

T_H1-mediated airway hyperresponsiveness independent of neutrophilic inflammation

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Background: T_H2-mediated allergic asthma is characterized by eosinophilia, mucus overproduction, and airway hyperresponsiveness (AHR). Although it is clear that T_H2 cells and their cytokines play an important role in AHR, the roles of T_H1 cells and neutrophils in AHR are controversial.

Objective: We sought to determine the roles of T_H1 cells and neutrophils in AHR.

Methods: Ovalbumin-specific CD4⁺ T cells were purified from DO11.10 mice, differentiated into T_H1 cells, and injected into naive BALB/c, IL-4RαKO, or IL-8RKO mice. After ovalbumin antigen challenge, cytokine mRNA levels in lung samples, as well as inflammatory cell types and numbers in bronchoalveolar lavage fluid (BALF), were determined. AHR was assessed by measuring resistance in tracheostomized mice and enhanced pause in freely moving mice.

Results: T_H1 cells induced AHR as robust as T_H2 cells. They also induced lung inflammation dominated by neutrophils.

Neither AHR nor inflammation were reduced when T_H1 cells were transferred into IL-4RαKO mice. When IL-8RKO mice were used as recipients of T_H1 cells, neutrophilia was greatly reduced, but the AHR was as strong as that seen in wild-type mice. On the other hand, dexamethasone treatment had no effect on neutrophilia but has significantly reduced AHR.

Reduction in AHR was accompanied by a reduction in the numbers of lymphocytes and macrophages in BALF.

Conclusions: T_H1 cells can induce strong AHR independent of IL-4 and IL-13. The AHR is associated with the presence of lymphocytes and macrophages, but not neutrophils, in BALF. Our results point to a pathway whereby T_H1 cells mediate AHR independent of neutrophilic inflammation. (J Allergy Clin Immunol 2005;115:309-15.)

Key words: Asthma, airway hyperresponsiveness, T_H1 cells, T_H2 cells, cytokines, neutrophils

Allergic asthma is a chronic inflammatory disease of the airways, and its incidence is on the increase in developed countries. The disease is characterized by pulmonary eosinophilia, mucus hyperproduction, increased serum

Abbreviations used

AHR: Airway hyperresponsiveness

BALF: Bronchoalveolar lavage fluid

GAPDH: Reduced glyceraldehyde-phosphate dehydrogenase

OVA: Ovalbumin

PenH: Enhanced pause

levels of allergen-specific IgE, and airway hyperresponsiveness (AHR).^{1,2} Several mouse models have been developed that closely resemble many aspects of the human asthmatic phenotype to understand mechanisms underlying disease pathogenicity. In particular, adoptive transfer of ovalbumin (OVA)-specific T_H2 effector cells into naive mice, followed by OVA challenge, has been shown to induce eosinophilic airway inflammation, hyperproduction of mucus, and AHR, thus demonstrating the capacity of these cells to induce asthma-like disease in mice.^{1,3} The T_H2-derived cytokines IL-4 and IL-13 seem to be critical for mucus production and induction of AHR, whereas IL-5 plays a pivotal role in inducing eosinophilic inflammation.⁴⁻⁶

Although it is clear that T_H2 cells and their cytokines play an important role in the development of asthma, the role of T_H1 cells and their cytokines is less clear. In a mouse adoptive transfer model, OVA-specific T_H1 cells have been shown to induce airway inflammation dominated by neutrophils, but the ability of these cells to induce AHR has been controversial.^{1,7-9} Some reports showed no AHR after the adoptive transfer of T_H1 cells,^{1,8,9} whereas others reported AHR as strong as that induced by the T_H2-type cell transfer.⁷ Clearly, it is important to determine the potential role for T_H1 cells in asthma, not least because many experimental treatments are based on the hypothesis that induction of T_H1-type responses in lungs might alleviate asthma symptoms.¹⁰⁻¹³

In the present study we revisit the issue of induction of AHR through adoptive transfer of OVA-specific T_H1 cells. We find that neutrophil-dominated T_H1-induced inflammation can result in a strong AHR. Interfering with IL-4 and IL-13 signaling in this model had no effect on either inflammation or AHR. On the other hand, adoptive transfer of T_H1 cells into IL-8R (CXCR-2)-deficient mice resulted in dramatically reduced neutrophilia, but AHR

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remained unaffected, indicating that neutrophilia is not a prerequisite for T_H1 -mediated AHR. Finally, dexamethasone treatment of the recipient mice had no effect on neutrophilia but dramatically reduced the numbers of lymphocytes and macrophages in bronchoalveolar lavage fluid (BALF) and attenuated AHR. Our results point to a pathway whereby T_H1 cells mediate AHR and suggest that therapy directed toward modulation of the inflammatory responses away from T_H2 -mediated cells and toward therapies driven by T_H1 cells might actually facilitate AHR, albeit through different mechanisms.

