

STAT6 deficiency in a mouse model of allergen-induced airways inflammation abolishes eosinophilia but induces infiltration of CD8⁺ T cells

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Summary

Background The TH2-type cytokines have been reported to contribute to the asthmatic response. STAT6 has an essential role in IL-4 signalling and in production of TH2 cytokines from T cells and is involved in IgE and IgG1 responses after nematode infections, indicating that STAT6 has an important role in allergic diseases.

Objective In this study we investigated the effects of STAT6 deficiency on allergen-induced airways inflammation in mice.

Methods Both ovalbumin (OVA)-sensitized STAT6 deficient (STAT6^{-/-}) mice and wild-type C57BL/6 mice were challenged with aerosolized OVA. Changes in inflammatory cell infiltration and cytokine levels in lung tissue as well as serum immunoglobulin levels were analysed in OVA-challenged STAT6^{-/-} and wild-type mice.

Results The eosinophilia and lung damage normally resulting from aeroallergen challenge were not seen in STAT6^{-/-} mice. Expression of TH2 cytokines (IL-4 and IL-5) in the lung tissue as well as IgE and IgG1 responses after OVA challenge were profoundly reduced in STAT6^{-/-} mice, whereas expression of IFN γ was the same in STAT6^{-/-} mice and wild-type mice after OVA challenge. Immunocytochemical analysis of T cells showed the infiltration of CD4⁺ T cells but not CD8⁺ T cells increased into the lung of wild-type mice after OVA challenge. However, the OVA-exposed STAT6^{-/-} mice demonstrated the infiltration of both CD4⁺ T cells and CD8⁺ T cells with a significant increase in percentage and total number of CD8⁺ T cells compared with OVA-exposed wild-type mice.

Conclusion These results indicate that factors which signal through STAT6 are important regulators of eosinophilia of allergic airway inflammation, regulating TH2-type cytokine production both in CD4⁺ T cells and CD8⁺ T cells.

Keywords: asthma, STAT6, IL-4, eosinophil, T cell, lung, mice

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Introduction

Atopic asthma is an allergic disease characterized by reversible obstruction of the airways or bronchi. Elevated numbers of activated CD4⁺ T cells, mast cells, and eosinophils, both in the bronchial mucosa and in bronchoalveolar

lavage (BAL) fluid, are constant features of asthma [1–4]. These inflammatory cells release mediators, including cytokines, that have the potential to augment the cell-mediated immune reaction and induce tissue damage and dysfunction [5]. It has been suggested that the onset of the asthmatic response is controlled by CD4⁺ T cells which produce T-helper (TH) 2-type cytokines [6] such as interleukin (IL)-3, IL-4, IL-5, IL-10, and granulocyte macrophage-colony stimulating factor [7–9]. Of these cytokines, IL-4 and IL-5 are thought to be most related to allergic asthma. IL-5

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regulates the growth, differentiation, and activation of eosinophils, the effector cells of the asthmatic response [10]. IL-4 stimulates the switching of B-cell isotypes to the production of IgE [11]. IL-4 also plays a crucial role in the differentiation of Th0 into TH2 lymphocytes *in vitro* [12] and *in vivo* [13]. Evidence in an asthma model of mutant mice indicates that IL-4 and IL-5 are essential for mounting aeroallergen-induced pulmonary eosinophilia [14–17]. However, a recent study using IL-4-deficient mice indicated that IL-4 is not essential for the development of IL-5-producing CD4⁺ T cells or for the induction of eosinophilic inflammation, airway damage or hyperreactivity [18]. Thus, there is conflicting evidence about the comparative importance of IL-4 in the molecular mechanism underlying aeroallergen-induced eosinophil inflammation and airway hyperreactivity [14,18,19].

Signal transducers and activators of transcription (STATs) are activated by tyrosine phosphorylation in response to cytokines, and are thought to mediate many functional responses [20,21]. STAT6 is activated in response to IL-4 [22,23] and may contribute to various functions including mitogenesis, T-helper cell differentiation, and immunoglobulin isotype switching [24]. Recently, we generated mice deficient in STAT6 by gene targeting [25]. In the STAT6^{-/-} mice, most of IL-4-mediated functions, including expression of CD23 and major histocompatibility complex (MHC) class II antigen in resting B cells, B cell proliferation, TH2 helper T-cell differentiation, and immunoglobulin class switching to IgE, were profoundly reduced, indicating that STAT6 plays an essential role in IL-4 signalling, despite the existence of multiple signalling pathways activated by IL-4 [26,27].

In this study we examined the importance of IL-4 signalling and eosinophils in the pathogenesis of asthma by using a mouse asthma model [17] and STAT6-deficient mice [25]. We examined whether pulmonary eosinophilia and aeroallergen-induced lung damage occur in STAT6^{-/-} mice *in vivo*. We also analysed the subsets of infiltrating lymphocytes in the lung of STAT6^{-/-} mice undergoing OVA challenge, because a recent study indicates that IL-4 signalling is important in the switching of CD8⁺ T cells, as well as CD4⁺ T cells, to a TH2 phenotype that produces IL-4, IL-5, and IL-10 [28–30].

Materials and methods

Animals, sensitization procedures and allergen exposure

Specific pathogen-free male C57BL/6 mice, 6–8 weeks old, were purchased from Charles River Laboratories (Sizuoka, Japan). Male homozygous STAT6 deficient (STAT6^{-/-}) C57BL/6 mice, 6–12 weeks old, and sex- and age-matched wild-type littermates were obtained from the department of Biochemistry of Hyogo College of Medicine.

