

Adoptive transfer of T cells induces airway hyperresponsiveness independently of airway eosinophilia but in a signal transducer and activator of transcription 6-dependent manner

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Background: Activated T cells, through the release of specific cytokines (ie, IL-4, IL-5, and IL-13), regulate effector cell recruitment and function. In this way T cells orchestrate the inflammatory response, which leads to airway hyperresponsiveness (AHR), a cardinal feature of allergic asthma.

Objective: In the present study the direct role of T cells and, in particular, the importance of signal transducer and activator of transcription 6 (STAT6) in T cells was investigated in the development of AHR.

Methods: In a murine model of allergen-driven AHR, the effects of adoptive transfer of STAT6-containing (STAT6^{+/+}) and STAT6-deficient (STAT6^{-/-}) T cells from sensitized mice into allergen-challenged mice were tested.

Results: Although greater in STAT6^{+/+} mice, both allergen-challenged STAT6^{+/+} and STAT6^{-/-} mice had AHR after transfer of T cells from sensitized STAT6^{+/+} mice. In contrast, AHR did not develop in allergen-challenged STAT6^{-/-} mice after transfer of T cells from sensitized STAT6^{-/-} mice. Reconstitution of AHR after T-cell transfer was not associated with airway eosinophilia.

Conclusions: The data indicate that the STAT6 status of the donor mice is critical to the development of AHR. Although not critical for the development of AHR, the STAT6 status of the recipient mice might play a contributory-regulatory role in the AHR response. The results identify a STAT6-dependent T-cell pathway capable of modulating airway responsiveness, even in the absence of a significant airway eosinophilia. (*J Allergy Clin Immunol* 2002;109:810-6.)

Key words: Airway hyperresponsiveness, T lymphocyte, STAT6, eosinophilia

Abbreviations used

AHR: Airway hyperresponsiveness
BALF: Bronchoalveolar lavage fluid
MBP: Major basic protein
OVA: Ovalbumin
STAT6: Signal transducer and activator of transcription 6

Airway hyperresponsiveness (AHR) is a major characteristic of allergic asthma. Clinical investigations indicate a relationship between the presence of activated airway inflammatory cells (particularly T cells and eosinophils), the morphologic changes in airway tissues, and the development and severity of AHR.¹⁻⁵

The eosinophil is thought to be a major effector cell in the pathogenesis of allergic AHR.⁶ T cells orchestrate the inflammatory response leading to AHR. Increased numbers of CD4⁺ and CD8⁺ T cells have been identified in the bronchoalveolar lavage fluid (BALF) and bronchial mucosa of allergic asthmatic patients expressing elevated levels of specific cytokines, particularly IL-4, IL-5, and IL-13, which is consistent with a T_H2 phenotype.⁷⁻¹⁰ Depletion of CD4⁺ T cells before allergen challenge of sensitized mice has been shown to abrogate airway inflammation and AHR, demonstrating a critical role for CD4⁺ T cells in the pathogenesis of the allergic airway response.^{11,12} There is also evidence that CD8⁺ T cells might play a role in allergic airway eosinophilia and AHR.¹³ More recent T-cell transfer studies, either of T_H2 or T_H1 clones, have further implicated T cells of the type 2 phenotype in the development of allergic airways eosinophilia and AHR.^{12,14-18}

Much effort has focused on the role of the type 2 cytokines, particularly IL-4, IL-5, and, more recently, IL-13, in the development and maintenance of allergic airway inflammation and AHR. IL-5 is known to regulate growth, differentiation, activation, and survival of eosinophils and appears critical in the development of allergic airway eosinophilia and AHR in mice.¹⁹⁻²¹ Conversely, IL-4 is critical for the commitment of T cells to the CD4⁺ T_H2 phenotype, and some of the functions of IL-4 can be subserved by IL-13.²²⁻²⁴ Experiments with signal transducer and activator of transcription 6 (STAT6)-deficient mice have identified that the STAT6 pathway is the principle signaling pathway involved in

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TABLE I. T-cell transfer key

Group	Donor mice (D)	Recipient mice (R)	Challenge
Naive D+/+ R−/− saline	Naive STAT6+/+	Naive STAT6−/−	Saline
Naive D+/+ R−/− OVA	Naive STAT6+/+	Naive STAT6−/−	OVA
D+/+ R−/− Saline	Sensitized STAT6+/+	Naive STAT6−/−	Saline
D+/+ R−/− OVA	Sensitized STAT6+/+	Naive STAT6−/−	OVA
D−/− R−/− OVA	Sensitized STAT6−/−	Naive STAT6−/−	OVA
D+/+ R+/+ Saline	Sensitized STAT6+/+	Naive STAT6+/+	Saline
D+/+ R+/+ OVA	Sensitized STAT6+/+	Naive STAT6+/+	OVA

D, Donor; R, recipient.