METHODS

Mice, induction of asthma, IL-13R α 2Fc, and dexamethasone treatment

BALB/c mice and induced mutants, DO11.10 (anti-OVA TCR Tg), IL4R α KO, and IL8RKO mice, all on A BALB/c background, were obtained from The Jackson Laboratory (Bar Harbor, Me).^{14,15} For induction of an asthma-like phenotype, 8-week-old female mice were injected intravenously (if no specification in the figure legend) with 3×10^6 *in vitro*-activated, OVA-specific T cells on day 0. Three days later, the mice were exposed to OVA (grade V; Sigma, St Louis, Mo) for 1 to 4 days. OVA was administered either intranasally (1% OVA in PBS, 50 μ L per mouse per day) or as aerosol (5% OVA in PBS) for 30 minutes every day. AHR was measured after 1 or 2 challenges, and BALF was collected after 3 to 4 challenges with OVA or PBS. For IL-13R α 2Fc or IgG treatment (both made at Wyeth, Cambridge, Mass), mice received the respective reagent (200 μ g) intraperitoneally on day 0 and again at the time of the first and second OVA exposure. For dexamethasone treatment, mice were injected with 4 mg/kg dexamethasone (Sigma) intraperitoneally once a day for a total of 4 doses; the first dose was administered on the day of cell transfer, and the following doses were given before each OVA challenge.

Generation of T_H1 and T_H2 cells

CD4⁺ cells were isolated from the spleens of DO11.10 mice by using anti-CD4 magnetic beads (Dynal, Oslo, Norway). CD4 cells were plated into 24-well plates that had been coated with 1 μ g/mL anti-CD3 and 5 μ g/mL anti-CD28 antibodies (BD Pharmingen, San Jose, Calif). The cultures were supplemented with 30 ng/mL rIL-12 (Wyeth), 10 U/mL rhIL-2 (Invitrogen), and 10 μ g/mL anti-mouse IL-4 antibodies (BD Pharmingen) to obtain T_H1 -skewed T cells. The cultures were supplemented with 40 ng/mL rIL-4 (R&D Systems, Minneapolis, Minn), 10 U/mL rhIL-2, and 10 μ g/mL anti-mouse IFN- γ antibodies (BD Pharmingen) to obtain skewed T_H2 T cells. Four days after the initiation of the cultures, cells were washed in PBS and injected into recipient mice.

Lung physiology

Airway responsiveness was measured either in anesthetized, tracheostomized mice or in conscious, unrestrained mice. Anesthetized, tracheostomized mice were mechanically ventilated, and lung function was assessed. The animal was placed in the plethysmograph and ventilated at a rate of 150 breaths/min. Resistance computations were derived from the tracheal pressure and airflow signals, using an algorithm of covariance.¹⁶ Methacholine aerosolization was performed by using a nebulizer inline with the inspiratory tubing from the ventilator. Responses in total resistance were analyzed over a 3-minute period after the last puff. In

conscious mice bronchial reactivity to aerosolized methacholine was measured by using Buxco whole-body plethysmography (Buxco Electronics, Troy, NY), as described by Hamelmann et al.¹⁷

BALF analysis

The lungs were lavaged 3 times with 0.7 mL of PBS, BALF cells were sedimented, and supernatants were stored at -20°C for later analysis of cytokine and mucin concentrations. Levels of IL-4, IL-5, IFN- γ , and TNF- α were quantified by using a cytometric bead array kit from BD Pharmingen. IL-13 levels were determined with an ELISA kit (R&D Systems). Mucin content was determined with a modified ELISA, as described previously.¹⁸ Differential cell counts of cytospin preparations were performed after HEMA3 staining (Biochemical Sciences Inc, Swedesboro, NJ).