Immunization and exposure of mice were carried out according to the method of Foster *et al.* [17]. Briefly, mice were sensitized by intraperitoneal (i.p.) injection with 50 µg OVA/1 mg aluminium hydroxide in 0.9% saline on day 0 and 12. Nonsensitized mice received 1 mg of aluminium hydroxide in 0.9% saline. On day 24, the animals were placed individually in a chamber and inhaled aerosolized OVA (10 mg/mL) in 0.9% saline (sensitized, OVA-challenged group) or saline only (sensitized, non-challenged group) for 30 min three times (at 1-h intervals), and then every second day thereafter, for 8 days. Non-sensitized mice received saline only (non-sensitized, non-challenged group). The aerosols were generated by ultrasonic nebulization. The mice were killed by cervical dislocation 3 h and 24 h after the last aeroallergen challenge. Mice were treated according to the Hyogo College of Medicine Guidelines for the Care and Use of Laboratory Animals and were housed in an approved containment facility.

Histological evaluation of eosinophils and lymphocytes

The lungs were excised and frozen quickly by dry ice, and 10 µm thick sections were cut on a cryostat and thaw-mounted on gelatinized glass slides. Lung sections were fixed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4), and were subjected to Giemsa's staining. For immunohistochemical analyses of CD4⁺ or CD8⁺ T cells that migrated into the lung tissue, the sections were fixed with acetone at room temperature (RT) for 5 min. After washing with phosphate-buffered saline (PBS; pH 7.4), all sections were treated with 0.3% H₂O₂ in methanol overnight at RT to quench the endogenous peroxidase, and then incubated for 30 min in 5% normal goat serum (NGS) diluted in PBS. Immunostaining was performed overnight at 4 °C with the following commercially available antibodies: rat antimouse CD4 (L3T4) or rat antimouse CD8 (Ly-2) monoclonal antibodies (PharMingen, San Diego, CA, USA) diluted 1:200 with 1% NGS in PBS. After incubation with a secondary biotinylated antibody (goat antirat IgG; Tago, Camarillo, CA, USA) diluted 1:500 with 1% NGS in PBS, the positive immunoreaction was detected by means of an avidin-biotin peroxidase complex technique (Vectastain ABC Kit, Vector, Burlingame, CA, USA) using 3,3'-diaminobenzidine and 0.005% H₂O₂ as a peroxidase substrate. The specificity of each antibody was confirmed by replacement of first antibodies to the nonimmunized rat IgG2a, κ (Clone, G155–178).

The number of eosinophils, CD4⁺ and CD8⁺ T cells were counted in all medium airways present in each section (at least 12 sections from each mouse) using a light microscope (x20 objective and x10 eyepiece) and analysed by Interaktive Bild-Analyse System (IBAS, Kontron-Bildanalyse, Germany). These counts reflected the number of cells per unit area

(number/mm²) of the subepithelial area of medium airways. Data represent the mean $\pm$ SD for groups of four or five mice.

Quantification of cytokine levels in lung tissue supernatants

The lungs were removed after retrieval of blood, snap-frozen in liquid nitrogen, and stored at -80°C . Later, the lungs were homogenized with 10 $\mu\text{mol/L}$ phosphate buffer (pH, 7.4) containing 0.1 mmol/L ethylene diaminetetra-acetic acid (Sigma), 0.1 mmol/L phenylmethanesulphonyl fluoride (Nacalai Tasque), 1 $\mu\text{mol/L}$ pepstatin (Peptide Institute, Osaka, Japan) and 2 $\mu\text{mol/L}$ leupepsin (Peptide Institute). The homogenates were then centrifuged at 105 000 *g* for 1 h. The supernatants were stored at -80°C .

The ELISA for IL-4, IL-5 and IFN γ were conducted using matching antibody pairs (Amersham, Buckinghamshire, UK) according to the manufacturers' instructions. The second antibodies were conjugated to horseradish peroxidase. Subtractive readings of 550 nm from the readings at 450 nm were converted to pg per mL using values obtained from standard curves generated with varying concentrations of recombinant IL-4, IL-5, and IFN γ . The limits of detection in these assay were less than 5 pg/mL, 5 pg/mL, and 15 pg/mL, respectively.

Measurement of serum IgE and IgG1

The blood was collected in a tube to measure serum IgE and IgG1. Serum IgE and IgG1 were detected by ELISA as previously reported [25]. Sera were obtained from mice 3 h after challenge either with saline or OVA (four mice per group).

Results

Lung histology

Histological analysis of lungs taken from both non-sensitized and sensitized groups (wild-type and STAT6^{-/-} mice) exposed to saline aerosol showed normal lung histology (Fig. 1a,d). In contrast, examination of lungs from aerosolized OVA-challenged wild-type mice showed severe widespread inflammatory infiltrates at 3 h (Figs 1b and 2a) and at 24 h after the challenge (Fig. 1c). These infiltrates localized primarily around airway walls (bronchi and bronchioli) and blood vessels. The peribronchial inflammatory cells consisted of eosinophils, lymphocytes and monocytes, infiltrating the bronchial epithelial and subepithelial area. Lung tissue in OVA-challenged wild-type mice also was characterized by gross alteration in the structural integrity of the airway walls, epithelial cell shedding and extensive mucosal oedema, and particulate exudates in the airway lumens and alveolar septae (Figs 1b, 1c and 2a).

Histological assessment of lungs from the OVA-challenged STAT6^{-/-} mice also showed clear widespread inflammatory infiltrates. However, eosinophilia was not induced either at 3 h (Figs 1e and 2b) or 24 h (Fig. 1f) after the OVA challenge. Cellular infiltrates were localized predominantly around the airway wall and blood vessels. These inflammatory cells included lymphocytes and monocytes, but not eosinophils. Despite the presence of cellular infiltration, OVA aerosolization of STAT6^{-/-} mice induced minimal morphologic changes in pulmonary structure (Figs 1e, 1f and 2b).

