Airway Hyperresponsiveness in the Absence of CD4+ T Cells after Primary but Not Secondary Challenge

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CD4+ T cells have been shown to play a role in the development of airway hyperresponsivness (AHR) and airway eosinophilia in mice using ablation as well as adoptive transfer experiments. However, as other T cell subsets (CD8, NKT) may play a role in these models, we examined the responses of sensitized CD4-deficient mice after either primary or secondary airway allergen challenge. After sensitization, CD4-deficiency in mice was not associated with airway eosinophilia, allergen-specific IgE, or elevated levels of interleukin (IL)-4 or IL-13. Increases in lung CD8 T cells and IL-5 were observed and shown to be essential for AHR as demonstrated after CD8 T cell depletion or anti–IL-5 treatment. In contrast to the response of sensitized CD4-deficient mice to primary allergen challenge, they failed to develop AHR after secondary allergen challenge. Although the importance of this CD4+ T cell–independent pathway in normal mice is unclear at this time, these studies identify the diversity of the cellular pathway, which may contribute to the development of AHR after primary allergen exposure of sensitized mice.

Keywords: airway hyperresponsiveness; CD4 T cells; inflammation; secondary challenge

T cells expressing the α/β T cell receptor are primarily divided on the basis of expression of CD4 or CD8 coreceptors and recognize antigenic peptides in conjunction with major histocompatibility complex (MHC) class II. CD4+ T cells are further subdivided based on their ability to enhance an inflammatory response through the synthesis of cytokines such as interferon (IFN)-γ or tumor necrosis factor-α. A second subset of CD4+ T cells synthesize cytokines such as interleukin (IL)-4, IL-5, and IL-13 and potentiate humoral immune responses (1, 2). These latter CD4+ Th2 cells have been posited to play a central role in the pathogenesis of asthma, mediating allergic inflammatory responses and allergen-specific IgE through the activities of Th2 cytokines (3). Indeed, activated CD4+ T cells are found in the bronchial mucosa of individuals with asthma, and allergen challenge of allergic individuals or mice induces a selective recruitment of CD4+ T cells into the airways (4, 5).

Support for an essential role for CD4+ T cells in the development of allergen-induced airway inflammation and airway hyperresponsiveness (AHR) derives from studies of T cell depletion where ablation of CD4+ T cells prior to allergen sensitization reduces AHR and airway inflammation, especially eosinophilia (6). Further, adoptive transfer of antigen-primed CD4+ T cells can induce an allergic response in the lungs (7, 8). However, *in vivo* depletion of CD4+ T cells may be less effective or complete than depletion of CD3+ T cells, suggesting that the role for CD4+T cells in allergic disease may not be as essential or complete as often is invoked (9). To the contrary, several investigations have identified roles for CD8+ T cells and NK T cells in the regulation of lung eosinophilia or AHR in murine models of allergen-induced inflammation and AHR (10, 11).

In the present study, we examined the response of sensitized CD4-deficient mice to primary and secondary allergen challenge. The responses elicited by these two challenge protocols were strikingly different and identify a role for CD4+-independent pathways in the development of allergen-specific AHR following primary but not secondary challenge of sensitized mice.

METHODS

Mice

Age-matched (8–12 wk old) female CD4+/+ and CD4−/− C57BL/6 mice bred in the animal facility at National Jewish Medical and Research Center were used. The CD4−/− mice were originally derived after disruption of the CD4 gene in embryonic stem cells (12) and were kindly provided by Dr. P. Marrack (Denver, CO). In each experiment, groups of four mice were used in each experimental condition, and each experiment was performed two to three times (n = 8–12). The mice were maintained on an ovalbumin (OVA)-free diet, and all studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Sensitization and Challenge

Sensitization to OVA was achieved after two intraperitoneal injections of 20 μg of OVA (grade V; Sigma-Aldrich, St. Louis, MO) emulsified in 2.25 mg of alum hydroxide (AlumInject; Pierce, Rockford, IL) in a total volume 100 μl, 14 d apart. Primary allergen challenge was on days 26, 27, and 28 with aerosol challenges of 1% OVA for 20 min each day using an ultrasonic nebulizer (DeVilbiss, Somerset, PA). A single secondary aerosolized challenge was administered 6 wk after completion of the primary challenge, after all of the responses to the primary challenge subsided (13). Endotoxin levels in the OVA solution were below 12.5 endotoxin U/mg protein. In some experiments, sensitization and/or challenge were performed in a similar manner using ragweed (RW) extract (Greer Laboratories, Lenoir, NC).

