

STAT6-mediated signaling in Th2-dependent allergic asthma: critical role for the development of eosinophilia, airway hyper-responsiveness and mucus hypersecretion, distinct from its role in Th2 differentiation

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Abstract

When wild-type BALB/c mice were transferred with OVA-specific Th2 cells followed by OVA inhalation, a severe eosinophilia, mucus hypersecretion and airway hyper-responsiveness (AHR) was induced in parallel with a marked elevation of IL-4, IL-5 and IL-13 levels in bronchoalveolar lavage fluid (BALF). However, neither eosinophilia, AHR nor mucus hypersecretion was induced in Th2 cell-transferred STAT6^{-/-} mice. The failure of eosinophilia was not due to the defect of Th2 cytokine production in BALF of STAT6^{-/-} mice transferred with Th2 cells, but because of the defect of STAT6-dependent eotaxin production. Indeed, intranasal administration of eotaxin reconstituted pulmonary eosinophilia but not AHR and mucus hypersecretion in OVA-inhaled STAT6^{-/-} mice. These results initially provided direct evidence that STAT6-dependent eotaxin production is essential for pulmonary eosinophilia. We also dissociated the role of STAT6 for eosinophilia from that for AHR and mucus hypersecretion. Thus, STAT6 also plays a critical role at late phase of Th2-dependent allergy induction.

Introduction

Allergic asthma can be characterized by reversible airway obstruction, elevated levels of IgE, chronic airway inflammation and airway hyper-responsiveness (AHR) to bronchoconstrictor stimuli (1). A considerable body of evidence has demonstrated that activation of T cells and eosinophilic infiltration of the airways play a critical role in the pathophysiology of allergic asthma (2,3). Activated CD4⁺ Th2 cells that are capable of producing IL-4, IL-5 and IL-13 are present in both the bronchoalveolar lavage fluid (BALF) and lung biopsies of atopic asthmatic patients. The presence of these cells and their inflammatory products in the lung often correlate with disease severity and the degree of AHR (4–6).

STAT6 is an essential transcription factor for IL-4 and IL-13 signaling. Using STAT6-deficient (STAT6^{-/-}) mice, it

was recently shown that STAT6 is essential for the induction of allergic asthma. The failure of these animals to develop allergic asthma has been considered to be due to defect in IL-4- and/or IL-13-dependent Th2 differentiation and Ig class switching to IgE (7–9). Thus, the importance of STAT6 for the initial induction phase of Th2-dependent allergic responses has been emphasized using STAT6^{-/-} mice (10,11). However, recently, several groups have reported that STAT6 also plays, in addition to its role in Th2 differentiation, a critical role at the late phase of allergic asthma. Kuperman *et al.* (10), using BALB/c background STAT6^{-/-} mice, reported that AHR and IgE production were totally dependent on STAT6 signaling, but that eosinophilia was only partially dependent upon STAT6 signaling. In contrast, Akimoto *et al.* (11) demonstrated that

both AHR and pulmonary eosinophilia were fully dependent on STAT6 signaling in C57BL/6 background mice. Thus, the precise role of STAT6 in the late phase of Th2-dependent allergic asthma is still controversial (12).

To evaluate the precise role of STAT6-mediated signaling in the late effector phase of allergic asthma, we utilized an adoptive transfer model of allergic disease. We adoptively transferred wild-type and STAT6^{-/-} mice with OVA antigen-specific T cells that were differentiated *ex vivo* into Th2 cells. This model permitted us to dissect the role of STAT6 during the late effector phase of allergic asthma, independently of its role in Th2 differentiation. In the present paper, we demonstrate that STAT6 plays a pivotal role not only in the initial phase of Th2 development but also in the late effector phase of allergic asthma. STAT6 is essential for AHR, mucus hypersecretion and eosinophilia, but not for VCAM-1 expression on lung vascular cells. We provide direct evidence that the failure of STAT6^{-/-} mice to develop eosinophilia is due to defective eotaxin production in the lung. We also dissociated the role of STAT6 for eosinophilia from that for AHR and mucus hypersecretion.

Methods

Mice

STAT6-deficient (STAT6^{-/-}) mice were generously provided from Tularik (South San Francisco, CA) (8). BALB/c mice were purchased from Charles River Laboratories (Shizuoka, Japan) and used as wild-type mice. OVA₃₂₃₋₃₃₉-specific I-A^d-restricted T-cell receptor transgenic mice (DO11.10) on a BALB/c background were kindly donated by Kenneth Murphy (Washington University, School of Medicine, St Louis, MO) (13). All mice were female and were used at 5–6 weeks of age. All studies reported here have been reviewed by the Taisho Pharmaceutical Animal Care Committee and have met the Japanese Experimental Animal Research Association standards, as defined in the Guidelines for Animal experiments (1987).

