American Journal of Pathology, Vol. 160, No. 2, February 2002

Stat6-Deficient Mice Develop Airway Hyperresponsiveness and Peribronchial Fibrosis during Chronic Fungal Asthma

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Signal transducer and activator of transcription 6 (Stat6) is critical for Th2-mediated responses during allergic airway disease. To investigate the role of Stat6 in fungus-induced airway hyperresponsiveness and remodeling, Stat6-deficient (Stat6−/−) and Stat6-wildtype (Stat6+/+) mice were sensitized to *Aspergillus fumigatus* and airway disease was subsequently assessed in both groups at days 21, 30, 38, and 44 after an intratracheal challenge with live *A. fumigatus* conidia. At all times after conidia, histological analysis revealed an absence of goblet cell hyperplasia and markedly diminished peribronchial inflammation in Stat6−/− mice in contrast to Stat6+/+ mice. Airway hyperresponsiveness and peribronchial fibrosis in Stat6−/− mice were significantly reduced at day 21 after conidia compared with Stat6+/+ mice, but both groups exhibited significant, similar increases in these parameters at all subsequent times after conidia. In separate experiments, IL-13-responsive cells in Stat6−/− mice were targeted via the daily intranasal administration of 200 ng of IL-13-PE38QQR (IL13-PE), comprised of human IL-13 and a derivative of *Pseudomonas* exotoxin, from days 38 to 44 after the conidia challenge. IL13-PE treatment abolished airway hyperresponsiveness, but not peribronchial fibrosis in Stat6−/− mice. Taken together, these data demonstrate that the chronic development of airway hyperresponsiveness during fungal asthma is IL-13-dependent but Stat6-independent. *(Am J Pathol 2002, 160:481–490)*

Allergic and asthmatic events elicited through interleukin (IL)-4 and IL-13 receptors involve a group of signal transducers and activators of transcription (Stat) proteins that, following their phosphorylation by cytokine receptor-associated Janus (JAK) kinases and migration into the nucleus, initiate cytokine responsive gene transcription.[1–3] Of the currently described Stat proteins, Stat6 appears to have the most prominent role during IL-4- and IL-13-mediated responses including Th2 differentiation[4] and immunoglobulin class switching to IgE.[5] Variants of Stat6 are present in clinical atopic asthma,[6] and linkage analysis suggests that Stat6 is situated in the chromosomal region containing candidate genes for atopy and asthma,[7] although the latter finding has garnered controversy.[8] At present, the most convincing data implicating Stat6 in allergic airway diseases is derived from experimental studies. Initial examination of mice lacking Stat6 function due to gene deletion revealed that Stat6-deficient (Stat6−/−) mice fail to generate IgE and a Th2 cytokine response and consequently failed to develop airway inflammation, airway hyperresponsiveness, or goblet cell hyperplasia during soluble ovalbumin (OVA)-induced allergic airway disease.[9–11] Subsequent studies in a similar model of OVA-induced allergic airway disease revealed that IL-5 could reconstitute many of the features of this disease in Stat6−/− mice.[12] More recent data suggests that Stat6-independent allergic inflammation can arise in the context of chronic OVA allergen challenge,[13] but it is currently unknown whether IL-4 and IL-13 contribute to Stat6-independent events during allergic airway disease.

Thus, the aim of the present study was to examine the role of Stat6 during chronic allergic airway disease induced by the fungus *Aspergillus fumigatus*.[14] This murine model recapitulates many of the features of clinical fungal asthma including chronic airway inflammation, airway hyperreactivity, and goblet cell hyperplasia.[15] In addition, these airway features persist for several weeks in *A. fumigatus*-sensitized mice that receive a single intrapulmonary challenge with *A. fumigatus* conidia.[16,17] Given

Supported by National Institutes of Health grants 1P50HL60289, HL35276, and P01-HL31963.

Accepted for publication October 17, 2001.

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that levels of IL-4 and IL-13 are markedly increased during the course of this model[14] and both contribute to the development of chronic fungal asthma in mice,[18] we examined the role of Stat6 in the airway inflammatory and remodeling events during the development of chronic fungal asthma.

Materials and Methods

Mice

The generation of Stat6−/− mice has been previously described in detail.[5] Stat6−/− were back-crossed 10 generations onto a BALB/c genetic background and were bred as homozygotes in the Indiana University Laboratory Animal Resource Center. Wild-type BALB/c (Stat6+/+) mice were purchased from Harlan Bioproducts (Indianapolis, IN).

A Chronic Model of A. fumigatus*-Induced Allergic Asthma*

We have previously described a model of chronic allergic airway disease induced by *A. fumigatus* conidia that is characterized by airway hyperreactivity, lung inflammation, eosinophilia, mucus hypersecretion, goblet cell hyperplasia, and subepithelial fibrosis.[14,16,17] Stat6+/+ or Stat6−/− mice were similarly sensitized to a commercially available preparation of soluble *A. fumigatus* antigens. Seven days after the third intranasal challenge, each mouse received 5.0×10^6 *A. fumigatus* conidia suspended in 30 μl of PBS Tween 80 (0.1%; v/v) via the intratracheal route.[14]

To determine the role of IL-4 and IL-13 in the development of fungus-induced allergic airway disease in the absence of Stat6, IL-13-PE38QQR (IL13-PE) was used to target IL-13 receptor-expressing cells. IL13-PE is a recombinant chimeric fusion protein comprised of human IL-13 and a mutated *Pseudomonas* exotoxin, and it has been previously used to target IL-13 receptor-expressing tumor cells.[19,20] Based on preliminary observations, a group of 10 *A. fumigatus*-sensitized Stat6−/− mice received 200 ng of IL13-PE dissolved in 20 μl of phosphate-buffered saline (PBS) containing 0.25% human serum albumin (HSA-PBS) via an intranasal bolus once daily. IL-13 receptor-positive cells were targeted with IL13-PE from days 37 to 44 after conidia challenge. Another group of ten *A. fumigatus*-sensitized Stat6−/− mice received 20 μl of IL13-PE vehicle alone once daily via the same route beginning at day 37 and concluding at day 44 after conidia.