IL-4- and IL-13-induced responses, including the commitment of CD4⁺ T cells to the T_H2 phenotype and IgE isotype switching in B cells.^{23,25,26} This suggests that the STAT6 pathway is an integral part of the allergic response. Indeed, we have previously demonstrated the critical role of STAT6 in the development of a T_H2 response, allergic airway eosinophilia, and AHR in ovalbumin (OVA)-sensitized and OVA-challenged mice.²⁷

In the present study we further addressed the role of the T cell in the development of AHR in mice after transfer of purified splenic T cells from either naive or sensitized STAT6-sufficient mice into naive STAT6-sufficient or STAT6-deficient mice before challenge with allergen. The data indicate a role for STAT6-positive T cells in the regulation of airway tone independent of eosinophils.

METHODS

Animals

Male and female STAT6-deficient (STAT6−/−) mice of a B6/129 background were obtained from Dr J. N. Ihle (St Jude Children's Research Hospital) and bred at National Jewish Medical and Research Center.²⁵ B6/129 STAT6-sufficient (STAT6+/+) mice were obtained from Jackson Laboratories. The mice were maintained on OVA-free diets. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Sensitization and airway challenge

Groups of donor mice, 10 to 12 weeks of age, were sensitized by means of intraperitoneal injection of 20 µg of OVA (Grade V, Sigma Chemical Co) emulsified in 2.25-mg aluminum hydroxide (Alum-Imuject, Pierce) in a total volume of 100 µL on days 1 and 14. Sensitized donor animals were killed on day 28, spleens were removed, and T cells were isolated. The isolated T cells (10 × 10⁶ cells administered intravenously) were administered to naive recipient mice that were then challenged (20 minutes) through the airways twice daily (morning and afternoon) with OVA (1% in PBS) or vehicle (PBS) aerosol (AeroSonic ultrasonic nebulizer, DeVilbiss) for 5 days (days 28, 29, 30, 31, and 32). Forty-eight hours after the last OVA challenge (day 34), AHR was assessed, and tissues were obtained for further analysis. Preliminary experiments established this time frame to result in maximum development of AHR and lung inflammation after T-cell transfer in these mice. The different T-cell transfers performed are shown in Table I.

Isolation of spleen mononuclear cells and FACS analysis

Spleens were harvested, and mononuclear cells were obtained by passing the tissue through a stainless steel mesh. Red cells were lysed, and the remaining cells were purified through a nylon wool column. FACS analysis was performed to assess the purity of the T-cell population. Cells were preincubated with mouse serum followed by incubation with either FITC-conjugated (anti-CD3 clone 145-2C11) or isotype control for 30 minutes on ice. After washing, cells were examined (10,000 gated events analyzed) with an EPICS XL analyzer (Coulter Electronics). Typically, the purified cell population contained greater than 90% CD3⁺ T cells.

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine. Anesthetized (intraperitoneal pentobarbital sodium, 70-90 mg/kg) and tracheostomized mice were mechanically ventilated, and lung function was assessed by using methods described by Takeda et al.²⁸ Methacholine aerosol was administered in increasing concentrations, and lung resistance and dynamic compliance were measured and expressed as the percentage change from baseline values after saline aerosol administration.

Bronchoalveolar lavage

Lungs underwent lavage through the tracheal tube with HBSS (1 × 1 mL at 37°C). Total leukocyte numbers were measured (Coulter Counter, Coulter Corp). Differential cell counts were performed by counting at least 300 cells on cytocentrifuged preparations (Cytospin 2, Shandon Ltd) stained with Leukostat (Fisher Diagnostics).

Immunohistochemistry

Lungs were fixed by means of inflation (1 mL) and immersion in 10% formalin. Cells containing eosinophilic major basic protein (MBP) were identified by means of immunohistochemical staining, as previously described, by using rabbit anti-mouse MBP (kindly provided by Dr J. J. Lee, Mayo Clinic, Scottsdale, Ariz).²⁹ The slides were examined in a blinded fashion with a Nikon microscope equipped with a fluorescein filter system.