Generation of Th2 cells

Th2 cells were generated as previously reported (14). Briefly, Th2 cells were induced from CD4⁺CD45RB⁺ naive Th cells isolated from DO11.10 TCR Tg mice. Cells were cultured in RPMI1640 medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal calf serum (Nichirei, Tokyo, Japan) with 5 µg/ml OVA peptide (OVA₃₂₃₋₃₃₉) in the presence of IL-4 (1 ng/ml; PM-19231W, Pharmingen, San Diego, CA) plus anti-IL-12 mAb (10 µg/ml; C15.6, Pharmingen) and anti-IFN-γ mAb (10 µg/ml; R4-6A2, Pharmingen). Functional differentiation of Th2 cells was confirmed by intracellular staining with FITC-conjugated anti-IFN-γ and PE-conjugated anti-IL-4 mAb or measuring cytokine production patterns using ELISA kits (Endogen, Cambridge, MA).

Induction of allergic asthma in Th2-cell transferred mice by OVA inhalation

The adoptive transfer model of Th2-dependent allergic asthma was established as described previously (15). Briefly, Th2 cells (1×10^7) in 0.2 ml of PBS were injected into the tail vein of wild-type or STAT6^{-/-} recipient BALB/c mice. Two days after the cell

transfer, mice were exposed daily to aerosolized OVA (100 mg/ml in 0.9% saline, 30 min) for 3 days. The aerosol was generated by a nebulizer (DeVilbiss 646 nebulizer, DeVilbiss Corp., Somerset, PA) driven by compressed air at 18 l/min. Seventy-two hours after the first OVA inhalation, mice were subjected to pulmonary function testing and sacrificed for BAL analysis.

Measurement of AHR

AHR was measured by acetyl-β-methylcholine (Mch)-induced airflow obstruction as previously reported (15). Briefly, mice were placed into whole body plethysmographs (Buxco Electronics, Inc., Troy, NY), interfaced with computers using differential pressure transducers. Measurements were performed of respiratory rate, tidal volume and enhanced pause. Airway resistance is expressed as: $Penh = [(Te/0.3Tr) - 1] \times [2Pef/3Pif]$, where Penh = enhanced pause, Te = expiratory time (s), Tr = relaxation time (s), Pef = peak expiratory flow (ml) and Pif = peak inspiratory flow (ml/s). Increasing doses of Mch were administered by nebulization (for 1 min), and Penh were calculated over the subsequent 3 min.

Characterization of cells in BALF

After measuring airway reactivity, the trachea was cannulated with a polyethylene tube through which the lungs were gently lavaged three times with 0.8 ml of PBS containing 0.1% BSA. Cells were stained with hematoxylin peroxidase and differentials were performed based on morphology and staining characteristics. Supernatants of BALF were kept frozen at -20°C until use. Cytokine levels were measured in samples of BALF from each animal by ELISA as described above.

Administration of mouse recombinant eotaxin

To evaluate the role of eotaxin, in some experiments, Th2 cell-transferred mice received intranasal mouse recombinant eotaxin (PeproTech, Rocky Hill, NJ: 4 µg in 50 µl of PBS), 3 h after each antigen challenge.

Histological examination of lung sections

Lungs were prepared for histology by perfusing the animal via the right ventricle with 5 ml of PBS. Lungs were then perfused with 1.0 ml of 10% neutralized formalin. Samples for Technovit 7100 resin sectioning (Heraeus Kulzer, Wehrheim, Germany) were formalin fixed and sectioned in the coronal plane at 2 µm, ensuring that central airways were visible. Sections were stained with hematoxylin-eosin and periodic acid-Schiff (PAS).

For the immunohistochemical detection of VCAM-1, lungs were excised and placed in 2% paraformaldehyde fixative overnight at 4°C. They were then soaked in 30% sucrose for 2 days at 4°C and then snap frozen in isopentane cooled with liquid nitrogen and stored at -80°C. Cryostat sections (5 µm) were cut and melted onto coated slides. Nonspecific reactions were minimized by blocking with 1% BSA/PBS. Sections were incubated with either rat anti-mouse VCAM-1 antibodies (M/K-2, rat IgG; Serotec, Oxford, UK; 1 mg/ml, 1:2000) or an isotype-matched control antibody (rat IgG; Vector Laboratories, Burlingame, CA) at the same dilution, overnight at 4°C. After washing, sections were incubated with mouse-absorbed goat anti-rat IgG antibodies (Vector Laboratories, 10 µg/ml) for 1 h

at room temperature. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Positive VCAM-1 staining was detected using the avidin-biotin-peroxidase complex method (ABC kit; Vector Laboratories) using 3-amino-9-ethylcarbazole (AEC chromogen, Sigma, St Louis, MO).

Statistical analysis

Data are reported as mean $\pm$ SE. Differences between means were determined using the two-tailed Student's *t*-test and significance was assumed at *P*-values of <0.05 .

Results

STAT6 is essential for the induction of AHR and eosinophilia in an allergic asthma model established by OVA-specific Th2 cell transfer and OVA inhalation

To determine the role of STAT6 in the effector phase of antigen-induced AHR, airway reactivity to inhaled Mch was measured in Th2 cell-transferred BALB/c wild-type and STAT6^{-/-} mice (Fig. 1). Th2 cell-transferred BALB/c wild-type mice exposed to aerosolized OVA developed a significant increase in airway reactivity to Mch (Fig. 1A). In sharp contrast, no such increase of AHR was induced in similarly treated STAT6^{-/-} mice (Fig. 1B). These data indicated that STAT6-mediated signaling is essential for AHR induced by adoptive transfer of Th2 cells and OVA inhalation.