Measurement of Bronchial Hyperresponsiveness

Immediately before and at days 21, 30, 38, and 44 after an intratracheal *A. fumigatus* conidia challenge, bronchial hyperresponsiveness was assessed in a Buxco plethysmograph (Buxco, Troy, NY) as previously described.[14] Sodium pentobarbital (Butler Co., Columbus, OH; 0.04 mg/g mouse body weight) was used to anesthetize mice before their intubation and ventilation was carried out with a Harvard pump ventilator (Harvard Apparatus, Reno, NV). Once baseline airway resistance was established, 5 μg of methacholine was introduced into each mouse via a cannulated tail vein, and airway hyperresponsiveness was monitored for approximately 3 minutes. The peak increase in airway resistance was then recorded. After the assessment of airway hyperresponsiveness, approximately 500 μl of blood was removed from each mouse via ocular bleed and centrifuged at $15{,}000 \times g$ for 10 minutes to yield serum. A bronchoalveolar lavage (BAL) was then performed using 1 ml of filter-sterilized normal saline. Finally, whole lungs were dissected from each mouse and snap frozen in liquid N_2 or fixed in 10% formalin for histological analysis (see below).

Morphometric Analysis of Leukocyte Accumulation in BAL Samples

Macrophages, eosinophils, neutrophils, and T cells were quantified in BAL samples cytospun (Shandon Scientific, Runcorn, UK) onto coded microscope slides. Each slide was stained with a Wright-Giemsa differential stain, and the average number of each cell type was determined after counting a total of 300 cells in 10 to 20 high-powered fields (HPF; ×1000) per slide. A total of 1×10^6 BAL cells were cytospun onto each slide to compensate for differences in cell retrieval.

ELISA, IgE, and Collagen Analysis

Murine IL-4, IL-13, transforming growth factor-β (TGF-β), macrophage chemoattractant protein-1 (MCP-1), regulated on activation, normal T-cell expressed and secreted (RANTES), and eotaxin protein levels were determined in 50-μl samples from whole lung homogenates using a standardized sandwich enzyme-linked immunosorbent assay (ELISA) technique previously described in detail.[21] Total IgE levels were measured in serum samples using a specific ELISA. All ELISAs were screened to ensure the specificity of each antibody used. Nunc-immuno ELISA plates (MaxiSorp) were coated with the appropriate polyclonal capture antibody (R&D Systems, Minneapolis, MN) at a dilution of 1 to 5 μg/ml coating buffer (in M: 0.6 NaCl; 0.26 H_3BO_4; 0.08 NaOH; pH 9.6) overnight at 4°C. The unbound capture antibody was washed away, and each plate was blocked with 2% BSA-PBS for 1 hour at 37°C. Each ELISA plate was then washed three times with PBS Tween 20 (0.05%; v/v), and 50 μl of undiluted or diluted (1:10) whole lung homogenate were added to duplicate wells and incubated for 1 hour at 37°C. Following the incubation period, the ELISA plates were thoroughly washed, and the appropriate biotinylated polyclonal detection antibody (3.5 μg/ml) was added. After washing the plates 45 minutes later, streptavidin-peroxidase (1:5000 dilution, Bio-Rad Laboratories, Richmond, CA) was added to each well, incubated for 30 minutes, and then thoroughly washed again. A chromagen substrate solu-

tion (Bio-Rad Laboratories) was added, and optical readings at 492 nm were obtained using an ELISA plate scanner. Recombinant murine cytokines and chemokines (R&D Systems, Rochester, MN) were used to generate the standard curves from which the sample concentrations were derived. The limit of ELISA detection for each cytokine was consistently above 50 pg/ml. The Sircol collagen assay (Biocolor Ltd., Belfast, Ireland) was used to measure the soluble forms of collagen present in the same lung homogenates. This assay was developed from the Sirius Red-based histochemical procedure. The cytokine and collagen levels in each sample were normalized to total protein levels measured using the Bradford assay.

Whole Lung Histological Analysis

Whole lungs from *A. fumigatus*-sensitized Stat6+/+ and Stat6−/− mice before and at various times after *A. fumigatus* conidia challenge were fully inflated with 10% formalin, dissected, and placed in fresh 10% formalin for 24 hours. Routine histological techniques were used to embed the entire lung with paraffin, and 5-μm sections of whole lung were stained with periodic acid-Schiff reagent (PAS) or Masson trichrome. Morphological evaluations of inflammatory infiltrates and structural alterations were determined around blood vessels and airways using light microscopy at a magnification of $\times$1000.

Statistical Analysis

All results are expressed as means ± SEM (SE). A Student's *t*-test or analysis of variance and a Student-Newman-Keuls Multiple Comparison test were used to determine statistical significance between Stat6+/+ and Stat6−/− mice at various times after the conidia challenge; $P < 0.05$ was considered statistically significant.

Results

Stat6-Deficient Mice Exhibit No IgE, Markedly Diminished Goblet Cell Hyperplasia, and Reduced Airway Inflammation during Chronic Fungal Asthma

Consistent with previous studies, Stat6−/− mice failed to show the generation of serum IgE at any time in the chronic fungal model. Total IgE levels in Stat6+/+ mice exceeded 5 μg/ml serum at days 21 to 44 after conidia, whereas total IgE levels in Stat6−/− mice were below the limit of ELISA detection at these same times (data not shown). Pronounced goblet cell hyperplasia and peribronchial inflammation are characteristically found in the airways following the induction of fungus-induced asthma.[14] PAS staining of whole lung sections from *A. fumigatus*-sensitized Stat6+/+ mice revealed a significant increase in goblet cells and mucus, as indicated by magenta staining 30 (A), 38 (C), and 44 (E) days after conidia challenge (Figure 1,A, C, and E). Major accumulations of leukocytes around these airways were also observed at all time points (Figure 1, A, C, and E). Compared with the Stat6+/+ group, little mucus and minor peribronchial accumulations of leukocytes were detected in the lungs of Stat6−/− mice at 30, 38, or 44 days after conidia challenge in *A. fumigatus*-sensitized mice (Figure 1, B, D and F). This data suggested that goblet cell hyperplasia and peribronchial inflammation during chronic fungal asthma is Stat6-dependent.

Whole Lung Deficits in MCP-1, RANTES, and Eotaxin in Stat6-Deficient Mice during Chronic Fungal Asthma

Previous *in vitro* studies have shown that Stat6 activation is required for the generation of the CC chemokine eotaxin by airway epithelial cells[22] and endothelial cells.[23] Other studies have shown that the synthesis of CC chemokines such as macrophage-derived chemokine and T cell activation gene-3 by primary Th2 cells in culture is also dependent on Stat6.[24] To determine whether Stat6 deficiency affected the lung levels of eotaxin and other pro-allergic CC chemokines such as MCP-1 and RANTES during chronic fungal asthma, whole lung samples from Stat6+/+ and Stat6−/− mice were analyzed at days 21, 30, 38, and 44 after the conidia challenge. As shown in Figure 2, whole lung levels of MCP-1, RANTES, and eotaxin were significantly decreased in Stat6−/− mice compared with Stat6+/+ mice at nearly all times examined after the conidia challenge. One exception to this trend was noted at day 30 after the conidia challenge when whole lung levels of eotaxin did not differ between the two groups of mice (Figure 2C). Furthermore, it is important to note that significantly higher levels of all three CC chemokines were present in the lungs of Stat6−/− mice at days 30 to 44 after conidia compared with whole lung levels of these chemokines measured in the day 21 group of Stat6−/− mice. These findings suggested that Stat6 was necessary, in part, for the full expression of MCP-1, eotaxin, and RANTES during chronic fungal asthma.