Analysis of cellular infiltrates in STAT6^{-/-} mice

To identify which subsets of T lymphocytes infiltrate into the lungs of wild-type and STAT6^{-/-} mice after OVA challenge, we examined immunohistochemically CD4⁺ and CD8⁺ T cell subsets in lung tissue. CD4⁺ and CD8⁺ cell numbers in lungs taken from mice exposed to saline aerosol were similar in wild-type and STAT6^{-/-} mice (Figs 3a, 3d, 4a, 4d, and Fig. 5). Aerosolized OVA challenge in wild-type mice induced a significant increase in CD4⁺ T cells ($P < 0.01$) (Fig. 3b) but not induced an increase in CD8⁺ T cells (Fig. 4b). Aerosol challenge of STAT6^{-/-} mice also induced an increase in CD4⁺ T cells ($P < 0.01$) (Fig. 3e,f). At both 3 h and 24 h, the OVA-challenged STAT6^{-/-} mice had a significantly greater number of CD4⁺ T cells compared with the OVA-challenged wild-type mice ($P < 0.01$) (Figs 3b, e, 3c, f, respectively). In more marked contrast to the results with wild-type mice, CD8⁺ T cell infiltration was induced in STAT6^{-/-} mice lung after OVA challenge ($P < 0.01$ vs each other group) (Figs 4e and 5a). The CD8⁺ T-cell infiltrates were localized primarily around airways and blood vessels. After 24 h, the number of CD8⁺ T cells was still greater in STAT6^{-/-} mice than that in wild-type mice ($P < 0.01$ vs each other group) (Figs 4f and 5b).

Analysis of the local expression of cytokines in STAT6^{-/-} mice

In supernatant from total lung tissue obtained from OVA-challenged wild-type mice, the levels of TH₂ cytokines such as IL-4 and IL-5 were significantly higher than those in supernatant from saline-challenged wild-type mice. In contrast, TH₂ cytokine levels in supernatant from OVA-challenged STAT6^{-/-} mice were not increased. The OVA-challenged wild-type mice showed a 6–10-fold increase in IL-4 in lung tissue supernatants compared with other groups (at 3 h, $P < 0.01$; at 24 h, $P < 0.05$ vs each other group). The OVA-challenged wild-type mice showed a 2–10-fold increase in IL-5 concentration in lung tissue supernatants compared with other groups (at 3 h, $P < 0.01$ vs saline-treated wild-type or STAT6^{-/-} mice,

$P < 0.05$ vs OVA-challenged STAT6^{-/-} mice; at 24 h, $P < 0.05$ vs saline-treated wild-type or STAT6^{-/-} mice). No significant difference in the level of IFN γ in lung tissue supernatants was found either between saline and OVA treatment or between wild-type and STAT6^{-/-} mice.

Analysis of serum IgE and IgG1 levels in STAT6^{-/-} mice

We measured serum IgE and IgG1 levels at 3 h after the last challenge with saline or OVA (Fig. 7). In saline-challenged STAT6^{-/-} mice there was no detectable IgE or IgG1, but