Measurement of BALF cytokines

Cytokine levels in the BALF supernatants were measured by means of ELISA (IFN-γ, IL-4, and IL-5, Pharmingen; IL-13, R&D Systems). The limit of detection was 10 pg/mL for IFN-γ, IL-4, and IL-5 and 1.5 pg/mL for IL-13.

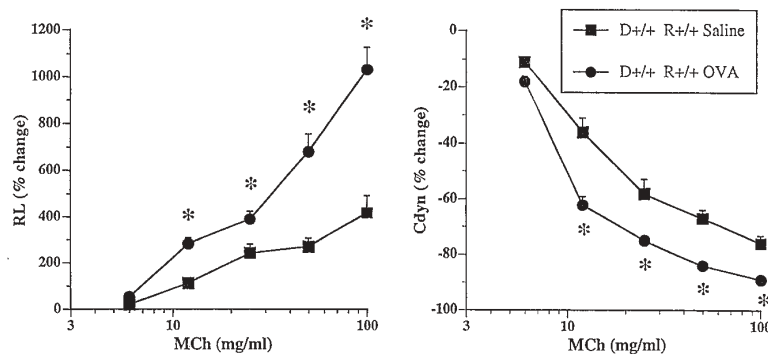


FIG 1. OVA-challenged naive STAT6^{+/+} mice have AHR after transfer of T cells from sensitized STAT6^{+/+} mice. *D+/+ R+/+ OVA*, OVA-challenged STAT6^{+/+} mice receiving T cells from sensitized STAT6^{+/+} mice; *D+/+ R+/+ saline*, saline-challenged STAT6^{+/+} mice receiving T cells from sensitized STAT6^{+/+} mice; *R_L*, lung resistance; *MCh*, methacholine. Data represent mean $\pm$ SEM ($n = 12-18$). *Significant difference ($P < .05$) between *D+/+ R+/+ OVA* and *D+/+ R+/+ saline* groups.

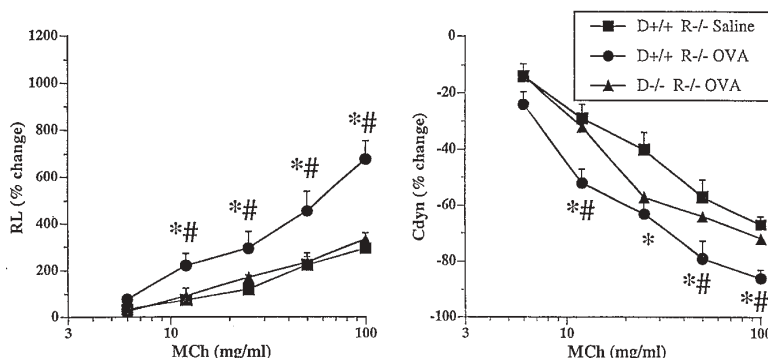


FIG 2. OVA-challenged naive STAT6^{-/-} mice have AHR after transfer of T cells from sensitized STAT6^{+/+} mice. *D+/+ R-/- OVA*, OVA-challenged STAT6^{-/-} mice receiving T cells from sensitized STAT6^{+/+} mice; *D+/+ R-/- saline*, saline-challenged STAT6^{-/-} mice receiving T cells from sensitized STAT6^{+/+} mice; *D-/- R-/- OVA*, OVA-challenged STAT6^{-/-} mice receiving T cells from sensitized STAT6^{-/-} mice; *R_L*, lung resistance; *MCh*, methacholine. Data represent mean $\pm$ SEM ($n = 8$). *Significant difference ($P < .05$) between *D+/+ R-/- OVA* and *D+/+ R-/- saline* groups. #Significant difference ($P < .05$) between *D+/+ R-/- OVA* and *D-/- R-/- OVA* groups.

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by using the Tukey-Kramer honest statistical difference test. P values for significance were set to .05. Values for all measurements are expressed as means $\pm$ SEM.

