger S, Karaghiosoff M, Muller M, Kovarik P: IFNs and STATs in innate immunity to microorganisms. J Clin Invest 2002;109:1271–1277.

9 Bogdan C: The function of type I interferons in antimicrobial immunity. Curr Opin Immunol 2000;12:419–424.

10 Nansen A, Randrup Thomsen A: Viral infection causes rapid sensitization to lipopolysaccharide: Central role of IFN-α/β. J Immunol 2001;166:982–988.

11 Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, Williams BR, Major J, Hamilton TA, Fenton MJ, Vogel SN: TLR4, but not TLR2, mediates IFN-β-induced STAT1α/β-dependent gene expression in macrophages. Nat Immunol 2002;3:392–398.

12 Schindler C, Darnell JE Jr: Transcriptional responses to polypeptide ligands: The JAK-STAT pathway. Annu Rev Biochem 1995;64:621–651.

13 Leonard WJ, O'Shea JJ: Jaks and STATs: Biological implications. Annu Rev Immunol 1998;16:293–322.

14 Darnell JE Jr, Kerr IM, Stark GR: Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264:1415–1421.

15 Ihle JN, Nosaka T, Thierfelder W, Quelle FW, Shimoda K: Jaks and Stats in cytokine signaling. Stem Cells 1997;15:105–111.

16 Rodig SJ, Meraz MA, White JM, Lampe PA, Riley JK, Arthur CD, King KL, Sheehan KC, Yin L, Pennica D, Johnson EM Jr, Schreiber RD: Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell 1998;93:373–383.

17 Shimoda K, Kato K, Aoki K, Matsuda T, Miyamoto A, Shibamori M, Yamashita M, Numata A, Takase K, Kobayashi S, Shibata S, Asano Y, Gondo H, Sekiguchi K, Nakayama K, Nakayama T, Okamura T, Okamura S, Niho Y: Tyk2 plays a restricted role in IFN-α signaling, although it is required for IL-12-mediated T cell function. Immunity 2000;13:561–571.

18 Karaghiosoff M, Neubauer H, Lassnig C, Kovarik P, Schindler H, Pircher H, McCoy B, Bogdan C, Decker T, Brem G, Pfeffer K, Muller M: Partial impairment of cytokine responses in Tyk2-deficient mice. Immunity 2000;13:549–560.

19 Shimoda K, Kamesaki K, Numata A, Aoki K, Matsuda T, Oritani K, Tamiya S, Kato K, Takase K, Imamura R, Yamamoto T, Miyamoto T, Nagafuji K, Gondo H, Nagafuchi S, Nakayama K, Harada M: Tyk2 is required for the induction and nuclear translocation of Daxx which regulates IFN-α-induced suppression of B lymphocyte formation. J Immunol 2002;169:4707–4711.

20 Suzuki K, Nakajima H, Watanabe N, Kagami S, Suto A, Saito Y, Saito T, Iwamoto I: Role of common cytokine receptor γ chain (γc)- and Jak3-dependent signaling in the proliferation and survival of murine mast cells. Blood 2000;96:2172–2180.

21 Fischer SM, Viaje A, Mills GD, Slaga TJ: Explant methods for epidermal cell culture. Methods Cell Biol 1980;21A:207–227.

22 Khan IA, MacLean JA, Lee FS, Casciotti L, DeHaan E, Schwartzman JD, Luster AD: IP-10 is critical for effector T cell trafficking and host survival in *Toxoplasma gondii* infection. Immunity 2000;12:483–494.

23 Sarafi MN, Garcia-Zepeda EA, MacLean JA, Charo IF, Luster AD: Murine monocyte chemoattractant protein (MCP)-5: A novel CC chemokine that is a structural and functional homologue of human MCP-1. J Exp Med 1997;185:99–109.

24 Bacon CM, McVicar DW, Ortaldo JR, Rees RC, O'Shea JJ, Johnston JA: Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: Differential use of Janus family tyrosine kinases by IL-2 and IL-12. J Exp Med 1995;181:399–404.

25 Trinchieri G: Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003;3:133–146.

26 Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M, Schreiber RD: Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 1996;84:431–442.

27 Durbin JE, Hackenmiller R, Simon MC, Levy DE: Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 1996;84:443–450.

28 Seto Y, Nakajima H, Suto A, Shimoda K, Saito Y, Nakayama KI, Iwamoto I: Enhanced Th2 cell-mediated allergic inflammation in Tyk2-deficient mice. J Immunol 2003;170:1077–1083.

29 Karaghiosoff M, Steinborn R, Kovarik P, Kriegshauser G, Baccarini M, Donabauer B, Reichart U, Kolbe T, Bogdan C, Leanderson T, Levy D, Decker T, Muller M: Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. Nat Immunol 2003;4:471–477.