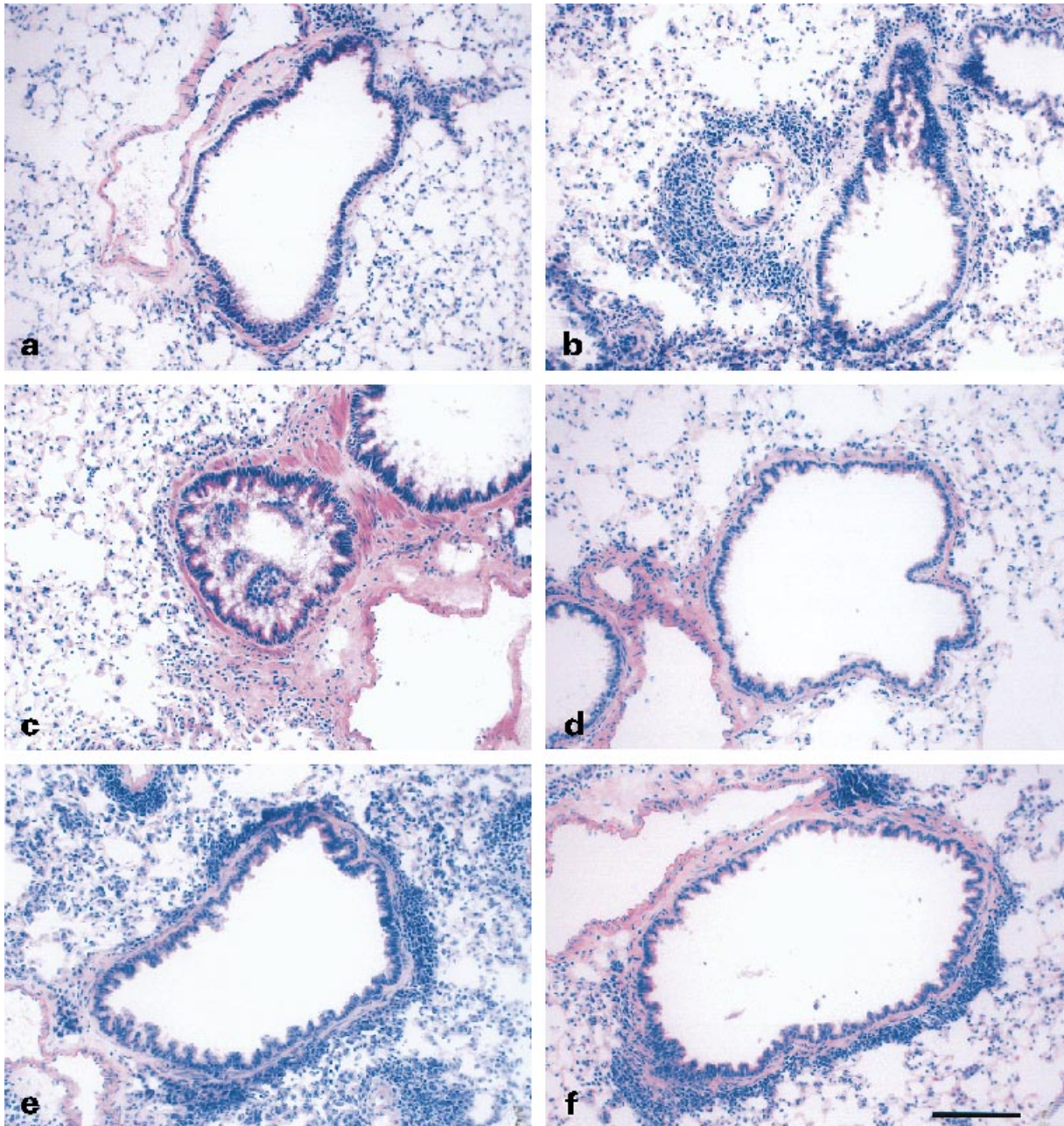


Fig. 1. Histological analysis of lung sections. (a) Wild-type (STAT6^{+/+}) mice that sensitized and exposed to a saline aerosol (non-sensitized wild or STAT6^{-/-} mice not shown). (b and c) Sensitized STAT6^{+/+} mice 3 h (b) and 24 h (c) after the last aerosolized OVA exposure. (d) STAT6^{-/-} mice that were sensitized and exposed to a saline aerosol. (e and f) Sensitized STAT6^{-/-} mice 3 h (e) and 24 h (f) after the last aerosolized OVA exposure. Sections shown are representative of 12 sections of lung per mouse from four mice in each group. Lung tissue was stained with Giemsa's solution. Bar = 100 μ m.

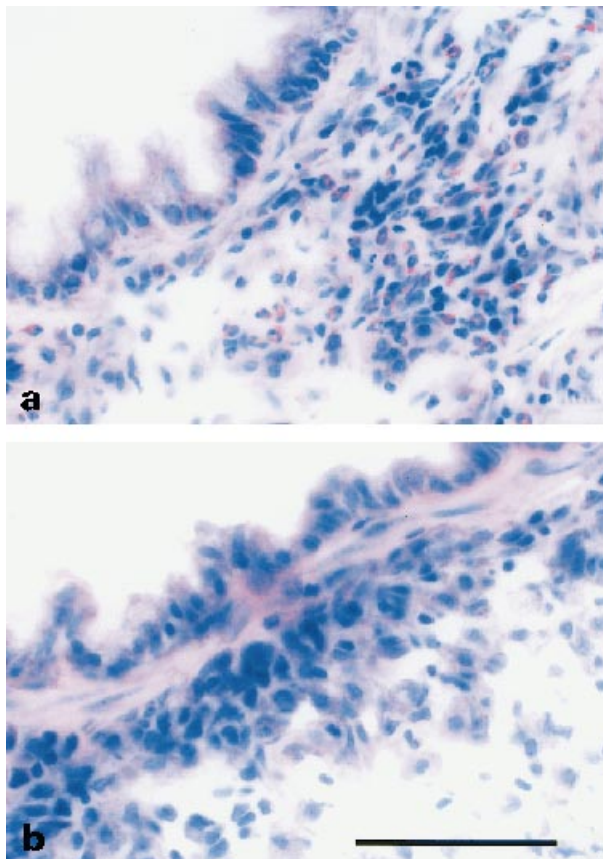


Fig. 2. Higher magnification of photomicrographs representing inflammatory cells that migrated in the bronchial walls of wild-type ($STAT6^{+/+}$) mice (a) and $STAT6^{-/-}$ mice (b) 3 h after the last aerosolized OVA exposure. Note that no eosinophils was detected around the bronchus of $STAT6^{-/-}$ mice. Bar = 100 μ m.

low level of IgG1 was detected in saline-challenged wild-type mice. Aerosol challenge of wild-type mice with OVA induced a dramatic increase in serum IgE and IgG1. Although IgG1 response was observed in OVA-challenged $STAT6^{-/-}$ mice, IgG1 levels were 50-fold lower than those in OVA-challenged wild-type mice. IgE remained undetectable even after OVA challenge in $STAT6^{-/-}$ mice.

Discussion

Role of *STAT6* in pulmonary eosinophilia

The present study clearly demonstrated that pulmonary eosinophilia was not induced anew in OVA aerosolization in sensitized $STAT6^{-/-}$ mice of C57BL/6 background, suggesting that *STAT6* activation plays a central role in pathogenesis of allergic airway inflammation with eosinophilia. Furthermore, the present study showed that production of TH2 cytokines in the lung as well as IgE and IgG1

responses after OVA challenge were profoundly reduced in $STAT6^{-/-}$ mice. Evidence has been accumulated that TH2 cytokines produced by $CD4^{+}$ lymphocytes are involved in the pathophysiology of bronchial asthma [31,32]. Our cytokine data also suggest that IL-4 and IL-5 were produced by T cells (and/or eosinophils) migrating into the lung tissue since the peak of local cytokine release occurred 3 h in wild-type mice after OVA challenge. Because *STAT6* plays a central role in exerting IL-4-mediated biological responses which include production of TH2 cytokines such as IL-4 and IL-5 from $CD4^{+}$ T lymphocytes and class switching of B cell immunoglobulin to IgE [25], these results further support the view that allergic eosinophilic inflammation predominantly correlates with the induction of TH2-type cytokine production and IgE synthesis [5,6,33].