RESULTS

OVA-challenged naive STAT6^{+/+} mice have AHR after transfer of T cells from sensitized STAT6^{+/+} mice

Baseline lung resistance and dynamic compliance measurements in saline-challenged naive STAT6^{+/+} mice receiving T cells from sensitized STAT6^{+/+} mice were 0.78 ± 0.07 cm H₂O·mL⁻¹·sec⁻¹ ($n = 8$) and 0.048 ± 0.006 mL/cm H₂O ($n = 8$), respectively, and were sim-

ilar ($P > .05$) across all transfer groups. OVA challenge of naive STAT6^{+/+} mice receiving T cells from sensitized STAT6^{+/+} mice resulted in the development of a significant AHR when compared with results in saline-challenged animals receiving the same T-cell transfer (Fig 1). This demonstrated the ability of allergen-primed T cells to modulate airway responsiveness in naive recipient mice when subsequently challenged with the same allergen. The role of STAT6 in the T-cell transfer activity was then determined. OVA-challenged naive STAT6^{-/-} mice did not have AHR after transfer of T cells from sensitized STAT6^{-/-} mice (Fig 2). In contrast, OVA-challenged naive STAT6^{-/-} mice had AHR after transfer of T cells from sensitized STAT6^{+/+} mice, suggesting STAT6 was important in the allergen priming of the donor T cells and that STAT6 deficiency in the recipient mice was

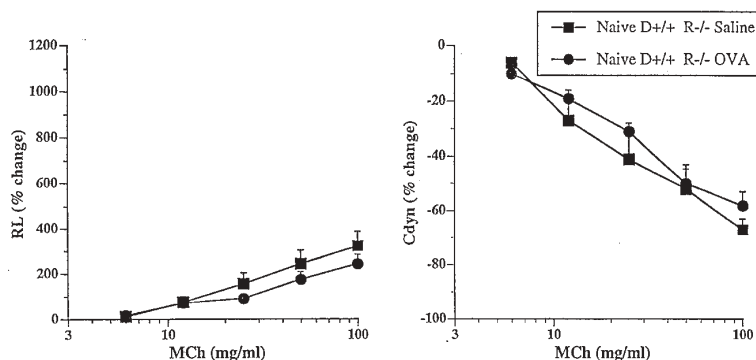


FIG 3. AHR does not develop in OVA-challenged naive STAT6^{-/-} mice after transfer of T cells from naive STAT6^{+/+} mice. Naive D^{+/+} R^{-/-} OVA, OVA-challenged STAT6^{-/-} mice receiving T cells from naive STAT6^{+/+} mice; naive D^{+/+} R^{-/-} saline, saline-challenged STAT6^{-/-} mice receiving T cells from naive STAT6^{+/+} mice; R_L, lung resistance; MCh, methacholine. Data represent mean ± SEM (n = 6-8 per group).

not critical for AHR (Fig 2). Interestingly, the level of AHR achieved was less than that observed in OVA-challenged naive STAT6^{+/+} mice after transfer of T cells from sensitized STAT6^{+/+} mice (Fig 1). This suggests that although STAT6 in the recipient mice was not critical for AHR, it nonetheless might play a contributory or regulatory role in the level of AHR achieved. Furthermore, OVA-challenged naive STAT6^{-/-} mice did not have AHR after transfer of T cells from naive STAT6^{+/+} mice, confirming that prior allergen priming of the donor T cells was necessary (Fig 3).

AHR after T-cell transfer was independent of BALF and tissue eosinophilia

The number and type of inflammatory cells in the BALF was assessed after allergen challenge of recipient mice (Fig 4). Total cell numbers recovered in BALF were essentially the same in all groups of mice. Moreover, the inflammatory cell type recovered in the BALF of all transfer groups consisted almost entirely of macrophages. A small number of lymphocytes was observed and was significantly increased in the OVA-challenged STAT6^{-/-} mice receiving T cells from sensitized STAT6^{+/+} mice when compared with that seen in saline-challenged STAT6^{-/-} mice receiving T cells from sensitized STAT6^{+/+} mice. However, very few neutrophils and even fewer eosinophils were observed in any of the transfer groups.

Eosinophilic inflammation was further assessed in airway tissues of recipient mice by using an antibody directed against MBP. Very few eosinophils were found in the airway tissues of any of the recipient mice, confirming the observations in the BALF (data not shown).

BALF cytokine levels

Cytokine levels were measured in BALF supernatants after allergen challenge of recipient mice. No significant differences in levels of IL-4, IL-5, IL-13, or IFN-γ were

detected between any of the different experimental and control groups (data not shown).

DISCUSSION

Activated T cells regulate allergic airway inflammation and AHR in human subjects through the release of specific cytokines, including IL-4, IL-5, and IL-13.^{1-5,7-9} In the present study we delineated a direct role of T cells in the regulation of allergic AHR in a murine model by using adoptive T-cell transfer. The data demonstrated that transfer of STAT6^{+/+} T cells from OVA-sensitized mice to naive STAT6^{-/-} mice resulted in AHR when the naive recipients were subsequently challenged with OVA. Moreover, the development of AHR was not associated with significant BALF or tissue eosinophilia.