We also examined the role of STAT6 in airway inflammation. The profile of inflammatory cells in BALF obtained from adoptively transferred and OVA-exposed BALB/c wild-type and STAT6^{-/-} mice was compared. As clearly shown in Fig. 2, BALB/c wild-type mice developed profound eosinophilia whereas little eosinophilia was observed in STAT6^{-/-} mice. These data indicated that, in addition to its role in AHR, STAT6-mediated signaling is critical for the induction of eosinophilia.

Production of significant levels of Th2 cytokines but not eotaxin in BALF of STAT6^{-/-} mice adoptively transferred with Th2 cells and exposed to OVA

Our experimental model involves the adoptive transfer of *ex vivo*-induced Th2 cells into STAT6^{-/-} or wild-type mice, followed by OVA inhalation. It is therefore unlikely that the defect of AHR and eosinophilia in STAT6^{-/-} mice is independent of defects in Th2 immunity in these animals. To confirm this, we examined whether transferred Th2 cells were able to respond to inhaled antigen in the lung to produce Th2 cytokines. For this purpose, we determined the levels of Th2 cytokines (IL-4, IL-5 and IL-13) in BALF of wild-type and STAT6^{-/-} mice by ELISA. As illustrated in Fig. 3, increased levels of IL-4, IL-5 and IL-13 were detected in BALF of STAT6^{-/-} mice, which were similar to those detected in wild-type mice (Fig. 3A–C), indicating that transferred Th2 cells respond equally well to inhaled antigen in wild-type and STAT6^{-/-} mice. This finding confirmed that the failure of STAT6^{-/-} mice to develop AHR and eosinophilia in this model of allergic asthma was independent of the defect in Th2 development during the initiation phase, but instead due to defects during the late effector phase of allergic asthma.

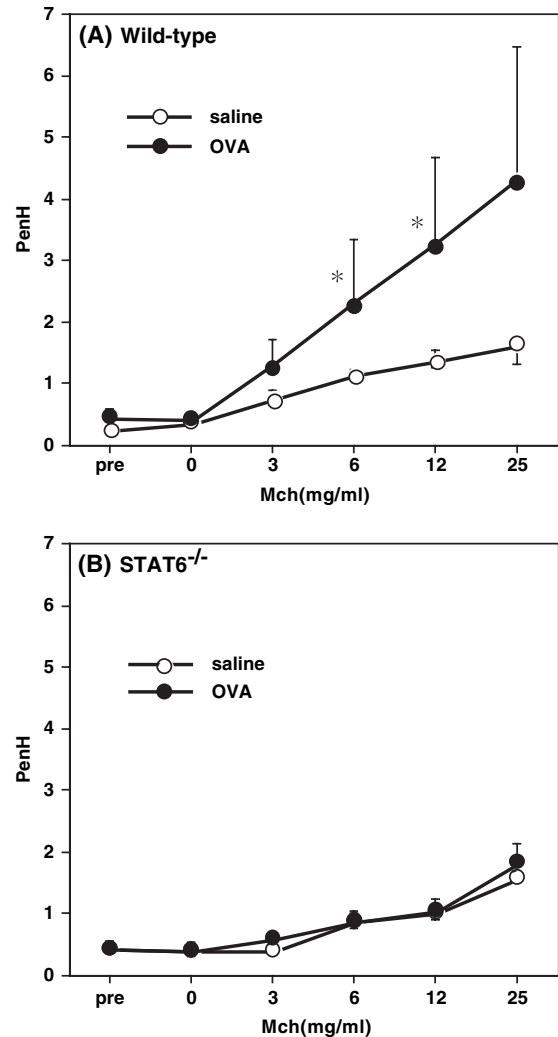


Fig. 1. STAT6 is essential for the induction of AHR in an allergic asthma model established by OVA-specific Th2 cell transfer and OVA inhalation. Th2 cells (1×10^7), which were induced from naive CD4⁺ T cells from DO11.10 transgenic mice, were intravenously injected into normal or STAT6^{-/-} recipient mice. Two days after the cell transfer, wild-type (A) and STAT6^{-/-} (B) mice were exposed to saline (open circles) or OVA (closed circles). Twenty-four hours after three consecutive OVA exposures, mice were placed in whole body plethysmographs and underwent methacholine inhalation. Basal values (pre) were measured, followed by measuring the response to nebulized PBS (0) and to increasing concentrations of methacholine (3–25 mg/ml). Values expressed are mean $\pm$ SE; *n* = 5 for each group; **P* < 0.05 as compared with saline-challenged mice.