Stat6-Deficient Mice Develop Airway Hyperresponsiveness during Chronic Fungal Asthma

Airway hyperresponsiveness is a persistent and complicating feature of fungus-induced asthma.[15] Stat6+/+ mice sensitized to *A. fumigatus* antigen exhibited significantly elevated airway hyperresponsiveness in response to methacholine 21 days after conidia challenge compared with airway responses to methacholine measured in these mice before the conidia challenge (Figure 3). Furthermore, airway hyperresponsiveness remained significantly elevated in Stat6+/+ mice at days 30, 38, and 44 after the conidia challenge. In contrast, Stat6−/− mice exhibited significantly less airway hyperresponsiveness at day 21 after the conidia challenge compared with

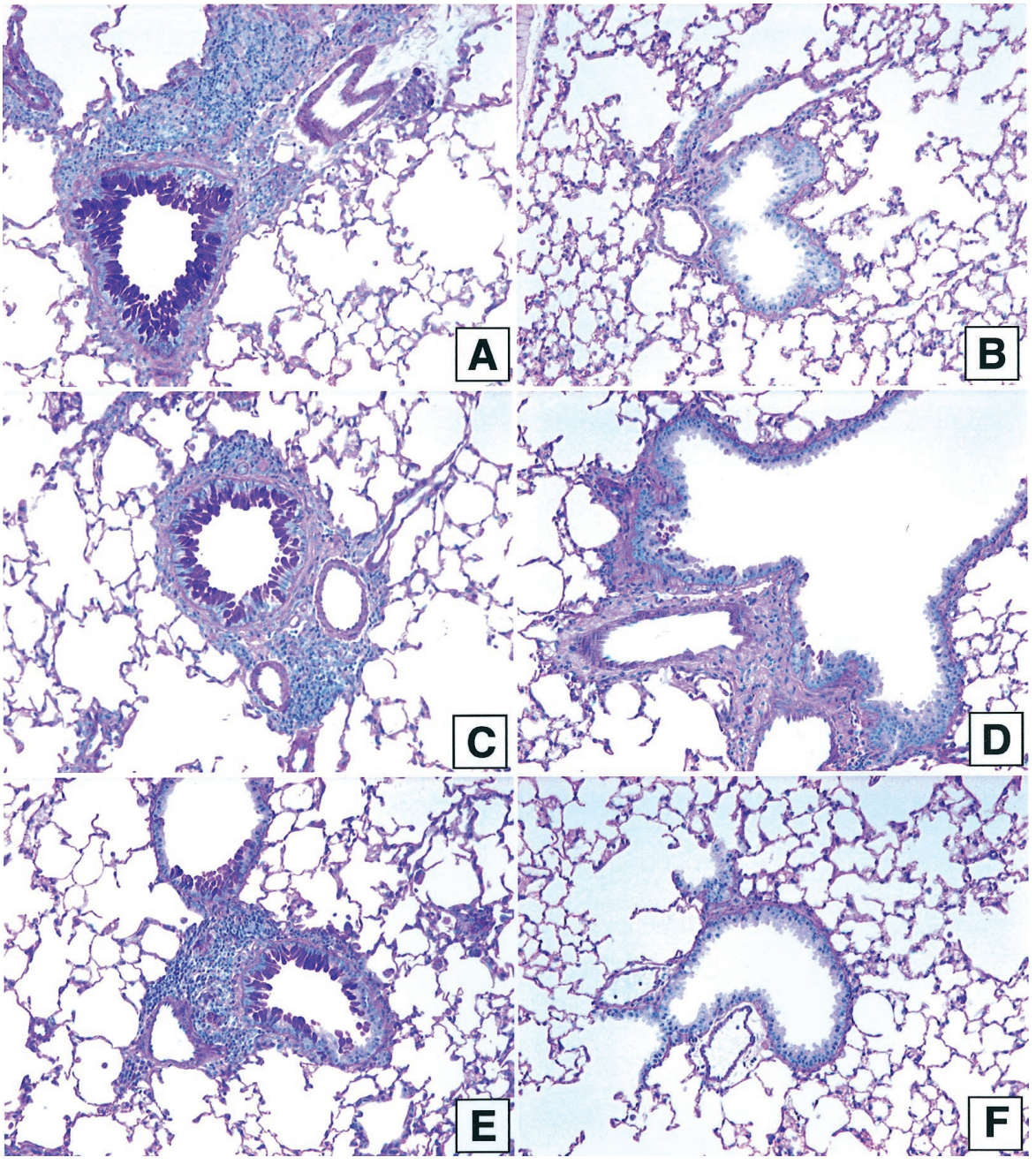
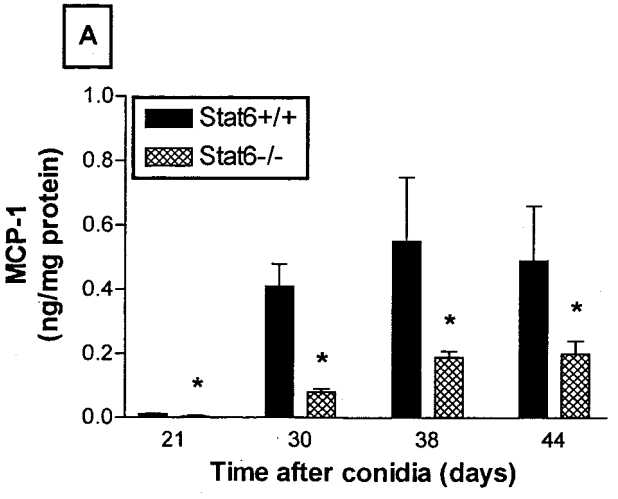