STAT6 in TH2 response

Clinical and experimental observations have identified IL-4 and IL-5 as key molecules in the regulation of the effector function of mast cells and eosinophils, which underlie the development of allergic airway inflammation. However, there is conflicting evidence about the comparative importance of IL-4 in the events which initiate the structural and functional changes in the airways in response to aeroallergens [14,17–19]. Corry *et al.* have reported that IL-4 but not IL-5 or eosinophils is required in a murine model of acute airway hyperreactivity [19], whereas Foster's group has indicated IL-5 but not IL-4 is essential for the induction of eosinophilic inflammation, lung damage, and airway hyperreactivity [18,19]. Our data indicate the importance of activation of IL-4 signalling for the eosinophilia and structural changes in the airways.

IL-4 exerts various biological effects by regulating proliferation and differentiation of a wide variety of lymphoid and myeloid cells [11]. The role of IL-4 in TH2 differentiation has been indicated in studies using $IL-4^{-/-}$ mice [13]. Our previous data [25] have almost completely agreed with data from studies of $IL-4^{-/-}$ mice or using antibodies and the IL-4 receptor [13,34,35]. The similarity of $STAT6^{-/-}$ mice to $IL-4^{-/-}$ mice suggests that *STAT6* evolved for IL-4-specific functions. However, activation of the IL-4 receptor involves interaction of the ligand with both the IL-4 receptor α -subunit and the common γ -chain, which is also a component of the IL-2 and IL-7 receptors [36,37]. In addition, both IL-3 and IL-13 activate *STAT6* [23,37]. Thus, although no TH2 differentiation by IL-4 signalling occurs, the presence of other alterations which is not apparent in IL-4 or its receptor deficiency, also is expected in $STAT6^{-/-}$ mice.

Lymphocyte infiltration in *STAT6*^{-/-} mice with OVA challenge

We have shown in this study that $STAT6^{-/-}$ mice with

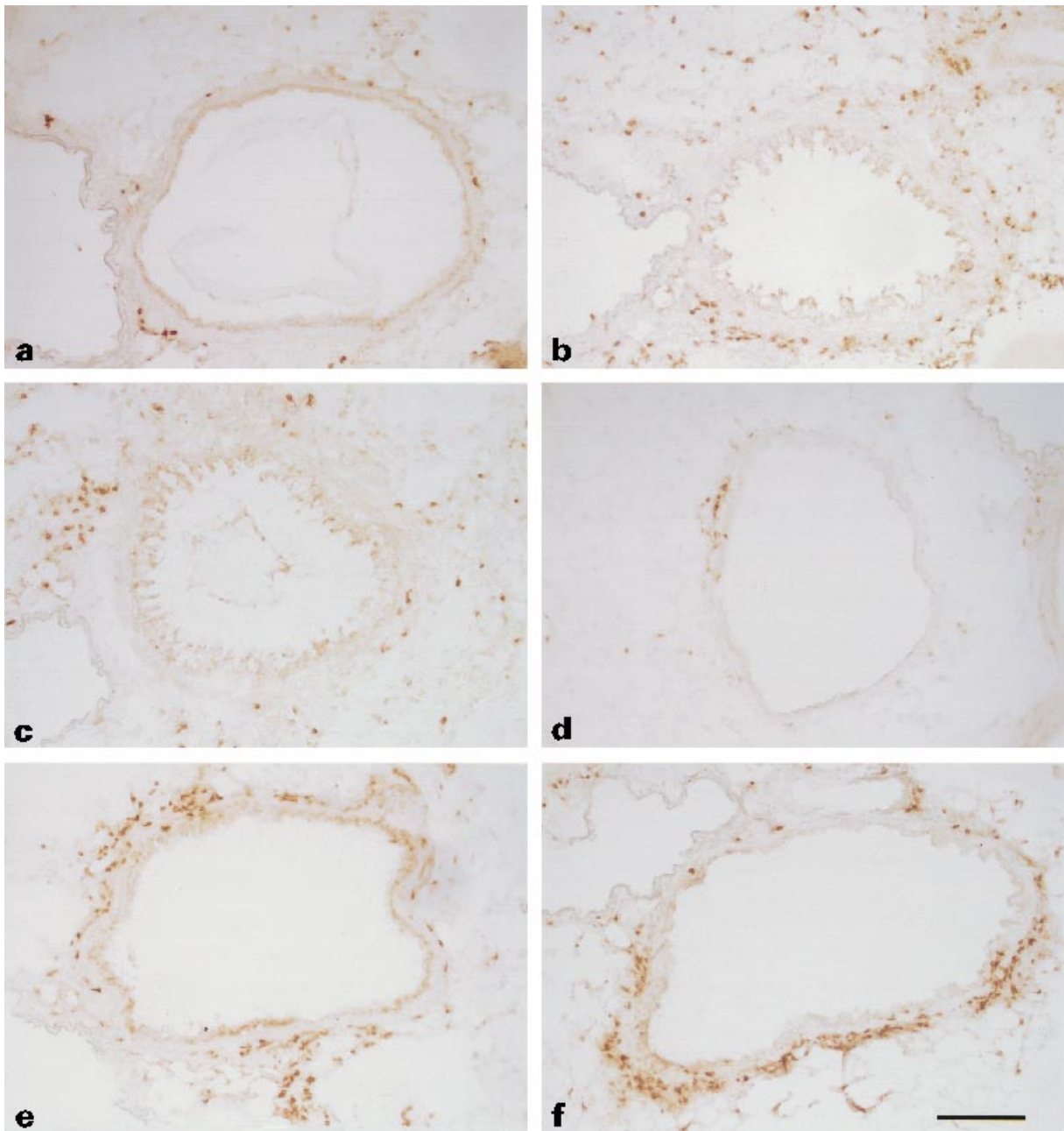


Fig. 3. Immunohistochemical identification of CD4⁺ T cells in the lung from wild-type (STAT^{+/+}) mice (a – c) or STAT6^{-/-} mice (d–f) given aerosolized saline (a and d) or OVA (b, c, e and f). Sections shown are representative of 12 sections of lung per mouse from four mice in each group. Lung tissue was obtained from mice 3 h (b and e) or 24 h (c and f) after the last OVA exposure and was subjected to immunohistochemistry as described in the text. Bar = 100 μ m.