TaqMan analysis

The lungs were collected, and RNA was isolated with the RNeasy kit (Qiagen Inc, Valencia, Calif). Purified RNA was treated with DNase and adjusted to a concentration of 50 ng/ μ L before quantitative mRNA analysis with the TaqMan PCR. Primer pairs and probes specific for murine reduced glyceraldehyde-phosphate dehydrogenase (*GAPDH*), *IL4*, *IL5*, *IL13*, and *IFNG* were designed with PrimerExpress software, and the labeled probes were generated by using Perkin-Elmer Applied Biosystems (Foster City, Calif). Cytokine mRNA expression in the lungs was normalized to *GAPDH* and presented as relative units of mRNA.

Statistical analysis

Data are represented as means $\pm$ SE. Statistical analysis was performed on parametric data by using *t* tests, with differences between means considered significant at a *P* value of less than .05.

RESULTS

Cytokine mRNA expression in inflamed lungs after T_H1 and T_H2 adoptive transfer

To determine the efficacy of T_H1 - T_H2 skewing, the cytokine production by these cells was first measured. The cells were washed after 3 days of culture and restimulated with anti-CD3/anti-CD28. The supernatants were collected after 24 hours and analyzed. T_H1 cells produced high levels of IFN- γ (72.1 ± 3.5 , 64.8 ± 7.3 , and 39.4 ± 9.1 ng/mL) and low levels of IL-13 (0.5 ± 0.01 , 0.2 ± 0.01 , and 0.3 ± 0.01 ng/mL), IL-4 (0.55 ± 0.07 , 0.42 ± 0.01 , and 0.22 ± 0.06 ng/mL), and IL-5 (0.6 ± 0.07 , 0.1 ± 0.01 , and 0.1 ± 0.01 ng/mL). T_H2 cells produced high levels of IL-4 (4.0 ± 0.01 , 4.1 ± 1 , and 4.9 ± 0.06 ng/mL), IL-5 (1.4 ± 0.07 , 1.7 ± 0.03 , and 0.8 ± 0.07 ng/mL), and IL-13 (5.5 ± 0.04 , 5.3 ± 0.09 , and 4.1 ± 0.13 ng/mL) and low levels of IFN- γ (0.7 ± 0.03 , 0.8 ± 0.03 , and 0.3 ± 0.03 ng/mL). All data are expressed as means $\pm$ SE of quadruplicate samples and are representative of 3 independent experiments.

After 4 days of culture, T_H1 - or T_H2 -skewed cells were washed and injected intraperitoneally into BALB/c mice. Three days later, the mice were challenged with aerosolized OVA or PBS, and lungs were collected 24 hour after the challenge. mRNA was isolated, and the levels of mRNA expression for IFN- γ , IL-4, IL-5, and IL-13

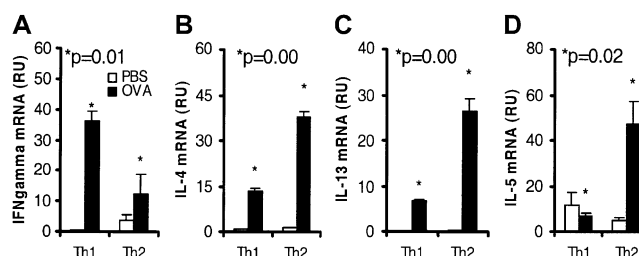


FIG 1. Lung cytokine gene expression in mice injected with T_H1 or T_H2 cells. Mice were injected with T_H1 or T_H2 cells intraperitoneally at 5×10^6 cells per mouse. Twenty-four hours after challenge, RNA was isolated from lungs, and *IFNG* (A), *IL4* (B), *IL13* (C), and *IL5* (D) mRNA levels were determined by means of quantitative PCR and expressed as relative units (RU). Values are presented as means $\pm$ SE ($n = 4$). Similar results were obtained from 2 independent experiments.

cytokines were determined by using real-time PCR (TaqMan). The levels of mRNA were expressed as relative units of *GAPDH* mRNA. Higher levels of *IFNG* mRNA were detected in the lungs in which inflammation was induced by T_H1 cells, whereas higher levels *IL4* and *IL13* mRNA were detected in the lungs in which the inflammation was induced by T_H2 cells (Fig 1, A-C). Significant amounts of *IL5* mRNA were detected only in the lungs in which the inflammation was induced by T_H2 cells (Fig 1, D). Similar results were obtained when cytokine mRNA expression was evaluated in lungs 24 hours after the second OVA or PBS challenge (data not shown).