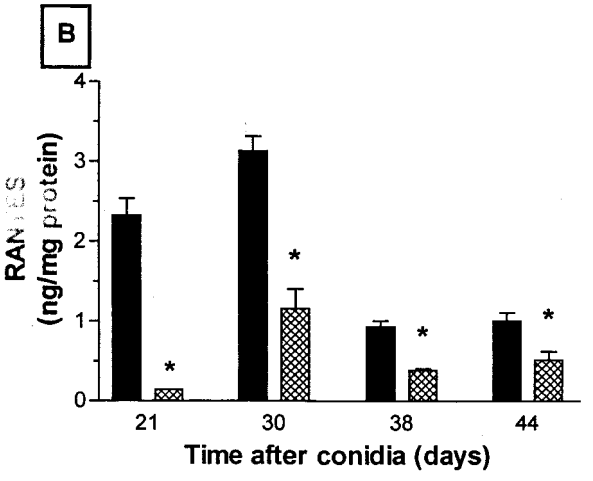
Figure 1. Representative photomicrographs of PAS-stained whole lung sections from *A. fumigatus*-sensitized Stat6-wildtype (+/+) (**A**, **C**, and **E**) and Stat6-deficient (−/−) (**B**, **D**, and **F**) mice at days 30 (**A** and **B**), 38 (**C** and **D**), and 44 (**E** and **F**) after a live *A. fumigatus* conidia challenge. Goblet cells (stained dark magenta) were prominent in the airways of Stat6+/+ mice at all times after the conidia challenge. Peribronchial accumulation of inflammatory leukocytes was also observed at all times after the conidia challenge in the group. In contrast, goblet cells were not detected in the airways of Stat6−/− mice at days 30 (**B**) and 44 (**F**) after conidia and were only rarely detected in Stat6−/− mice at day 38 (**D**) after conidia. Furthermore, markedly fewer leukocytes were detected around the airways of Stat6−/− mice compared with the wild-type groups at all times after the conidia challenge. Original magnification was ×200 for each photomicrograph.

Stat6+/+ mice at the same time (Figure 3). However, Stat6−/− mice exhibited airway hyperresponsiveness similar to that measured in Stat6+/+ mice at days 30, 38, and 44 after conidia (Figure 3). Thus, these data suggested that airway hyperresponsiveness ultimately manifests in Stat6−/− mice during chronic fungal asthma.

Increased T Cell Recruitment into the Airways of Stat6-Deficient Mice at Day 30 after Conidia

The infiltration of eosinophils and lymphocytes into the airways has been shown to play a key role in the development of allergic airway disease.[25] Given that airway hyperresponsiveness and peribronchial fibrosis were





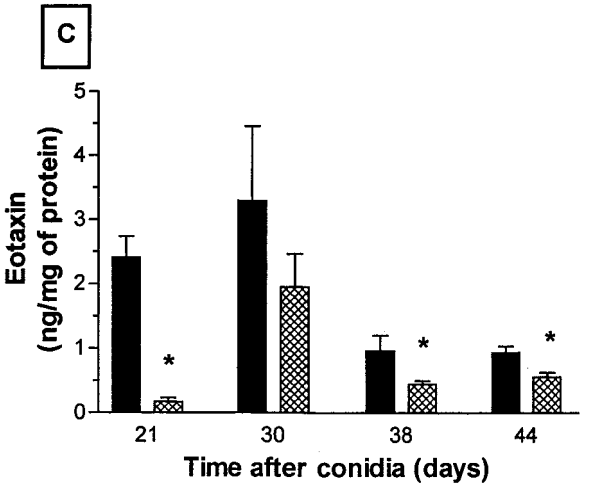


Figure 2. Whole lung levels of MCP-1 (**A**), RANTES (**B**), and eotaxin (**C**) in *A. fumigatus*-sensitized Stat6-wildtype (+/+) and Stat6-deficient (−/−) mice at various times after an *A. fumigatus* conidia challenge. CC chemokine levels were measured using specific ELISAs as described in the Materials and Methods. Values are expressed as mean ± SE; $n = 5$/group/time point. *, $P < 0.05$, demonstrate significant differences between Stat6+/+ and Stat6−/− mice at the same time after conidia challenge.

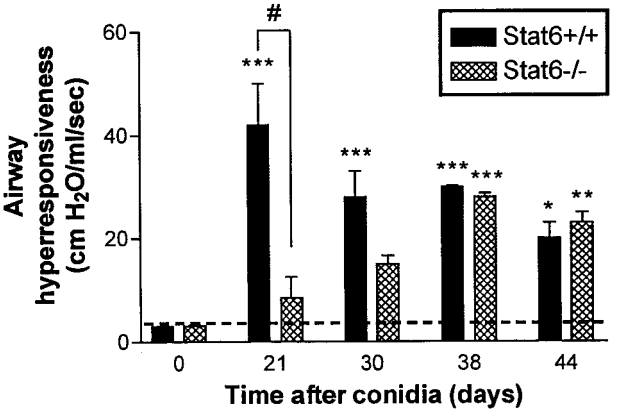


Figure 3. Airway hyperresponsiveness in *A. fumigatus*-sensitized Stat6-wildtype (+/+) and Stat6-deficient (−/−) mice before and at various times after an *A. fumigatus* conidia challenge. Airway resistance (units = cm H_2O/ml/s) was calculated at each time point before (**dashed line**) and after methacholine (5 μg iv). Values are expressed as mean ± SE; $n = 5$/group/time point. *, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ demonstrate significant differences in airway resistance compared to levels before conidia challenge (time = 0). #, $P < 0.05$ demonstrates a significant difference in airway resistance between Stat6+/+ and Stat6−/− mice at day 21 after conidia.

present at day 30, but not at day 21 after conidia in Stat6−/− mice, we next examined whether the appearance of these features of allergic disease was associated with increased leukocyte numbers in the BAL. No significant differences in eosinophil, neutrophil, or macrophage numbers were measured between Stat6+/+ or Stat6−/− mice at any time after conidia (data not shown). At day 21 after the conidia challenge in *A. fumigatus*-sensitized Stat6+/+ mice, approximately 50% of the cells in the BAL were T cells (Figure 4), whereas T cells comprised less than 1% of the cells in the BAL from Stat6−/− mice at this time. However, at day 30 after the conidia challenge, T cells were prominent in the BAL from both Stat6+/+ and Stat6−/− mice, comprising greater than 20% of the total cells in the BAL (Figure 4). Thus, these findings suggest that the appearance of airway hyperresponsiveness in Stat6−/− mice subsequent to day 30 after conidia may be related to the augmented recruitment of T cells into the airways of these mice.