OVA challenge revealed clear T cell (especially CD8⁺ T cell) infiltrates in the lung, although they showed minimal morphological changes in airway structure. Lung histology from these animals may represent pulmonary inflammation characterized by T-cell infiltration. These findings have not

been reported in IL-4^{-/-} mice [14,16], but similar distinct lung pathology has been reported in the murine model of viral infection, in which mice sensitized to the attachment glycoprotein of respiratory syncytial virus exhibit a strong IL-4 and IL-5 response and develop pulmonary eosinophilia,

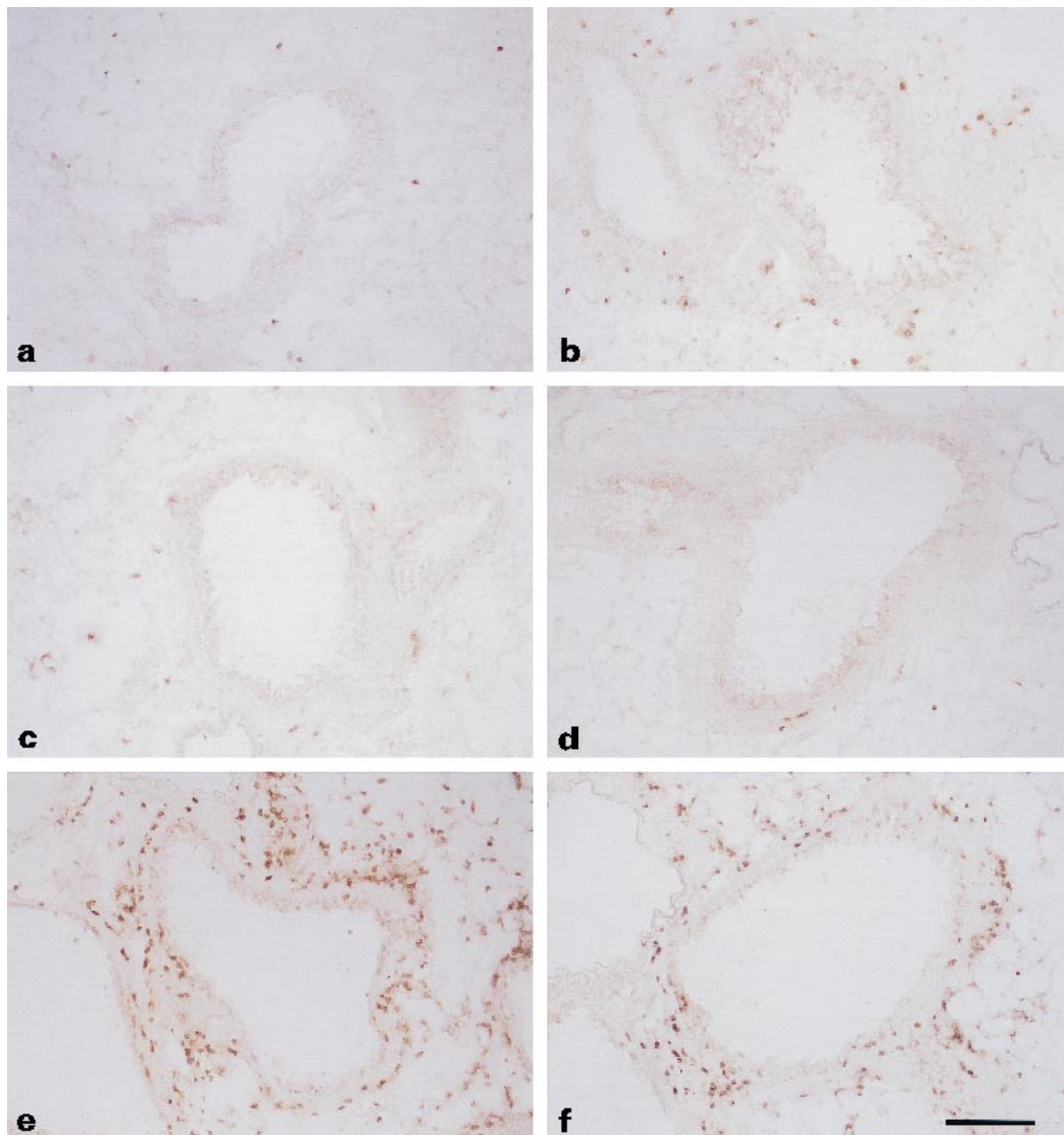


Fig. 4. Immunohistochemical identification of CD8⁺ T cells in the lung from wild-type (STAT^{+/+}) mice (a – c) or STAT6^{-/-} mice (d – f) given aerosolized saline (a and d) or OVA (b, c, e and f). Sections shown are representative of 12 sections of lung per mouse from four mice in each group. Lung tissue was obtained from mice 3 h (b and e) or 24 h (c and f) after the last OVA exposure and was subjected to immunohistochemistry as described in the text. Bar = 100 μ m.

whereas mice sensitized to the fusion glycoprotein of the virus develop a predominantly TH1 response and pulmonary inflammation characterized by a mononuclear cell infiltration [38,39]. These findings may suggest that TH1 predominant responses are concomitant with