T_H1 cells induce lung inflammation dominated by neutrophils, low mucus production, and robust AHR

To assess the lung inflammation induced by adoptive transfer of OVA-specific T_H1 and T_H2 cells, we collected and analyzed BALF after 3 to 4 daily challenges with OVA or PBS. In accordance with what has been described previously,¹ OVA-challenged mice that had received T_H1 cells had inflammation dominated by neutrophils (Fig 2, A), whereas the mice that had received T_H2 cells had inflammation dominated by eosinophils (Fig 2, B). Measurement of mucin content in BALF revealed increased mucus production in OVA-challenged mice that had received T_H2 , but not T_H1 , cells (data not shown).

Conflicting reports exist on whether T_H1 -mediated lung inflammation is accompanied by AHR.^{1,7-9} In our experiments AHR was assessed by measuring airway resistance in anesthetized, tracheostomized mice (Fig 2, C), as well as by determining enhanced pause (PenH) in conscious, freely moving mice (Fig 2, D). In each of 2 independent experiments with anesthetized, tracheostomized mice to determine airway resistance and 10 independent experiments with whole-body plethysmography of freely moving mice to determine PenH, a very robust bronchoconstriction was observed after methacholine challenge in OVA-challenged mice that had received OVA-specific T_H1 cells. In all experiments, T_H1 -induced AHR was at the very least as pronounced as that induced by adoptive transfer of T_H2 cells (Fig 2, E).

AHR detected after the adoptive transfer of T_H1 cells is not dependent on IL-13

A potent AHR inducer, IL-13, was detected in some T_H1 clones.¹⁸ Also, we have detected relatively low levels of IL-13 in the supernatant of activated T_H1 cells (<3 ng/mL). Finally, increased *IL13* mRNA levels were detected in the lungs of OVA-challenged mice that had been injected with T_H1 cells (Fig 1, C). We therefore first attempted to determine whether T_H1 -induced AHR in our model is IL-13 dependent. It has been previously shown that a soluble murine IL13R α 2Fc protein effectively blocks both AHR and inflammation in a number of antigen-induced asthma models.^{19,20} We show here that administration of IL-13R α 2Fc had no effect on inflammation or AHR in mice that had received T_H1 cells (Fig 3, A), whereas it was sufficient to reduce eosinophilic infiltration and AHR induced by the transfer of T_H2 cells (Fig 3, B).

T_H1 -skewed cells were injected into IL-4R α KO mice to further explore the possible roles of either IL-4 or IL-13 on AHR development in the T_H1 -transfer model. IL-4R α KO mice lack the signaling receptor α -chain used by both IL-4R and IL-13R. When these mice were used as recipients of T_H1 cells, both inflammation and AHR after OVA challenge was as robust as that observed in wild-type control animals (Fig 3, C).

Dexamethasone treatment significantly reduces T_H1 -mediated AHR

Dexamethasone treatment has been shown to reduce both inflammation and AHR in T_H2 -mediated asthma models.^{21,22} In our T_H1 model treatment with dexamethasone (4 mg/kg) did not significantly reduce the total numbers of BALF cells in comparison with the numbers observed in the vehicle-treated group. However, the analysis of the inflammatory cells in these mice revealed that the treatment dramatically reduced the numbers of lymphocytes and macrophages while not affecting the numbers of neutrophils in BALF (Fig 4, A). Mice treated with 4 mg/kg dexamethasone also had a significant reduction in AHR, as measured on the basis of airway resistance, in anesthetized, tracheostomized mice and PenH in conscious, freely moving mice (Fig 4, B and