Peribronchial Fibrosis Occurs in the Absence of Stat6 during Chronic Fungal Asthma

Previous studies have demonstrated a role for Stat6 in the development of granulomas and collagen deposition;[26] therefore, we investigated the role of Stat6 in the development of peribronchial fibrosis following the induction of chronic fungal asthma. Total collagen levels measured in whole lung homogenates were significantly attenuated in Stat6−/− mice compared to Stat6+/+ mice 21 days after

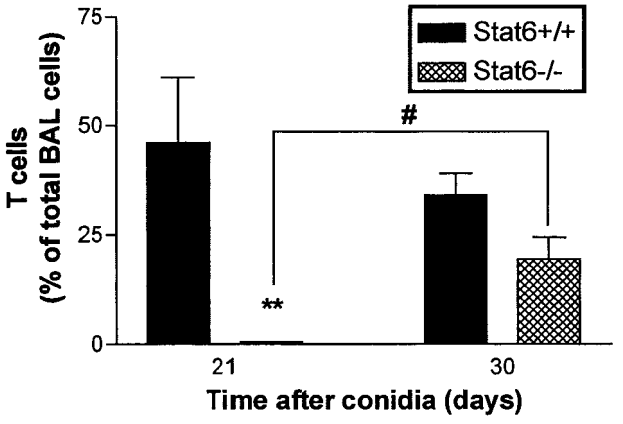


Figure 4. T cell counts in bronchoalveolar lavage (BAL) samples at days 21 and 30 after *A. fumigatus* conidia challenge in *A. fumigatus*-sensitized Stat6-wildtype (+/+) and Stat6-deficient (−/−) mice. BAL cells were dispersed onto microscope slides using a cytospin, and T lymphocytes were differentially stained with Wright-Giesma stain. A minimum of 15 high-powered fields or 300 cells was examined in each cytospin. A total of 1×10^6 BAL cells were cytospun onto each slide to compensate for differences in cell retrieval from each mouse. Values are expressed as mean ± SE. **, $P \leq 0.01$ compared with lymphocyte numbers counted in the Stat6+/+ mice at day 21 after conidia. ≠, $P < 0.05$ demonstrates a significant difference in lymphocyte numbers between Stat6−/− mice at days 21 and 30 after conidia.

conidia challenge. However, total collagen levels in the lungs of Stat6−/− mice were markedly increased at later time points after conidia challenge. No significant differences in collagen levels were detected between Stat6+/+ and Stat6−/− mice 30, 38, or 44 days after conidia challenge in *A. fumigatus*-sensitized mice (Figure 5A). Histological analysis of whole lung sections taken from Stat6+/+ and Stat6−/− mice at 30, 38, and 44 days after conidia challenge confirmed these findings (not shown). At day 30 after conidia, the increase in total collagen in whole lung samples from Stat6−/− mice coincided with a significant increase in levels of the profibrotic cytokine, TGF-β (Figure 5B). Thus, the appearance of peribronchial fibrosis was delayed in Stat6−/− mice compared with Stat6+/+ mice, and changes in total lung collagen in the former group coincided with an increase in lung levels of TGF-β.

Temporal Changes in Whole Lung Levels of IL-4 and IL-13 in Stat6−/− Mice during Chronic Fungal Asthma

To determine whether the absence of Stat6 during chronic fungal asthma influenced levels of Th2 cytokines such as IL-4 and IL-13, whole lung levels of both cytokines were measured at days 21, 30, 38, and 44 after the conidia challenge in *A. fumigatus*-sensitized Stat6+/+ and Stat6−/− mice. Whole lung IL-4 protein levels are shown in Figure 6A. It was evident that IL-4 levels increased in a time-dependent manner in both groups. At days 21 and 30 after conidia, IL-4 levels in Stat6−/− mice were significantly lower than levels measured in Stat6+/+ at the same time points. However, at days 38 and 44, whole lung IL-4 levels were comparable in both groups (Figure 6A). Whole lung IL-13 levels are shown in Figure 6B. The greatest levels of IL-13 in Stat6+/+ mice

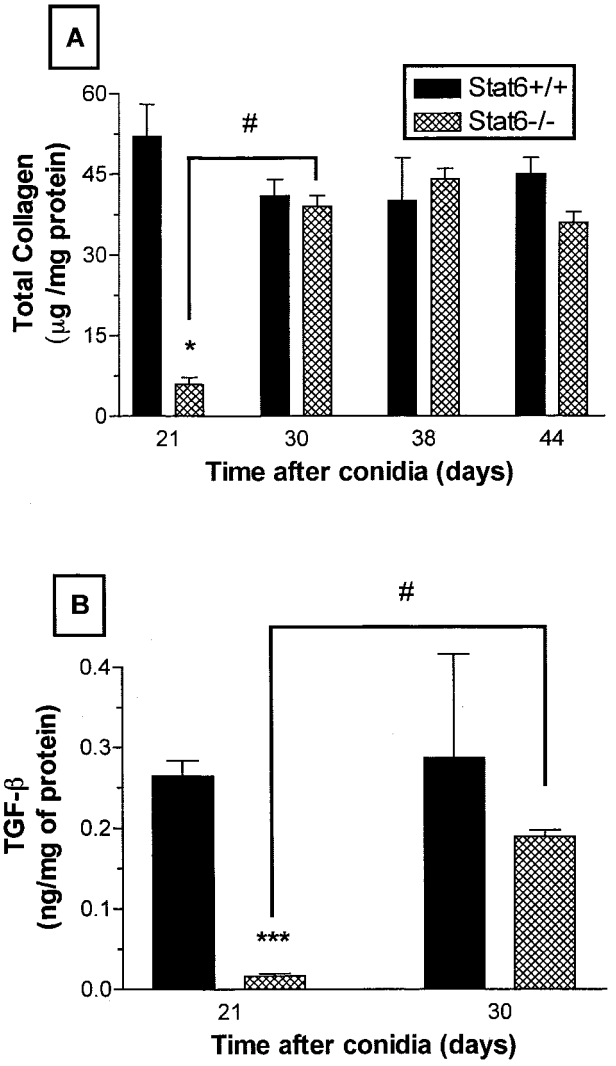


Figure 5. Total soluble collagen levels (**A**) and whole lung TGF-β (**B**) in *A. fumigatus*-sensitized Stat6-wildtype (+/+) and Stat6-deficient (−/−) mice at various times after an *A. fumigatus* conidia challenge. Whole lung levels of collagen and TGF-β were measured in both groups as described in the Materials and Methods section. Values are expressed as mean ± SE; n = 5/group/time point. *, $P < 0.05$, ***, $P < 0.001$ demonstrate significant differences between Stat6+/+ and Stat6−/− mice at the same time after conidia challenge. ≠, $P < 0.05$ demonstrates a significant difference in total collagen and TGF-β levels in Stat6−/− mice at days 21 and 30 after conidia.

were detected at day 30 after conidia. Whole lung IL-13 levels in Stat6−/− mice were significantly lower than levels measured in their wild-type counterparts at the day 21 following the conidia challenge. However, at all subsequent times examined, IL-13 levels were similar in the two groups (Figure 6B).