‘TH1-like’ CD8⁺ cytotoxic T-cell infiltration. The present study suggested that OVA-challenged STAT6^{-/-} mice also develop a predominantly TH1 response, in which TH2 cytokine levels were not increased. However, it is possible that OVA-challenged STAT6^{-/-} mice developed neither Th1

response nor TH2 response, because there were no changes in IFN γ levels between the saline- and OVA-challenged mice. Although the precise mechanism is unknown, the aeroallergen-induced lymphocyte infiltration in STAT6 $^{-/-}$ mice may suggest

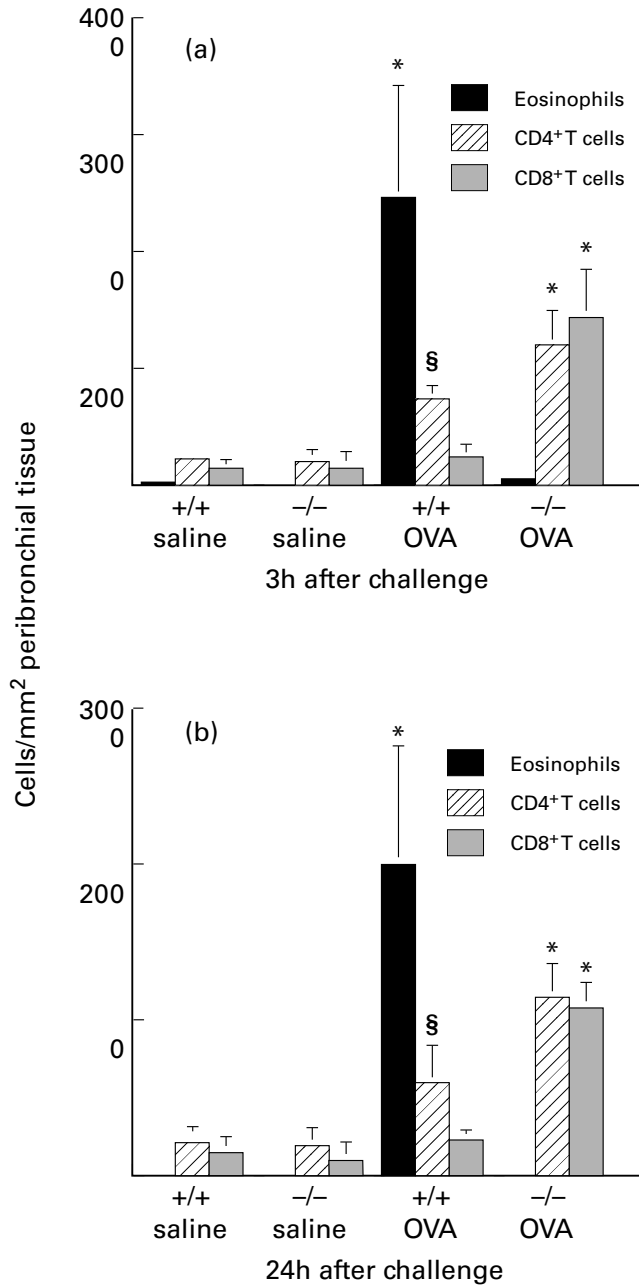


Fig. 5. Eosinophil, and CD4⁺ and CD8⁺ T cell numbers in lung sections in saline and aeroallergen-challenged, sensitized STAT6^{+/+} and STAT6^{-/-} mice. Data represent the mean $\pm$ SD for groups of four mice. The significance of differences between experimental groups was analysed using Scheffe-type multiple comparison test. Cellular infiltrates were assessed at 3 h (a) and 24 h (b) after the last challenge. * $P < 0.01$ compared with all other groups; † $P < 0.01$ compared with +/+ saline and -/- saline groups; § $P < 0.01$ compared with +/+ saline and -/- saline groups.