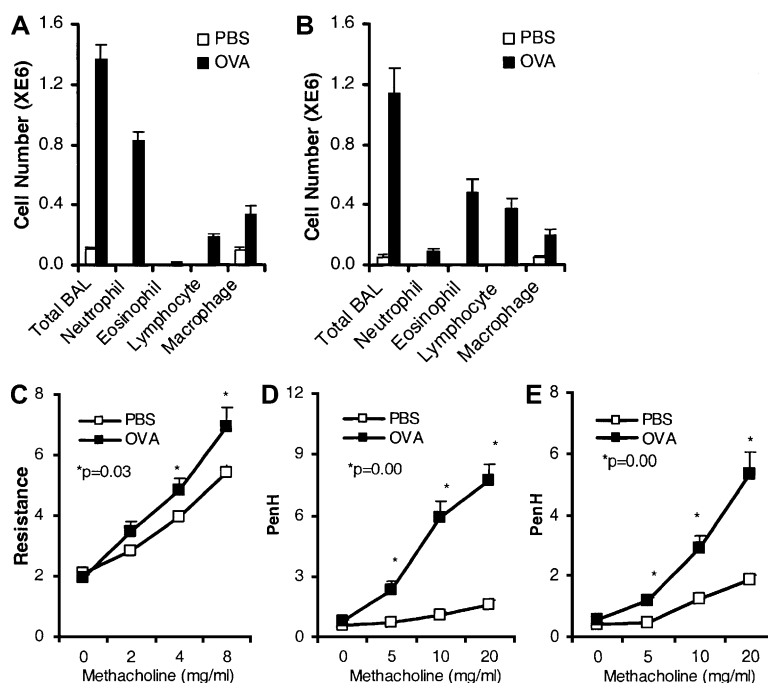


FIG 2. Lung inflammation and AHR in mice injected with T_H1 or T_H2 cells. BALF cell and differential counts from mice injected with T_H1 (A) or T_H2 (B) cells are shown. Airway resistance (C) and PenH (D) were measured in T_H1 cell-injected mice (cells were injected intraperitoneally at 5×10^6 in Fig 2, A and D), and PenH was measured in T_H2 cell-injected mice (E). Values are presented as means $\pm$ SE (n = 10 mice).

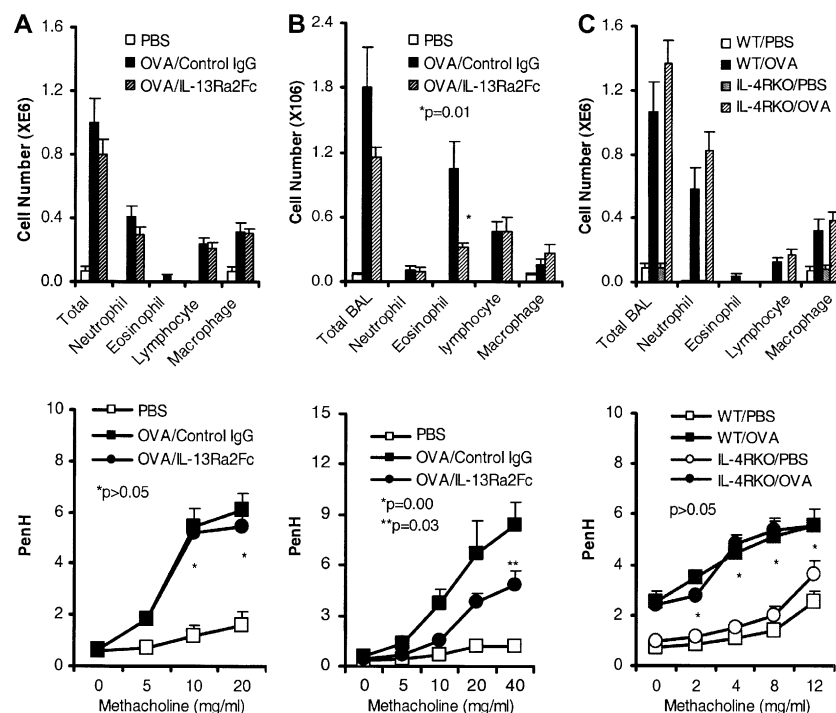


FIG 3. Effects of IL-13 and IL-4 on inflammation and AHR mediated by T_H1 cells. Total BALF cell and differential counts (upper panels) and AHR (lower panels) from T_H1 cell-transferred (A) and T_H2 cell-transferred (B) mice with IgG control antibody or IL-13Ra2Fc treatment are shown. C, The inflammation and AHR in wild-type and IL-4RKO mice injected with T_H1 cells (n = 8-12) are shown.