In contrast to IL-4, IL-5 and IL-13, the levels of eotaxin, which is essential for the development of eosinophilia, were greatly decreased in BALF of STAT6^{-/-} mice compared with wild-type mice (Fig. 3D). These results indicated that STAT6-dependent signaling might play a crucial role at the late effector phase of allergic responses to produce eotaxin, which is essential for development of eosinophilia.

Reconstitution of eosinophilia but not AHR in STAT6^{-/-} mice by intranasal administration of recombinant eotaxin

To determine the precise role of STAT6-dependent eotaxin production for the induction of airway eosinophilia and AHR,

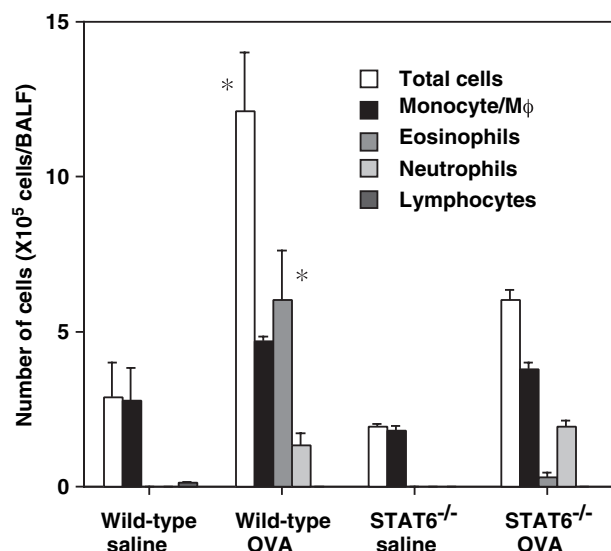


Fig. 2. STAT6-mediated signaling is essential for eosinophilia in the Th2 cell transfer model of allergic asthma. Wild-type BALB/c and STAT6^{-/-} BALB/c mice were transferred with Th2 cells and treated with OVA as described in the legend to Fig. 1. Twenty-four hours after OVA inhalation, cells harvested from BALF were stained with hematoxylin peroxidase and differentials were performed based on morphology and staining characteristics. The data represent mean $\pm$ SE; $n = 5$ for each group; * $P < 0.05$ as compared with saline-challenged mice.

STAT6^{-/-} mice were intranasally administered with recombinant eotaxin (4 μ g in 50 μ l of PBS) 3 h after each antigen challenge. As shown in Fig. 4(A), Th2 cell-transferred STAT6^{-/-} mice exhibited no significant eosinophilia in response to OVA inhalation. However, administration of eotaxin into Th2 cell-transferred STAT6^{-/-} mice reconstituted significant levels of OVA-induced eosinophilia. Such increase in eosinophilia by eotaxin administration was not seen in the absence of OVA inhalation. Thus, together with the data shown in Fig. 3, these findings demonstrate that pulmonary eosinophilia is completely dependent upon STAT6-dependent eotaxin production.

In contrast to its effects on eosinophilia, administration of recombinant eotaxin did not reconstitute AHR in STAT6^{-/-} mice, even when these animals were exposed to OVA (Fig. 4B). These data suggest that an additional unknown STAT6-dependent factor(s) may be crucial for the development of AHR.

Role of STAT6-dependent signaling for the induction of VCAM-1 expression and mucus hypersecretion

Because VCAM-1 expression is induced by IL-4 and IL-13, and is responsible for eosinophil recruitment (16,17), we examined its expression in the lung. We found that VCAM-1 expression in Th2 cell-transferred and antigen-challenged STAT6^{-/-} mice is equivalent to wild-type mice (Fig. 5B and E). Interestingly, VCAM-1 expression was induced in parallel with eosinophil infiltration in wild-type mice (Fig. 5C), while no significant eosinophilia was observed in STAT6^{-/-} mice (Fig. 5F). These data indicated that VCAM-1 expression was