Development of Airway Hyperresponsiveness in Stat6−/− Mice during Chronic Fungal Asthma Is Dependent on IL-13 Receptor Expression

IL-13 and IL-4 have key roles in the development of airway hyperresponsiveness in this model of fungus-induced allergic airway disease.[18] We recently began to examine the therapeutic effect of IL13-PE, a fusion pro-

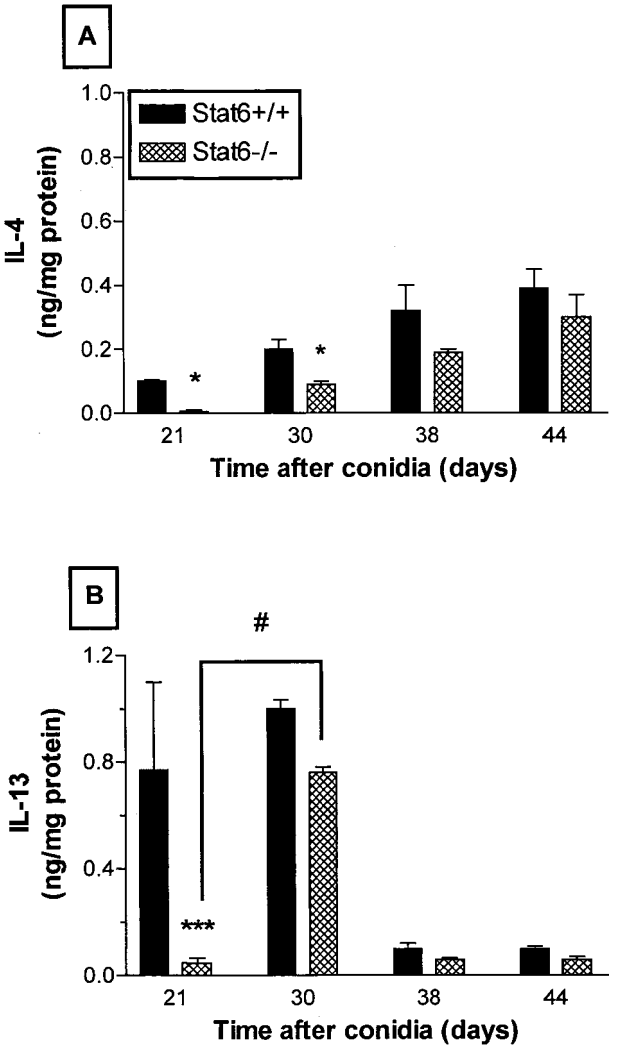


Figure 6. IL-4 (**A**) and IL-13 (**B**) levels in whole lung homogenates from *A. fumigatus*-sensitized Stat6-wildtype (+/+) and Stat6-deficient (−/−) mice at various times after an *A. fumigatus* conidia challenge. Cytokine levels were measured using a specific ELISA as described in Materials and Methods. Values are expressed as mean ± SE, $n = 5$ mice/group/time point. *, $P \leq 0.05$ ***, $P \leq 0.001$ denotes significant differences in cytokine levels compared with levels measured in Stat6+/+ mice at the same time point. ≠, $P < 0.05$ demonstrates a significant difference in IL-13 levels in Stat6−/− mice at days 21 and 30 after conidia.

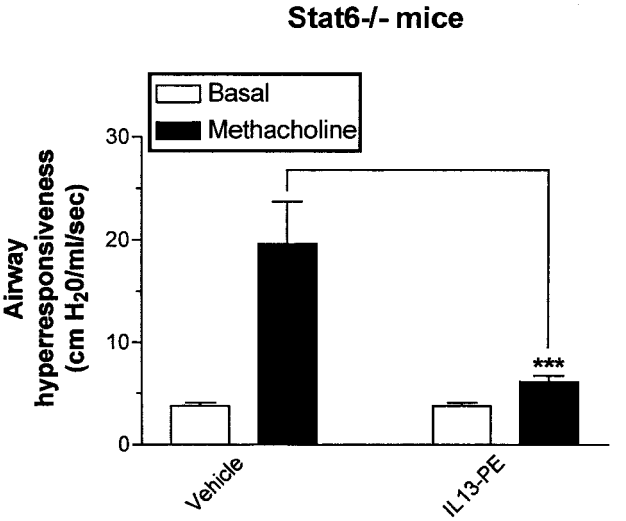


Figure 7. Therapeutic effects of IL-13-PE38QQR (IL13-PE) treatment on airway hyperresponsiveness in *A. fumigatus*-sensitized Stat6-deficient (−/−) at day 44 after conidia. Stat6−/− mice received IL13-PE (200 ng) or the vehicle for IL13-PE (PBS containing human serum albumin) daily from days 38 to 44 after the conidia challenge. Airway hyperresponsiveness (units = cmH_2O/ml/s) was measured before, and after a methacholine challenge (5 μg iv). Values are expressed as mean ± SE, $n = 5$ mice/group/time point. ***, $P < 0.001$ denotes significant differences in airway hyperresponsiveness in vehicle-treated mice compared with IL13-PE treatment at the same time point.

tein that targets and kills IL-4- and IL-13-responsive cells,[19,20] in this chronic fungal asthma model. Our preliminary findings show that IL13-PE reverses all of the airway features of chronic fungal asthma when administered from day 14 to 28 after the conidia challenge. In the present study, Stat6−/− mice were treated for seven consecutive days with IL13-PE, and airway hyperresponsiveness following methacholine was determined 44 days after conidia challenge. Airway hyperresponsiveness in Stat6−/− mice treated with vehicle increased significantly after methacholine administration (3.8 ± 0.1 to 19.6 ± 4.1 cm H_2O/ml/s; Figure 7). In contrast, mice treated with IL13-PE exhibited no significant increase in airway hyperresponsiveness from basal levels after methacholine administration (Figure 7). The IL13-PE treatment also completely abolished the presence of T cells, eosinophils, and neutrophils present in BAL samples from Stat6−/− mice at day 44 after conidia (Figure 8).