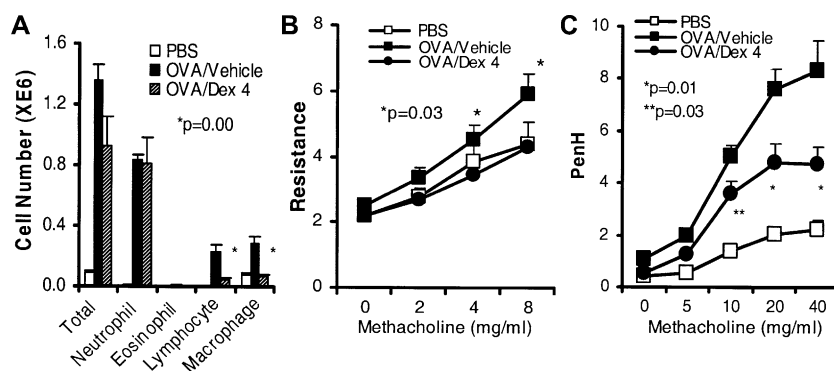


FIG 4. Effects of dexamethasone (Dex) on T_H1 cell-mediated inflammation and AHR. T_H1 cell-injected and OVA-challenged mice were treated with vehicle or dexamethasone at 4 mg/kg ($n = 10$). Total BALF cell and differential cell numbers (A), Airway resistance (B), and PenH (C) was measured.

C), although AHR was still significantly increased compared with that observed in PBS-challenged mice (Fig 4, C). Thus inhibition of AHR with dexamethasone is associated with a reduction in the numbers of lymphocytes and macrophages, but not neutrophils, in BALF.

IL-8RKO mice had significantly reduced inflammation but maintained AHR after T_H1 cell transfer and OVA challenge

Because T_H1 -induced inflammation is dominated by neutrophils and chemokines of the IL-8 family are the most important attractants of neutrophils *in vivo*,^{23,24} to further define the roles of neutrophils in our T_H1 model, we examined inflammation and AHR development in IL-8RKO mice. When T_H1 cells were transferred into IL-8RKO mice and these mice were challenged with OVA, the total number of BALF cells was significantly reduced in comparison with that seen in the wild-type control animals. This reduction was entirely caused by a dramatic reduction in the numbers of neutrophils because the absolute numbers of lymphocytes and macrophages remained unaltered in IL-8RKO mice (Fig 5). AHR in these mice was comparable with that observed in OVA-challenged wild-type animals that had received T_H1 cells (Fig 5).

DISCUSSION

Numerous studies have shown that allergen-specific T_H2 cells can initiate asthmatic inflammatory responses in lungs, which is characterized by eosinophilic inflammation, mucus hypersecretion, and AHR.^{5,19,25} The role of T_H1 cells, on the other hand, is much more controversial. Although various animal models have indicated that T_H1 cells invariably induce lung inflammation on local antigen challenge, investigations into their ability to induce AHR, a hallmark of asthma, have yielded conflicting results.^{1,7-9} Here we report that adoptive transfer of OVA-specific T cells that have been skewed along a T_H1 pathway induce both neutrophil-dominated inflammation and robust AHR after OVA airway challenge in normal recipients.

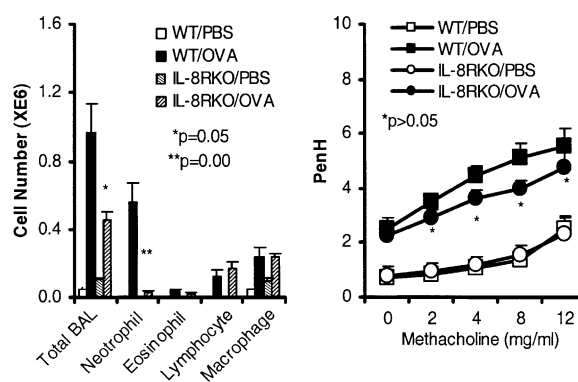


FIG 5. T_H1 cell-mediated inflammation and AHR in IL-8RKO mice. The total BALF cell and differential counts (left panel) and AHR (right panel) in wild-type and IL-8RKO mice injected with T_H1 cells is shown. Data represent means $\pm$ SE ($n = 8-10$ mice).

To ensure that the observed changes in AHR were not due to erroneous levels of sensitivity of the experimental methods, we used anesthetized, tracheostomized mice to measure airway resistance, as well as freely moving mice to determine PenH. With both methodologies, we consistently observed statistically significant increases in AHR in OVA-challenged mice that had received T_H1 -skewed cells compared with that seen in recipients that were challenged with PBS. Both inflammation and airway responsiveness were essentially identical in IL-4R α KO, IL-13R α 2Fc-treated, or control mice, indicating that AHR in this model was not dependent on small amounts of either IL-13 or IL-4 that might be present in the lungs after the adoptive transfer of T_H1 -skewed cells.