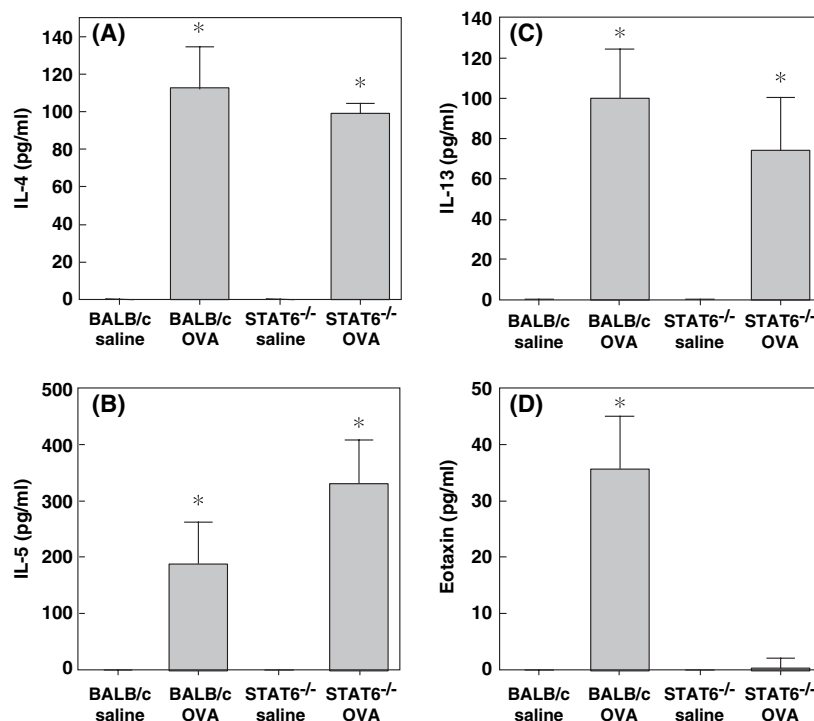


Fig. 3. Production of significant levels of Th2 cytokines but not eotaxin in BALF of STAT6^{-/-} mice following Th2 cell transfer and OVA challenge. Mice were treated as described in the legend to Fig. 1. Twenty-four hours after challenge with saline or OVA, cytokine levels (A, IL-4; B, IL-5; C, IL-13; D, eotaxin) were analyzed in BALF by sandwich ELISA. Values expressed are mean $\pm$ SE; $n = 5$ for each group; * $P < 0.05$ as compared with saline-challenged mice.

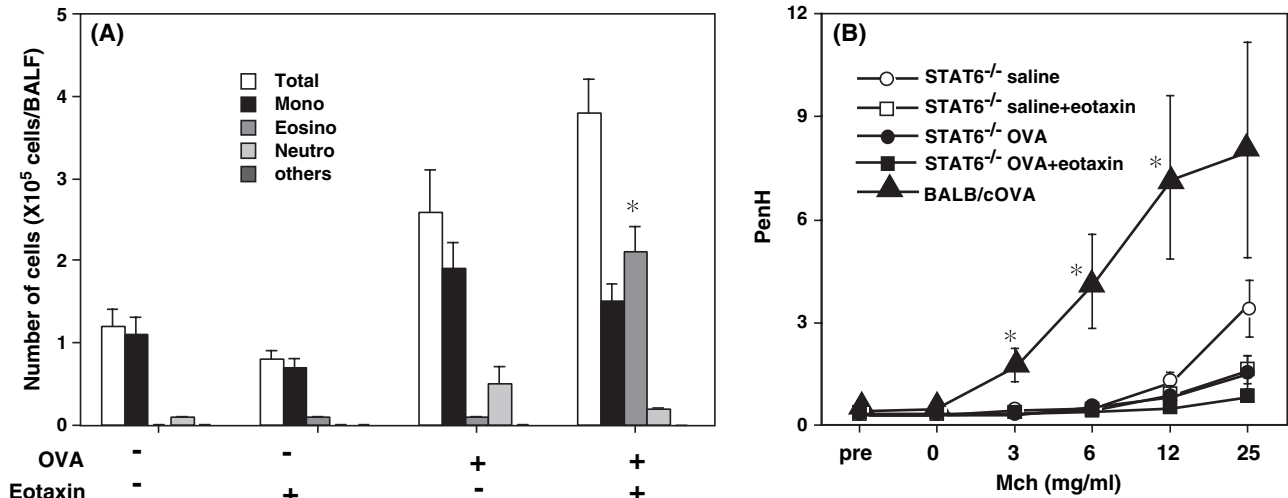


Fig. 4. Reconstitution of eosinophilia but not AHR in STAT6^{-/-} mice by intranasal administration of recombinant eotaxin. Mice were treated as described in the legend to Fig. 1. (A) Three hours after each OVA challenge, mice were treated intranasally with mouse recombinant eotaxin (4 µg). Twenty-four hours after three consecutive inhalations, BAL was conducted. Differential cell counts were performed as described in the legend to Fig. 2. Values expressed are mean ± SE; *n* = 5 for each group; **P* < 0.05 as compared with eotaxin-untreated mice. (B) Twenty-four hours after three consecutive inhalations, airway responsiveness to Mch was measured as described in the legend to Fig. 1. Values expressed are mean ± SE; *n* = 5 for each group; **P* < 0.05 as compared with saline-challenged mice.