STAT6 has been identified as the principle signaling pathway involved in IL-4- and IL-13-induced responses, including the commitment of CD4⁺ T cells to the T_H2 phenotype.^{23,25,26} OVA-challenged naive STAT6^{-/-} mice had AHR after transfer of T cells from sensitized STAT6^{+/+} mice. This suggests that STAT6 was not essential in the recipient mice for the development of AHR. Whether this reflects possible alternate signaling pathways by which IL-4 and IL-13 could induce AHR, if indeed they are the major cytokines derived from the donor T cells, or whether other T cell-derived cytokines, such as IL-5, are responsible for AHR is not clear at present. Interestingly, the AHR response was reduced in the STAT6^{-/-} recipient mice when compared with that for OVA-challenged naive STAT6^{+/+} mice after transfer of T cells from sensitized STAT6^{+/+} mice. This would suggest that STAT6 in the recipient mice, although not essential for the development of AHR, might nonetheless play some contributory role. This might be an indication of the contribution to AHR of the donor T cell-derived cytokines that require STAT6 in the recipient for induction of response (ie, IL-4 and IL-13 or even IL-9). Alter-

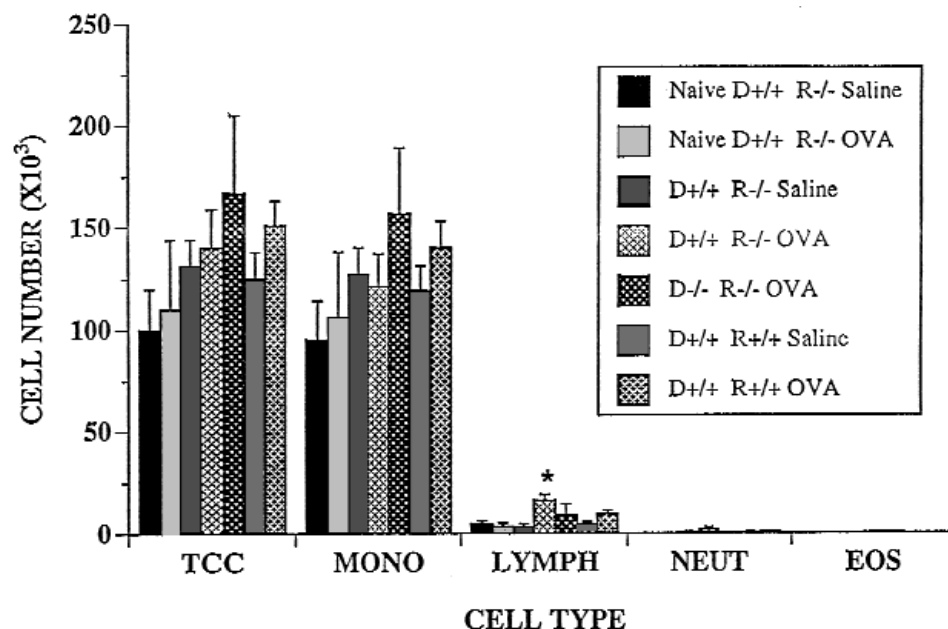


FIG 4. BALF differential cell counts. TCC, Total cell count; MAC, macrophages; LYMPH, lymphocytes; NEUT, neutrophils; EOS, eosinophils. Results of each group are expressed as mean $\pm$ SEM ($n = 6-18$). See Table I for T-cell transfer key.

natively, given the time from first OVA challenge to measurement of AHR (7 days), it might reflect the recipient's T-cell response to OVA. This, however, seems unlikely because previously we have demonstrated that there was no significant difference in the airway responsiveness of naive STAT6 $^{-/-}$ and STAT6 $+/+$ mice when subsequently challenged with OVA by using the same protocol.

In contrast, the STAT6 status in the donor mice was critical during the initial T-cell priming because OVA-challenged naive STAT6 $^{-/-}$ mice did not have AHR after transfer of T cells from sensitized STAT6 $^{-/-}$ mice. The AHR response appeared to be dependent on allergen provocation because saline challenge of the naive STAT6 $^{-/-}$ mice receiving T cells from sensitized STAT6 $+/+$ mice did not induce AHR. Furthermore, OVA-challenged naive STAT6 $^{-/-}$ mice did not have AHR after transfer of T cells from naive STAT6 $+/+$ mice. This observation indicated that the development of AHR in the STAT6 $^{-/-}$ recipient mice was dependent on prior priming with antigen of the donor T cells.