The present study demonstrates that T_H1 -initiated responses against allergen can lead to a severe inflammation and AHR, which are independent of T_H2 -type cytokines. Our results differ from those published in several other studies, in which T_H1 cells were shown to induce lung inflammation but not AHR.^{1,8,9} Several explanations are possible for this discrepancy. The timing and the length of exposure to antigen could explain the differences in results. In contrast to published experimental procedures, we rested animals for 3 days between

injection of antigen-specific T cells and antigen challenge and have observed that this rest period increases inflammation by several fold (as determined by the number of BALF cells, data not shown). It is possible that this increase in inflammation also led to an increase in AHR. In addition, in our model animals were exposed to only 1 to 2 airway antigen challenges, rather than 7, as reported in some other studies. It is possible that T_H1 cells, which are more susceptible to activation-induced cell death than T_H2 cells,²⁶ were largely eliminated from the lungs after 7 or more exposures to antigen, resulting in absence of AHR in these longer-term models. Finally, T_H1 cells used in other studies were either long-term cell lines, which might have lost some of their *in vivo* functions, or were short-term cultured cells that were activated by using antigen-presenting cells and antigen rather than anti-CD3/anti-CD28 stimulation, which we chose to use. It is possible that different modes of activation result in T cells with different capacities to induce AHR. Indeed, Sugimoto et al²⁷ recently published a study demonstrating that *in vitro* differentiated OVA-specific memory T_H1 cells injected into BALB/c mice induce strong AHR on OVA challenge if IL-18 is administered to the mice together with OVA. It is also possible that the differences observed in T_H1 -mediated AHR are due to the different level of IL-10 or other regulatory cytokines produced *in vitro* or *in vivo* in different models of T_H1 -induced inflammation. It has been demonstrated in multiple studies that overexpression of IL-10 or TGF- β 1 prevents allergen-induced AHR and inflammation.^{28,29}

We were interested in exploring the role of IL-8R and neutrophils in the induction of AHR in our model because at the time of completion of this work, the only report on T_H1 -induced AHR suggested that this is IL-8R mediated, neutrophil mediated, or both.⁷ To evaluate whether IL-8R plays a role in our model of T_H1 -induced AHR, we adoptively transferred T_H1 cells into IL-8RKO mice. In contrast to the previous report that anti-IL-8R treatment reduces AHR in a T_H1 -adoptive transfer model,⁷ we saw no reduction in AHR when we transferred T_H1 cells into IL-8R-deficient mice. It is possible that IL-8RKO mice are developmentally different from wild-type animals in a way that makes T_H1 -induced AHR independent of IL-8R in the former but not the later case. However, when we used IL-8R-deficient mice, we still observed an almost complete absence of neutrophils in BALF (similar to observations made using anti-IL8R antibodies), indicating that in IL-8RKO mice, IL-8R is still essential for lung neutrophilia, but neutrophilia is not essential for AHR development.

To understand the similarities between T_H1 - and T_H2 -mediated AHR, we investigated whether the inflammatory responses induced by T_H1 cells could be modified by glucocorticoid treatment. Glucocorticoids are among the first-line therapeutics for asthma, reducing both inflammation and AHR in T_H2 -induced asthma. Although the exact mechanism of action remains unclear, it has recently been demonstrated that dexamethasone does not affect AHR after direct administration of IL-13, suggest-

ing that glucocorticoids reduce AHR by acting upstream of IL-13 production.³⁰ In our T_H1 model dexamethasone treatment led to a severe reduction in the numbers of lymphocytes and macrophages in the BALF of OVA-challenged mice accompanied by a significant reduction in the maximal AHR to aerosolized methacholine (Fig 4), indicating that the level of neutrophilic inflammation is not associated with the severity of AHR. Consistent with this observation, results from IL-8RKO mice showed AHR comparable with that observed in wild-type animals, despite the fact that IL-8RKO mice had dramatically reduced neutrophilic infiltration (Fig 5), suggesting that neutrophils might not be critical for T_H1 -mediated AHR in this model.

Our clear demonstration that newly differentiated T_H1 cells can induce inflammatory responses and AHR after appropriate activation calls into question the validity of switching from T_H2 -type responses to those dominated by T_H1 cells as a potential asthma treatment. Our results clearly demonstrate that T_H1 cells are potentially important players in AHR induction and that their role should be further investigated in the pathogenesis of asthma.

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