Treatment

Monoclonal anti-CD8β antibody and anti–IL-5 antibody (53–5.8 [Ly3.2], TRFK-5, American Type Culture Collection, Manassas, VA) were prepared as described (14). Either antibody (200 μg) was administered intravenously before sensitization or before the first of the primary challenges. Depletion of cell subsets was verified by phenotypic analysis of cells prepared from lung tissue digests using flow cytometry.

Cell Preparation and Culture

Lung T cells were isolated by collagenase digestion of the lungs and enriched using nylon wool columns as described (15) which resulted in a population of cells that was > 90% CD3+.

Allergen-Specific T Cell Proliferation

Lung mononuclear cells ($5 \times 1\ 0^4$) were cultured together with 10 μg/ml OVA for 5 d in 96-well plates. Tritiated thymidine (1 μci) was added to each well 16 h before ending the culture.

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Adoptive Transfer

For adoptive transfer, 5×10^6 lung T cells were injected intravenously into each recipient mouse. Immediately after adoptive transfer, nonsensitized recipient mice received aerosol allergen challenges (OVA or RW) or phosphate-buffered saline (PBS) for 20 min on six consecutive days.

Measurement of Airway Responsiveness

Airway responsiveness was assessed as a change in airway function to aerosolized methacholine (MCh) 48 h after the last challenge as previously described (16). MCh was administered for 10 s (60 breaths/min, 500 μl tidal volume) in increasing concentrations. Lung resistance (RL) and dynamic compliance (Cdyn) were continuously computed (Labview; National Instruments, Austin, TX) by fitting flow, volume, and pressure to an equation of motion. Maximum values of RL and minimum levels of Cdyn were taken and expressed as a percentage change from baseline following PBS aerosol.

Bronchoalveolar Lavage

Immediately following measurement of AHR, lungs were lavaged with HBSS (1×1 ml 37°C). Total leukocyte numbers were analyzed (Coulter Counter, Hialeah, FL). Differential cell counts were performed under light microscope by counting at least 200 cells on cytocentrifuged preparations (Cytospin 2; Cytospin, Shandon Ltd., Runcorn, Cheshire, UK), stained with Leukostat (Fisher Diagnostics) and differentiated by standard hematological procedures in a blinded fashion.

Determination of Serum Antibody Titers

Serum levels of total IgE, OVA-specific IgE, IgG1, IgG2a, and IgG2b were measured by ELISA as previously described (17).

Measurement of Cytokines in Bronchoalveolar Lavage Fluid

Cytokine levels in the bronchoalveolar lavage (BAL) fluid were measured by ELISA using commercial kits for IL-4, IL-5, IL-10, and IFN-γ (Pharmingen, San Diego, CA). IL-13, and GM-CF, RANTES, and eotaxin ELISAs were performed according to the manufacturer's directions (R&D Systems, Minneapolis, MN). The limits of detection were 4 pg/ml for IL-4, and IL-5, 10 pg/ml for IL-10 and IFN-γ, and 15 pg/ml for RANTES and eotaxin, and 8 pg/ml for GM-CSF and IL-13.

Statistical Analysis

ANOVA was used to determine statistical significance. Comparisons for all pairs were performed by Tukey-Kramer honest significant difference test. The P values for significance were set to 0.05. Values for all measurements were expressed as the mean ± SEM.