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.

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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

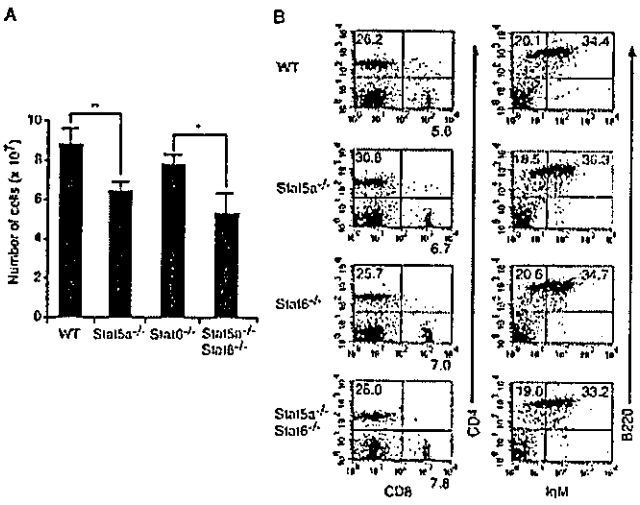

FIGURE 1. Normal T cell and B cell development in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. *A*, Number of splenocytes in WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, and Stat5a$^{-/-}$Stat6$^{-/-}$ mice. Data are the mean ± SD from eight mice for each genotype. *, $p < 0.05$; **, $p < 0.01$. *B*, Flow cytometric analysis 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