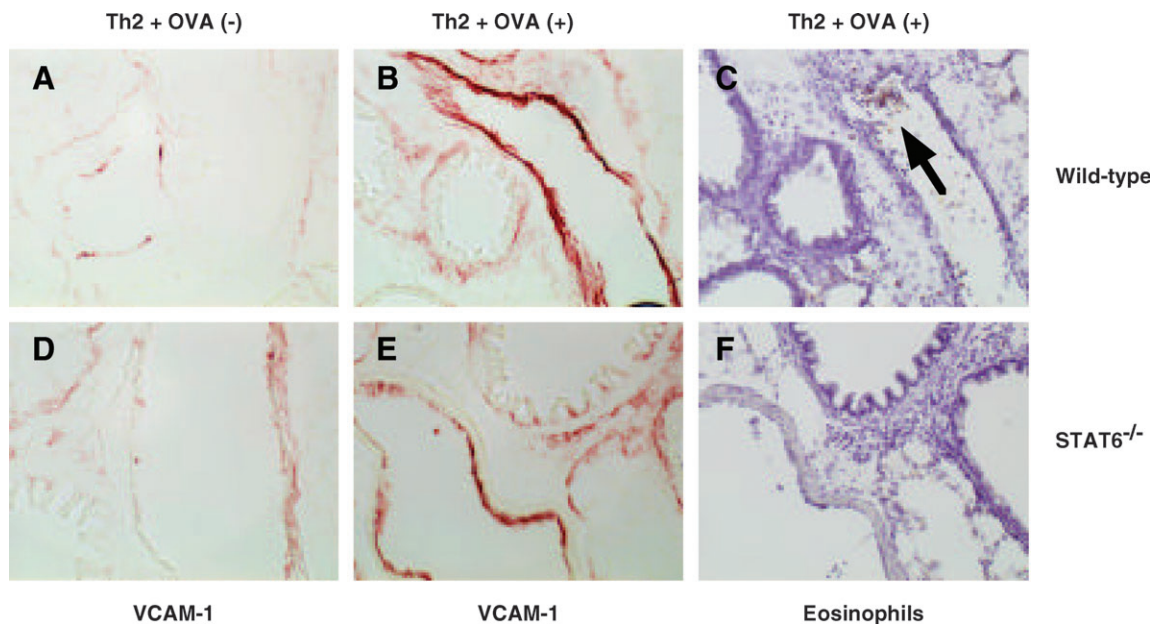


Fig. 5. STAT6-independent induction of VCAM-1 expression in lung sections of allergic mice transferred with Th2 cells. Two days after Th2 cell transfer, wild-type (A–C) or STAT6^{-/-} (D–F) mice were exposed to saline (A and D) or OVA (B, C, E and F). Twenty-four hours after OVA inhalation, immunohistochemical analysis of lungs was performed. Lung sections were incubated with anti-VCAM-1 mAb. Comparative VCAM-1-positive staining in sections from OVA-treated wild-type and STAT6^{-/-} mice was determined (B and E). Eosinophil infiltration was also examined by immunohistochemical staining with peroxidase (C and F). Infiltration of eosinophils is indicated by an arrow (C).

independent of STAT6-mediated signaling and that STAT6-dependent eosinophilia and AHR are not linked to the induction of VCAM-1 expression.

We also examined the role of STAT6 in mucus hypersecretion, which is responsible for the airway obstruction in asthma (18). As shown in Fig. 6, mucus positive goblet cells were increased

in OVA-challenged BALB/c wild-type mice (Fig. 6D). However, no significant increase of mucus positive cells was observed in STAT6^{-/-} mice (Fig. 6E). Administration of recombinant eotaxin was unable to reconstitute mucus hypersecretion in these animals (Fig. 6F). These data demonstrate that airway mucus production is induced in a STAT6-dependent manner but that

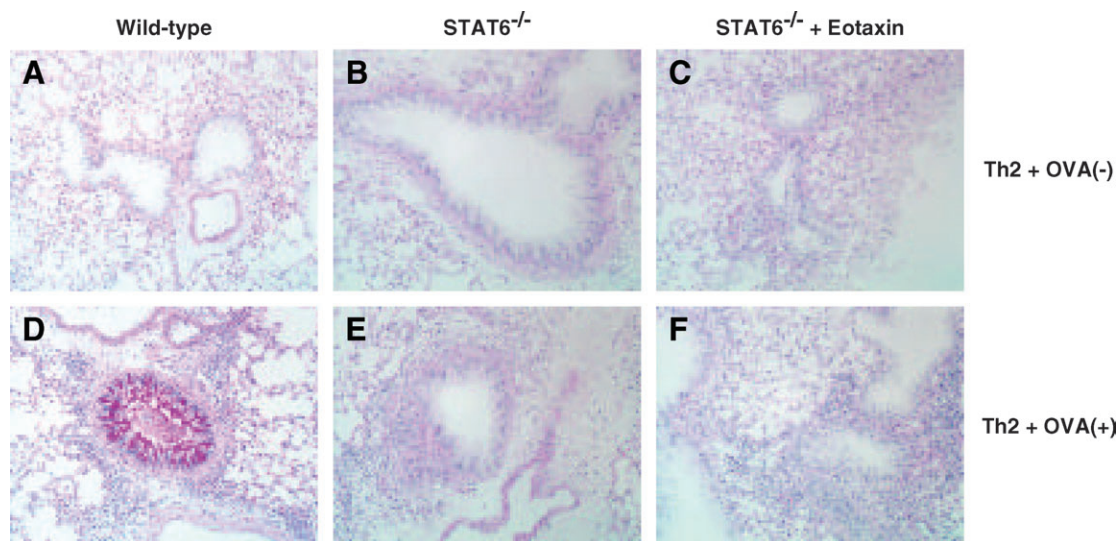


Fig. 6. Mucus hypersecretion is not reconstituted by eotaxin in STAT6^{-/-} mice. Lung sections from Th2 cell-transferred wild-type (A and D) and STAT6^{-/-} (B, C, E and F) mice were stained with periodic acid-Schiff. Mice were exposed to saline (A–C) or OVA (D–F). STAT6^{-/-} mice exposed to saline (C) or OVA (F) were treated intranasally with eotaxin. Only in the lung section of OVA-challenged wild-type mice was a large degree of goblet cell hyperplasia observed (D).