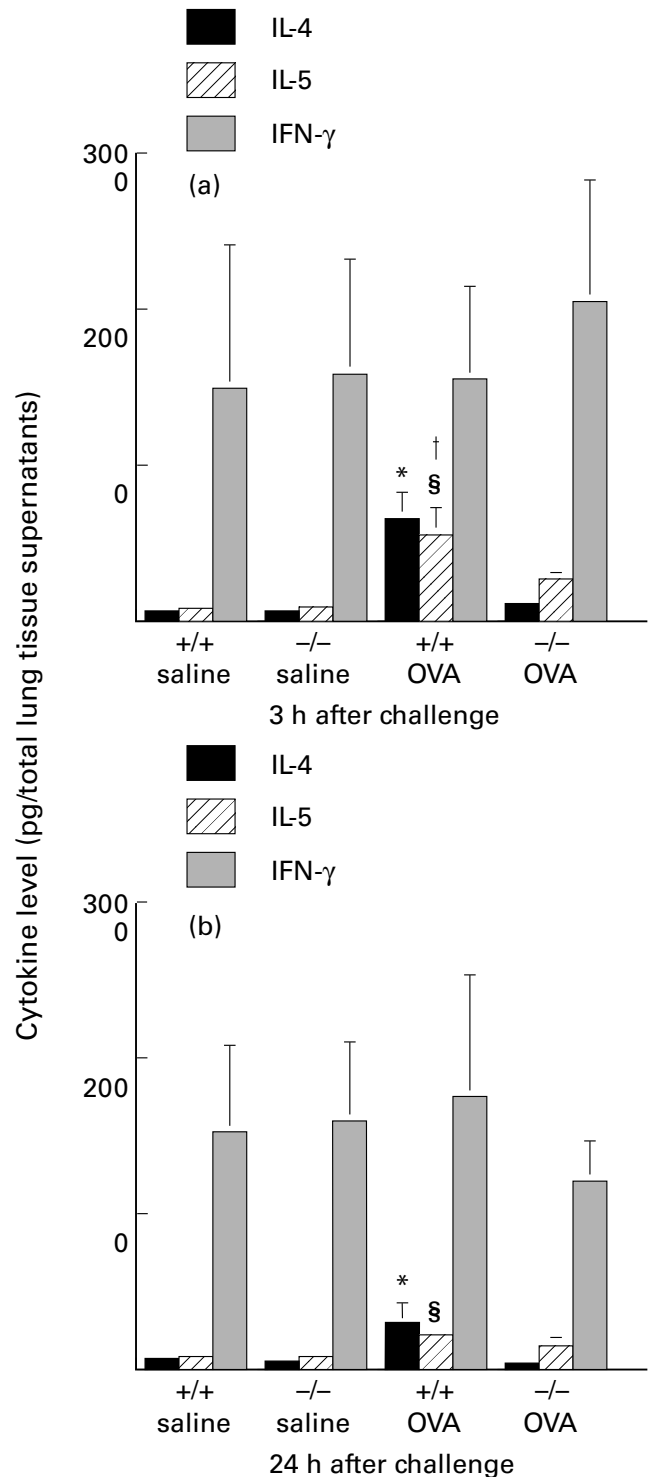


Fig. 6. Cytokine levels in lung tissue supernatants in saline and aeroallergen-challenged, sensitized STAT6^{+/+} and STAT6^{-/-} mice. Data represent the mean $\pm$ SD for groups of four mice. Cytokine levels were measured by ELISA at 3 h (a) and 24 h (b) after the last challenge. (a) * $P < 0.01$ compared with all other groups; § $P < 0.01$ compared with +/+ saline and -/- saline groups; † $P < 0.05$ compared with -/-OVA group. (b) * $P < 0.05$ compared with all other groups; § $P < 0.05$ compared with +/+ saline and -/- saline groups.

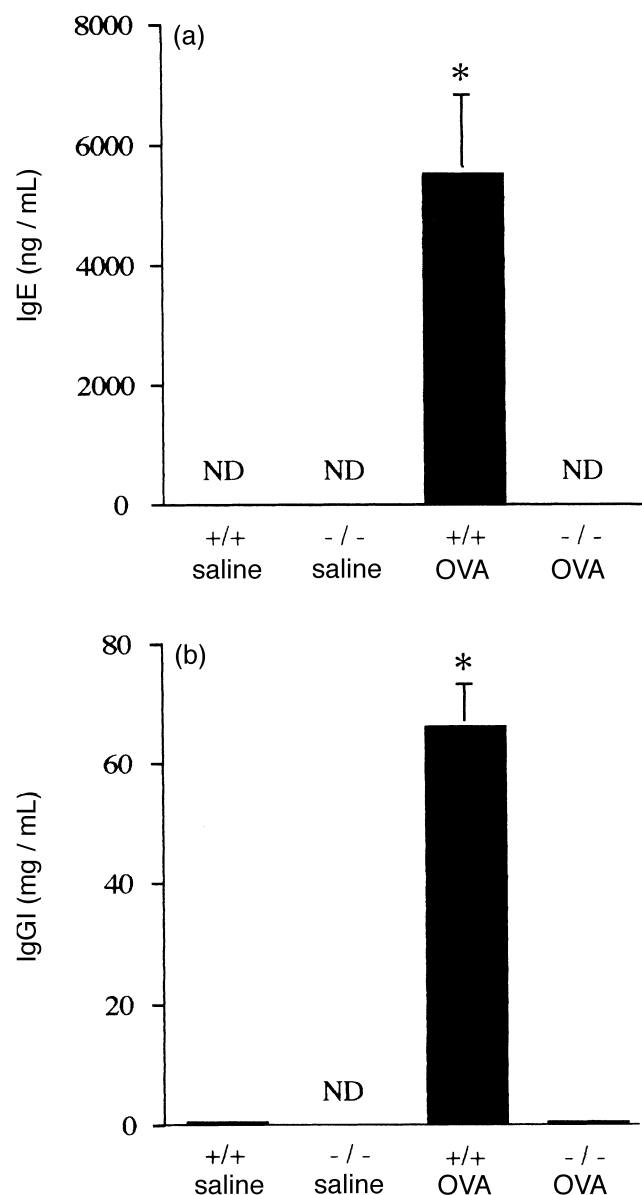


Fig. 7. Serum IgE (a) and IgG1 (b) levels in saline and aeroallergen-challenged, sensitized STAT6^{+/+} and STAT6^{-/-} mice. Data represent the mean $\pm$ SD for groups of four mice. Immunoglobulin levels were measured by ELISA at 3 h after the last challenge. * $P < 0.01$ compared with all other groups.

the involvement of activation of signalling other than STAT6 in developing the inflammation characterized by T-cell infiltration.

The functional significance on increasing number of CD8⁺ cells in the lung of STAT6^{-/-} mice undergoing OVA challenge, also remained to be clarified. In a murine model of viral infection triggered by virus peptide, the virus-specific CD8⁺ cells that help to control infection by

producing IFN γ and lysing virus-infected cells [29]. These cells can also switch to IL-5 production and induce airway eosinophilia. Recent studies have indicated that IL-4 signalling is important in the switching of CD8⁺ T cells as well as CD4⁺ T cells to a TH2 phenotype [28–30]. The CD8⁺ cells in the lung of OVA-challenged STAT6^{-/-} mice is not likely to switch to TH2 phenotype, because no increase in TH2 cytokine levels in their lung. The present results suggest that activation of STAT6 signalling has a crucial role in regulating TH2-type cytokine production both in CD4⁺ T cells and CD8⁺ T cells. In conclusion, the present study shows that STAT6 gene has an important role in the regulation of eosinophilia, and allows for further understanding of the contribution of STAT6 to allergic inflammation.

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