Histological analysis of whole lung sections from both treatment groups of Stat6−/− mice revealed that peribronchial inflammation was markedly diminished in lung sections from IL13-PE-treated Stat6−/− mice in contrast

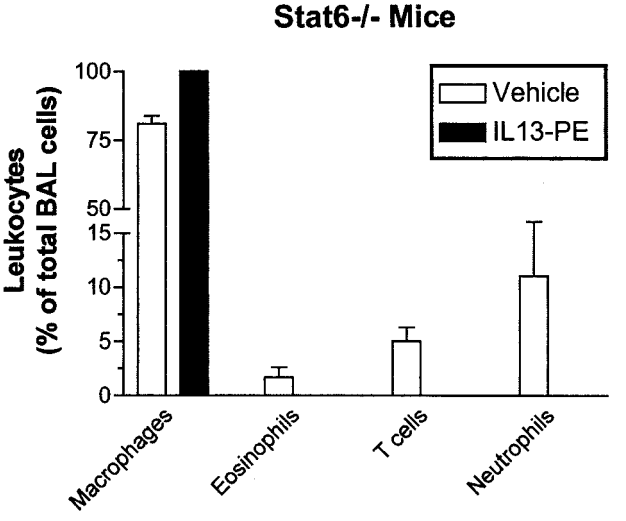


Figure 8. Leukocyte differentials in bronchoalveolar lavage (BAL) samples at day 44 after an *A. fumigatus* conidia challenge in *A. fumigatus*-sensitized Stat6-deficient (−/−) mice. Stat6−/− mice received IL13-PE (200 ng) or the vehicle for IL13-PE (PBS containing human serum albumin) daily from days 38 to 44 after the conidia challenge. BAL cells were dispersed onto microscope slides using a cytospin, and T lymphocytes were differentially stained with Wright-Giesma stain. A minimum of 15 high-powered fields or 300 cells was examined in each cytospin. A total of 1×10^6 BAL cells were cytospun onto each slide to compensate for differences in cell retrieval from each mouse. Values are expressed as means ± SE.

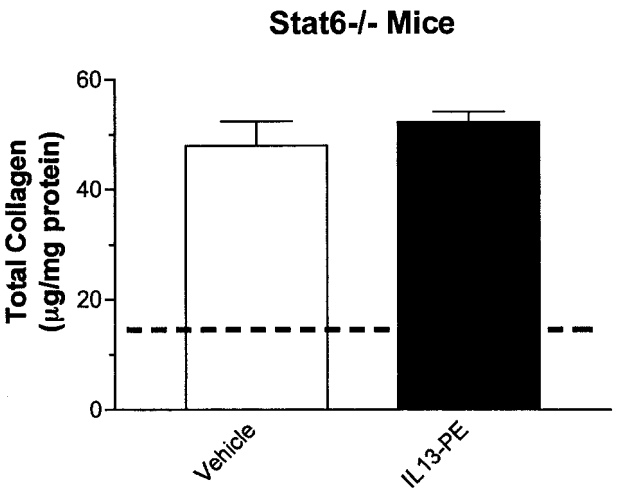


Figure 9. Total soluble collagen levels in *A. fumigatus*-sensitized Stat6-deficient (−/−) mice at day 44 after an *A. fumigatus* conidia challenge. Stat6−/− mice received IL13-PE (200 ng) or the vehicle for IL13-PE (PBS containing human serum albumin) daily from days 38 to 44 after the conidia challenge. Whole lung levels of collagen were measured in both groups as described in the Materials and Methods section. Values are expressed as means ± SE; $n = 5$/group/time point. The **dashed line** denotes total soluble collagen levels in whole lung samples from non-sensitized Stat6−/− mice that did not receive conidia.

to Stat6−/− mice that received the vehicle for IL13-PE alone (not shown). Examination of trichrome-stained whole lung sections revealed that peribronchial fibrosis was prominent in both Stat6−/− treatment groups (not shown). These qualitative findings were confirmed using a quantitative collagen assay (Figure 9). Essentially, IL13-PE therapy from days 38 to 44 after conidia did not reverse the peribronchial fibrosis evident at day 44 after conidia (Figure 9). These data suggest that the development of airway hyperresponsiveness, but not peribronchial fibrosis, in Stat6−/− mice is dependent on IL-13 receptor-expressing cells.

Discussion

Given the clinical and experimental relevance of IL-4 and IL-13 during the development of several features of allergic asthma,[27–29] the cellular and molecular pathways these Th2 cytokines exploit have received considerable research attention. A number of previous experimental studies have established a prominent role for Stat6 signaling during the development of various features of allergic asthma.[9–13] These studies clearly showed that the induction of Th2 responses were severely impaired during acute allergen challenge in previously sensitized Stat6−/− mice. However, more recent studies by Trifilieff et al[13] demonstrated that OVA-sensitized Stat6−/− mice could acquire some features of allergic airway disease following a chronic OVA challenge. Given these more recent findings in allergic Stat6−/− mice, we investigated whether Stat6-deficiency would affect the development of chronic fungus-induced asthma characterized by persistent airway hyperresponsiveness and airway remodeling. In the present study, we demonstrated that goblet cell hyperplasia and peribronchial inflammation were largely absent from *A. fumigatus*-sensitized Stat6−/− mice at all times examined after their challenge with *A. fumigatus* conidia. However, allergic Stat6−/− mice ultimately acquired many of the same features of allergic airway disease observed in Stat6-wildtype (+/+) mice after the conidia challenge, particularly airway hyperresponsiveness and peribronchial fibrosis. Additional experiments demonstrated that the selective targeting of IL-13-responsive cells in the lung using IL13-PE abolished airway hyperresponsiveness in Stat6−/− mice. Thus, the present study highlights that certain prominent characteristics of chronic allergic airway disease can eventually develop in the absence of Stat6, but Stat6-independent airway hyperresponsiveness relies on the presence of IL-13-responsive cells in the lung.

Stat6 appeared to modulate a number of features of chronic fungal asthma regardless of when these parameters were analyzed. First, the present study confirmed previous observations[9,12] that serum IgE generation and goblet cell hyperplasia during allergic airway disease unequivocally require Stat6 signaling. Mucus overproduction and goblet cell hyperplasia are characteristic of the remodeled asthmatic airway,[30] and the appearance of goblet cells in the airways is a Th2-mediated response.[31,32] Second, the intensity of allergic airway inflammation was markedly diminished in Stat6−/− mice compared with their wild-type counterparts. Although it was noted that T cell accumulation in the airways of allergic Stat6−/− mice did progressively increase during the course of fungal asthma, the lymphocyte counts in the BAL of Stat6−/− mice remained considerably lower than similar counts in BALs from Stat6+/+ mice. These finding were consistent with those of Trifilieff et al[13] who demonstrated that the airway inflammatory response was only partially mediated by Stat6 in the context of a chronic OVA challenge. Third, it was observed that Stat6 deficiency significantly attenuated the levels of major proallergic CC chemokines such as MCP-1, RANTES, and eotaxin.[33] These findings are consistent with results showing that Stat6 was necessary for TNF-α and IL-4 to promote eotaxin gene expression in human airway epithelial cells[22] and for IL-13 to induce MCP-1 in endothelial cells.[23] Our finding that RANTES/CCL5 was also decreased in Stat6−/− mice during chronic fungal asthma was novel; previous studies suggested that this chemokine was a product of Th1 cells.[24] The present study demonstrated that a number of major characteristics of chronic fungal asthma in Stat6+/+ mice were absent or diminished in Stat6−/− mice at all times after conidia challenge.