STAT6-dependent eotaxin production and eosinophilia are not linked with mucus hypersecretion.

Discussion

In this paper, we demonstrate that STAT6 plays a pivotal role for the development of pulmonary eosinophilia, AHR and mucus hypersecretion in a Th2 cell-transfer model of allergic asthma in mice. We provide direct evidence that STAT6-dependent eotaxin production, which is induced at the late effector phase of allergic airway responses, is essential for the development of eosinophilia but not for AHR or mucus hypersecretion.

It has been demonstrated that STAT6 is essential for early allergic responses through its role in Th2 cell development (7,8). However, our results indicate that STAT6 expressed in resident airway cells other than Th2 cells plays a critical role in the induction of AHR, eosinophilia and mucus hypersecretion.

Although the critical role of STAT6 for the induction of allergic asthma has been reported (10,11), it has been difficult to dissociate its role in the differentiation of Th2 cells from that of late allergic responses. To overcome this problem, we have examined the role of STAT6 in the late effector phase of allergic responses using an adoptive transfer model of allergic asthma (15). By transferring *ex vivo*-induced Th2 cells from wild-type animals into STAT6^{-/-} mice, it became possible to exclude the role of STAT6 for early Th2 cell development. Indeed, we demonstrated that the BALF of both wild-type and STAT6^{-/-} mice produced similar levels of Th2 cytokines such as IL-4, IL-5 and IL-13 (Fig. 3). Thus, Th2 immunity is equally induced at the local site of airway inflammation in both wild-type and STAT6^{-/-} mice treated with the adoptive transfer model. However, in contrast to wild-type mice, STAT6^{-/-} mice failed to develop a significant increase in AHR, eosinophilia and mucus hypersecretion (Figs 1, 2 and 6). These data indicate that STAT6 plays an essential role for inducing AHR, eosinophilia

and mucus hypersecretion by a mechanism that is distinct from its role for early Th2 cell development.

Recently Mathew *et al.* (19) have demonstrated a role of STAT6 for the induction of AHR using a Th2 adoptive transfer model. These investigators concluded that impaired trafficking of transferred Th2 cells and decreased IL-13 production are responsible for defective AHR induction in STAT6^{-/-} mice. However, in our model, transferred Th2 cells, which are functionally activated in the OVA-sensitized airway, produce similar levels of IL-4, IL-5 and IL-13 in STAT6^{-/-} and wild-type mice. These differences in experimental results might be due to differences in the number of transferred Th2 cells, concentration of inhaled OVA or frequency of OVA inhalation.

As shown in Fig. 3, BALF of Th2 cell-transferred wild-type and STAT6^{-/-} mice contained similar amounts of IL-4 and IL-13. Although the number of lymphocytes in the BALF was very limited (Fig. 2), the major cells that produce Th2 cytokines are considered to be transferred Th2 cells because a large number of transferred Th2 cells were reported to infiltrate in the lung tissue but not be detected in the BALF (20). Thus, it is possible that the Th2 cells infiltrated in the lung tissue were contributed to significant Th2 cytokines production in the BALF. In contrast to Th2 cytokines, eotaxin, which is essential for eosinophil migration, was detected only in the BALF of wild-type mice that received Th2 cells. Eotaxin was not produced by Th2 cells stimulated with antigen *in vitro* (data not shown). These data indicate that the resident cells, but not the transferred Th2 cells, might produce eotaxin following antigen inhalation. *In vitro*, both IL-4 and IL-13 could upregulate eotaxin production in airway epithelial cells, airway smooth muscle cells and airway fibroblasts (21–27). Thus, these cells that are stimulated with Th2 cytokines are considered to be responsible for the eotaxin production in the lung.

The finding that eotaxin was not detected in the BALF of Th2 cell-transferred STAT6^{-/-} mice is consistent with the study by Mathew *et al.* (19), which demonstrated that mRNA expression

for Th2-type chemokines (eotaxin, eotaxin-2, MDC and TARC) in the lung of STAT6^{-/-} mice was diminished. Therefore, we propose that STAT6 is essential for eotaxin production by IL-4- and/or IL-13-stimulated resident airway cells. This possibility is supported by the recent findings that (i) IL-4 and IL-13 induce eotaxin production *in vitro* and *in vivo* (28,29) and (ii) STAT6 is essential for eotaxin production triggered by IL-4 and TNF- α in fibroblasts (30). It has been assumed that STAT6-dependent eotaxin production and eosinophilia are linked with AHR and mucus hypersecretion. To answer these issues directly, we examined whether administration of recombinant eotaxin to STAT6^{-/-} mice can reconstitute AHR and mucus hypersecretion as well as eosinophilia. As indicated in Fig. 4(A), administration of eotaxin combined with OVA inhalation induced an increase in eosinophils in the BALF of STAT6^{-/-} mice. However, no significant increase of AHR (Fig. 4B) or mucus production (Fig. 6F) was induced by eotaxin. Thus, this reconstitution experiment directly demonstrates that STAT6-mediated signaling plays a critical role for the development of eosinophilia by regulating eotaxin production, but that this role of STAT6 is dissociated from its role in AHR and mucus hypersecretion.