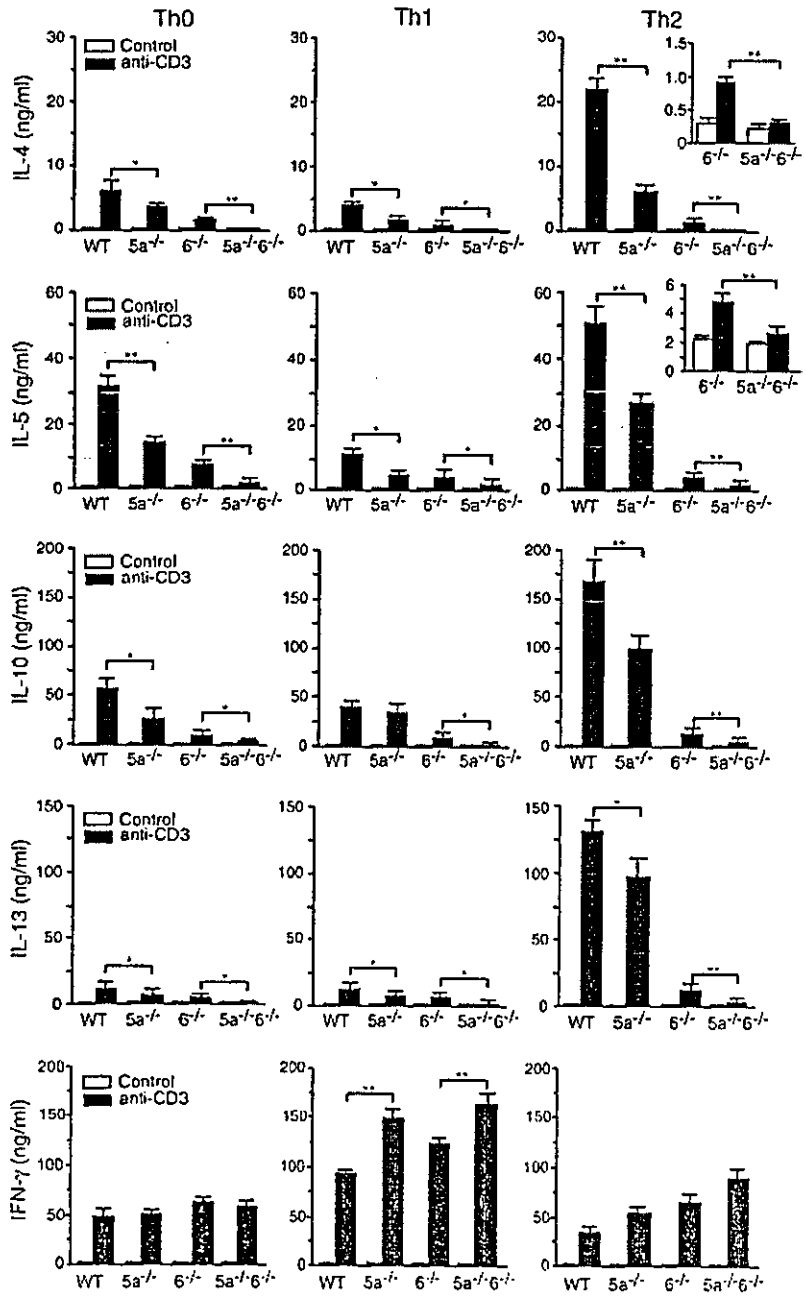

FIGURE 2. Th2 cytokine production is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. Splenocytes from WT, Stat5a$^{-/-}$ (5a$^{-/-}$), Stat6$^{-/-}$ (6$^{-/-}$), or Stat5a$^{-/-}$ Stat6$^{-/-}$ (5a$^{-/-}$ 6$^{-/-}$) mice were stimulated with plate-bound anti-CD3 mAb in the nonpolarizing Th0 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. *, $p < 0.05$; **, $p < 0.01$.

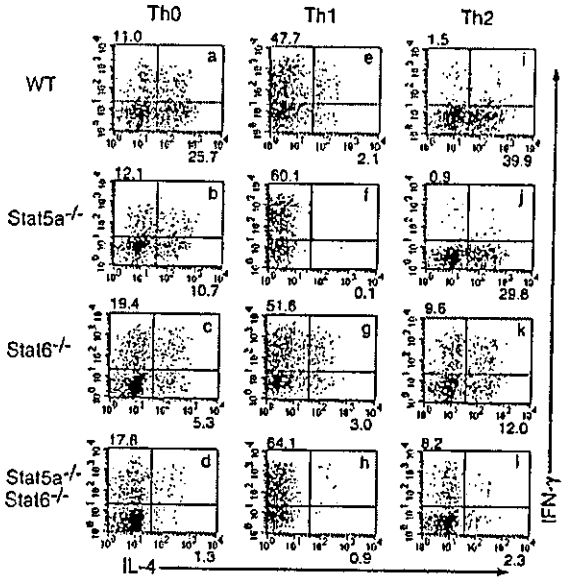

FIGURE 3. Th2 cell differentiation is severely decreased in Stat5a$^{-/-}$ Stat6$^{-/-}$ mice. Splenocytes from WT, Stat5a$^{-/-}$, Stat6$^{-/-}$, or Stat5a$^{-/-}$ Stat6$^{-/-}$ mice were stimulated with plate-bound anti-CD3 mAb for 48 h in Th0, Th1, or Th2 conditions and cultured for another 72 h in 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

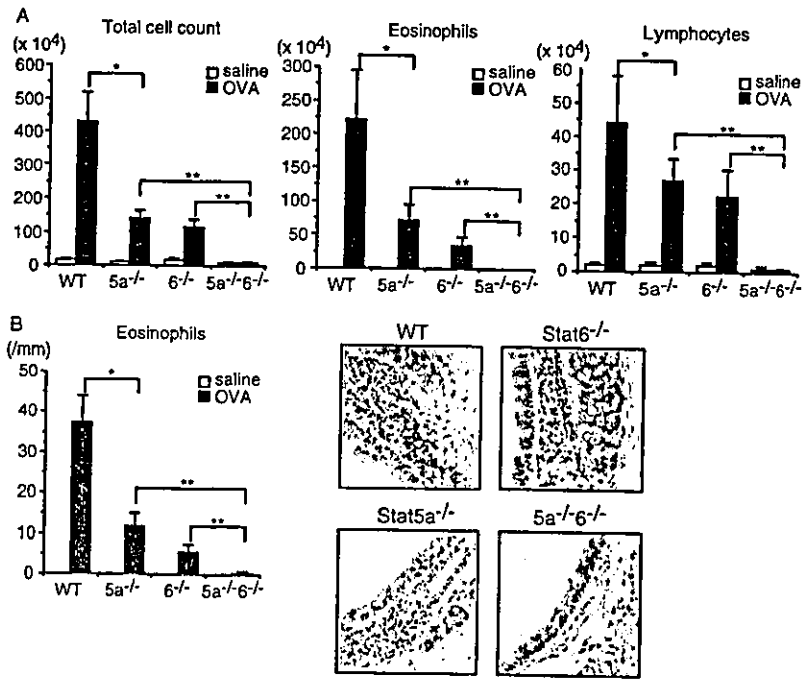

FIGURE 4. Ag-induced eosinophil and lymphocyte recruitment into the airways is severely decreased in Stat5a$^{-/-}$Stat6$^{-/-}$ mice. *A*, OVA-sensitized Stat5a$^{-/-}$, Stat6$^{-/-}$, Stat5a$^{-/-}$Stat6$^{-/-}$, and littermate WT mice were challenged with the inhalation of OVA or saline (as a control) 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).