Persistent airway hyperresponsiveness and airway remodeling due to excessive collagen deposition and subepithelial fibrosis characterize fungus-induced asthma.[14] In light of previous studies showing that OVA-sensitized and -challenged Stat6−/− mice remained hyporesponsive to a methacholine challenge,[9,10,13] the delayed appearance of methacholine-induced airway hyperresponsiveness in Stat6−/− mice during chronic fungal asthma was a novel and surprising finding from the present study. Additionally, the development of pulmonary fibrosis was unique to the present study, as it had been

previously reported that Stat6−/− mice failed to develop pulmonary and hepatic fibrosis in the context of granuloma formation induced by *Schistosoma mansoni* eggs.[26] Airway hyperresponsiveness and peribronchial fibrosis were significantly reduced at day 21 after conidia challenge in Stat6−/− mice compared with their wild-type controls, but at all subsequent times after conidia, Stat6−/− mice exhibited vigorous methacholine-induced bronchoconstriction and peribronchial fibrosis comparable to Stat6+/+ mice. Furthermore, the appearance of peribronchial fibrosis at day 30 in the Stat6−/− mice was associated with a significant increase in whole lung levels of the profibrotic cytokine, TGF-β. The discrepancy between our findings and those of previous investigators may be linked to the manner in which Stat6−/− mice were sensitized and challenged with allergen, the genetic background of the Stat6−/− mice examined (ie, BALB/c *versus* C57BL/6), and/or the duration over which the features of allergic airway disease was monitored.

Biological Th2 responses elicited by IL-4 and IL-13 binding to their appropriate receptors involves a complex array of signaling pathways and regulators of which Stat6 appears to be the major contributor.[34] The lack of Th2 responses in Stat6−/− mice during acute allergic airway disease was consistent with initial *in vitro* studies demonstrating the Stat6 requirement for IL-4-induced Th2 cell differentiation and immunoglobulin class switching to IgE.[5] However, more recently, it was shown that NK T cells, which do not require IL-4 for maturation, produce IL-4 in the absence of Stat6.[35] In the present study, Stat6-independent pathways were activated leading to the appearance of airway hyperresponsiveness and peribronchial fibrosis at time points after day 21 post conidia. Although these alternative pathways were not specifically examined in the present study, it is probable that cell-signaling pathways involving Stat3[36] and/or phosphatidylinositol 3-kinase[37] compensated for the absence of Stat6 at days 30 to 44 after the conidia challenge. Furthermore, Th2-mediated inflammatory disease progressed in the absence of Stat6 and IL-4 when the transcriptional repressor that shares DNA-binding motifs with Stats, Bcl-6, was deleted from mice.[38] Thus, the ultimate development of airway disease in *A. fumigatus*-sensitized Stat6−/− mice after an *A. fumigatus* conidia challenge presumably reflects the fact that other cell signaling pathways are activated in the chronic stages of this model. Presently, it is not apparent which cell signal pathway compensates for the lack of Stat6 during chronic fungal asthma, but this question is the focus of ongoing investigations.

The time-dependent increases in whole lung levels of IL-4 and IL-13 in Stat6−/− mice relative to Stat6+/+ mice appeared to correlate with the advent of airway hyperresponsiveness and peribronchial fibrosis in Stat6−/− mice. T cells were presumably the source of IL-4 and IL-13 since increased levels of both cytokines correlated with an increased accumulation of lymphocytes in the airways of Stat6−/− mice at days 30 to 44 after the conidia challenge. Previous experimental evidence from this chronic fungal asthma model strongly suggested that IL-13-responsive T cells were the major effectors in this chronic fungal asthma model.[18] Therefore, we investigated whether the targeting of IL-13-responsive cells with IL13-PE, a chimeric protein consisting of IL-13 and *Pseudomonas* exotoxin, would reverse the Stat6-independent features of the chronic asthma model. Accordingly, Stat6−/− mice were treated daily with IL13-PE from days 38 to 44 after the conidia challenge, and airway hyperresponsiveness and peribronchial fibrosis were examined at day 44 after conidia. The IL13-PE treatment abolished the presence of T cells, eosinophils, and neutrophils in the BAL and significantly reduced the airway hyperresponsiveness to methacholine in Stat6−/− mice. However, the IL13-PE treatment did not appear to have an effect on the peribronchial fibrosis manifest in Stat6−/− mice at day 44 after the conidia challenge. These data differ from our previous findings that showed IL13-PE treatment from days 14 to 28 after the conidia challenge in *A. fumigatus*-sensitized CBA/J mice prevented the appearance of peribronchial fibrosis at day 28. The discrepancy between the previous findings and those from the present study may be related to the fact that IL13-PE therapy is necessary during the time of fibroblast activation and matrix deposition. It appears that the peribronchial fibrotic process is complete in Stat6−/− mice by day 38 after the conidia challenge and is consequently impervious to IL13-PE therapy. Thus, the Stat6-independent events leading to airway inflammation and hyperresponsiveness in Stat6−/− mice were dependent on the presence of lung cells that responded to IL-13.

In conclusion, while IL-4 and IL-13 have been shown to be central mediators in the development of fungus-induced asthma, the role of Stat6-mediated signaling had not been previously investigated during the course of this disease. The data presented herein demonstrate that Stat6 was absolutely required for goblet cell hyperplasia but other features of chronic fungal asthma including airway inflammation, airway hyperresponsiveness, and peribronchial fibrosis could develop in the absence of Stat6. However, all Stat6-independent events required IL-13 since the depletion of IL-13-responsive cells in the lung reversed many prominent features of chronic fungal asthma in Stat6−/− mice. The IL13-PE treatment strategy obviates the need to target multiple signal transduction pathways involved in the Th2 responses leading to chronic fungal asthma. Furthermore, these findings suggest that the prolonged targeting of Stat6 in the context of allergic or asthmatic airway disease may provide diminishing therapeutic effects.

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