Our results are supported by the previous finding that gene transfer of IL-5 and/or eotaxin to the lungs of naive mice induces a pronounced and selective eosinophilia, but does not result in AHR (31). However, conflicting results were reported by Hisada *et al.* (32), who demonstrated that eotaxin induces both lung eosinophilia and AHR when administered intratracheally to IL-5 transgenic mice. These contradictory results may be due to the abnormal hypereosinophilia that is observed in IL-5 transgenic mice. Indeed, in the IL-5 transgenic mouse model, as compared to our Th2 transfer model, eotaxin induced ~10-fold more potent pulmonary eosinophilia and these animals also showed enhanced baseline eosinophilia.

In addition to eotaxin production, several other IL-4- and/or IL-13-mediated processes have been postulated to play a role in regulating eosinophil recruitment. For example, IL-4 and IL-13 upregulate VCAM-1 expression on endothelial cells, leading to preferential eosinophil recruitment (16,17). In our Th2 cell-transfer model, however, VCAM-1 expression in antigen-challenged STAT6^{-/-} mice is equivalent to wild-type mice (Fig. 5). This result is consistent with findings by Kuperman *et al.*, demonstrating that VCAM-1 expression in STAT6^{-/-} and wild-type mice in an active model of asthma sensitization are indistinguishable (10). These data indicate that VCAM-1 expression, which is regulated in a STAT6-independent manner, might be important for airway asthma but not sufficient for pulmonary eosinophilia and AHR.

We have demonstrated that STAT6-dependent signaling plays a critical role for the late effector phase of airway responsiveness, including AHR, eosinophilia and mucus hypersecretion. Although the precise role of STAT6 for eosinophilia is directly demonstrated by eotaxin reconstitution experiments (Fig. 4), the mechanism by which STAT6 regulates AHR and mucus hypersecretion remains unclear. Airway epithelial cells express both IL-4R α and IL-13R α 1 and both IL-4 and IL-13 could induce STAT6 activation (33,34) and mucus secretion (35) *in vitro*. The role of IL-4 for the development of AHR has been extensively studied. Administration of neutralizing mAb against IL-4 during the antigen challenge only partly reduced the development of AHR (36–38). On the other hand, blockade

of either IL-13 or IL-4R α at the time of allergen challenge inhibited AHR (29,39,40). Furthermore, in Th2 cell transfer model, Th2 cells derived from IL-4 deficient mice have a potential to induce mucus secretion and AHR (41); in contrast, IL-13 deficient Th2 cells could not (42,43). Consistently with these findings, intratracheal administration of anti-IL-4 mAb could not modify the allergic responses in the airway in our Th2 cell-transfer model (data not shown). Thus, IL-13 is considered to be more important than IL-4 for the induction of AHR and mucus secretion in our asthma model. Using IL-4R α ^{-/-} and STAT6^{-/-} mice adoptively transferred with OVA-specific IL-13^{-/-} or IL-13^{+/+} CD4⁺ T cells, it was recently shown that IL-13 induces AHR and eosinophilia but not mucus hypersecretion in an IL-4R α -independent and STAT6-dependent manner (44). On the other hand, Kuperman *et al.* (45) demonstrated a critical role of STAT6 in epithelial cells for IL-13-induced AHR and mucus hypersecretion but not for eosinophilia. Thus, the role of STAT6 for development of airway asthma at the late effector phase is still controversial. This may be due to different airway asthma models with differential mechanisms that are used: (i) airway asthma induced by IL-13 or Th2 cell conditioned medium (45,46) and (ii) Th2 cell-transfer (44). The former model is totally dependent on IL-4R α but the latter is independent of IL-4R α . In future experiments, it will be interesting to investigate whether epithelial cells from STAT6^{-/-} mice reconstituted with STAT6 (45) induce AHR and mucus hypersecretion but not eosinophilia in an IL-4R α -independent and STAT6-dependent manner in the Th2 cell-transfer model.

The present work highlights the critical but complex role of STAT6-mediated signaling at the late effector phase of airway responsiveness. We provide direct evidence that STAT6-mediated signaling is involved in eosinophilia through the regulation of eotaxin production. Further evaluation of the role of STAT6 at the late effector phase of allergic responsiveness may provide new avenues for the development of effective immunomodulating drugs for allergic asthma.

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Abbreviations

AHR	airway hyper-responsiveness
BALF	bronchoalveolar lavage fluid
Mch	acetyl- β -methylcholine
PAS	periodic acid–Schiff
VLA-4	very late antigen 4

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