The ability to reconstitute AHR after T-cell transfer is consistent with recent reports in which AHR was observed in OVA-challenged mice receiving OVA-specific T_H2 clones.^{12,14-16,18} Previously, we have shown that the sensitization of STAT6 $+/+$ mice, using an identical protocol, induces a distinct T_H2-type response.²⁷ Sensitized STAT6 $^{-/-}$ mice, in contrast, have a predominant T_H1 response and did not have AHR when challenged.²⁷ Thus the sensitization or priming of T cells in STAT6 $^{-/-}$ and STAT6 $+/+$ donor mice might result in a different

skewing of the T-cell populations toward a T_H1- or T_H2-type response. This would agree with the results of studies in which mice transferred with T_H1 T-cell clones did not have AHR when challenged with allergen.^{14,15,17} In the present study levels of T_H1 and T_H2 cytokines in the BALF of challenged recipient mice were low (low picograms per milliliter) and did not differ between any of the groups of mice. However, this does not rule out the possibility that cytokines play a role in the development of AHR. It is possible that the release of cytokines occurred at an earlier time point before the time at which changes in airway responsiveness were detectable. This would be consistent with the observations of Kaminuma et al.¹⁵ In their transfer study, although they were able to detect an increase in T_H2 cytokines after antigen challenge, the increase peaked within 24 hours of challenge, but by 48 hours, levels had returned to baseline values. Alternatively, the local release of cytokines at low levels or in a time-dependent manner in the tissues, levels that are below detection in the BALF, might contribute to the differences in AHR between the different transfer groups.

The reconstitution of AHR in STAT6 $^{-/-}$ recipients by means of STAT6 $+/+$ T cells was not associated with significant BALF or tissue eosinophilia. Indeed, no significant eosinophilia in BALF or airway tissue was observed in any of the transfer studies. Although many clinical and experimental studies support the notion that the eosinophil is a major effector cell in the development of allergic AHR, there are a growing number of studies that have failed to demonstrate such a relationship.^{6,17,21,29-33}

A number of transfer studies with T_H2 clones show the development of airway eosinophilia associated with AHR.^{12,14-18} However, discrepancies exist as to what role the eosinophils might play in the development of AHR in some of these studies. Severe combined immunodeficient mice or BALB/c mice receiving T_H2 cell lines generated from OVA-specific T-cell receptor transgenic mice and challenged with OVA also had AHR and airways eosinophilia.¹⁴ The transfer of T_H1 cells did not attenuate T_H2 cell-induced AHR but significantly reduced airway eosinophilia, suggesting that alternative mechanisms might be involved in the T_H2-induced AHR. Using T-cell receptor transgenic T_H2 cells, Cohn et al¹⁷ were able to reconstitute AHR and airways eosinophilia in BALB/c mice. Interestingly, BALB/c or IL-4-deficient mouse recipients of IL-4-deficient (IL-5 and IL-13 producing) T_H2 cells also generated AHR but with minimal airway eosinophilia, indicating that the T-cell dependent AHR was independent of IL-4 and, possibly, eosinophils. In addition, sensitization and challenge of recombinase-activating gene-deficient mice (C57BL/6 background) after transfer of CD4⁺ T cells from wild-type mice induced AHR and airway eosinophilia.¹⁶ However, neutralizing antibody to IL-5, although inhibiting airway eosinophilia, had no effect on AHR in B cell-deficient mice (C57BL/6 background), confirming a T cell-dependent but eosinophil-independent mechanism for the development of AHR, as indicated by the observations in the present study. Given the strain background (B6/129) used in the current study and those described above (BALB/c and C57BL/6), it appears that strain differences per se are not a major contributing factor to the eosinophil-independent nature of the T cell-mediated response, although there are clear differences in the extent and location in which eosinophils accumulate in the lung tissue and BALF.³⁴ The sum of these data implicate several pathways, both eosinophil-dependent and eosinophil-independent pathways, in the development of altered airway responsiveness.

In summary, our data demonstrate that T cells transferred from previously primed animals into recipients challenged with the same antigen induce AHR. These transfer studies establish a critical role for STAT6 in the donor T cells but not in recipient mice. Moreover, the lack of significant airway eosinophilia in any of the transfer studies performed implies that a pathway might exist by which STAT6⁺ T cells can modulate AHR independently of eosinophils.

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