RESULTS

Development of AHR and Airway Inflammation following Primary Challenge

Following sensitization of CD4+/+ mice, allergen challenge via the airways 2 wk later resulted in an increase in RL and decrease in Cdyn to inhaled MCh in a dose-dependent manner (Figures 1A and 1B). Nonsensitized but challenged wild-type (WT) mice showed a much lower dose-dependent increase in MCh-induced AHR. When CD4-deficient mice (CD4−/−) were examined under the same conditions, the results were virtually identical to those in the normal mice, with significant increases in RL and decreases in Cdyn throughout the MCh dose–response curve.

In contrast to the changes in airway function, when inflammatory cell accumulation in the BAL fluid was examined, major differences were observed. CD4+ mice developed a marked increase in BAL eosinophilia, whereas the CD4−/− mice had few eosinophils in the BAL fluid, with levels similar to nonsensitized but challenged controls; increases in lymphocyte numbers were observed in both strains of mice (Figure 1C). Similarly, when lung tissue cell numbers were examined (following collagenase digestion), only small numbers of eosinophils ($0.9 \pm 0.3 \times 10^6$) could be detected in the deficient mice, whereas more than $13 \pm 3.2 \times 10^6$ eosinophils/lung were isolated from the lungs of CD4+ mice.

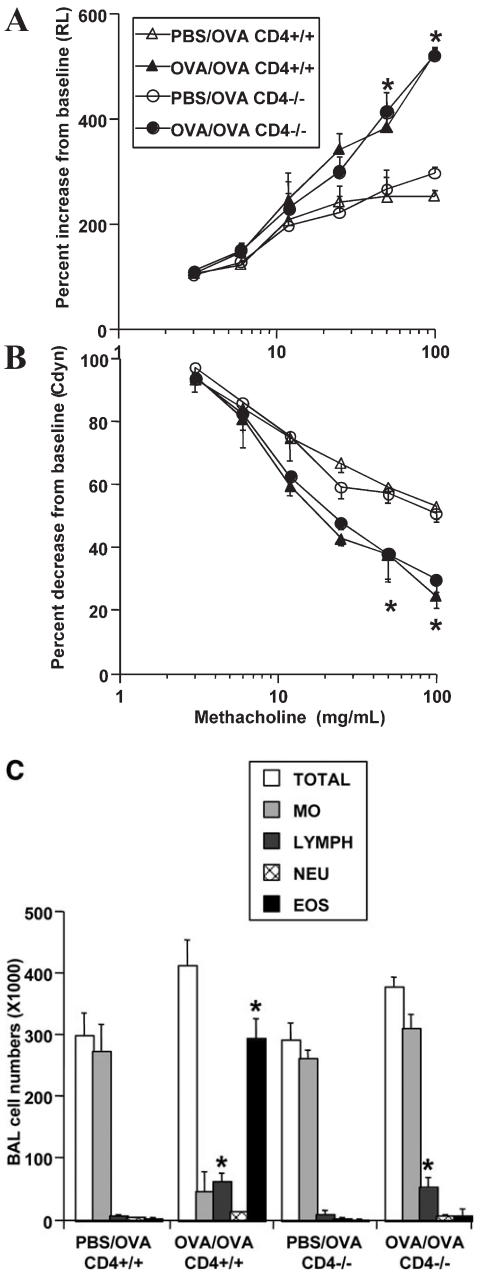

Figure 1. Development of AHR and airway inflammation in CD4+/+ and CD4−/− mice following sensitization and primary challenge. Mice were sensitized to OVA (or received PBS injections) and subsequently exposed to three consecutive days of airway allergen challenges as described in MATERIALS AND METHODS. (*A*) Airway resistance. (*B*) Dynamic compliance. (*C*) BAL inflammatory cell number. $n = 12$ in each group. *$P < 0.01$ compared with the corresponding challenged alone control. PBS/OVA, no sensitization but challenged only; OVA/OVA, sensitized and challenged to OVA. There were no significant differences in baseline RL or Cdyn between any of the groups.

Following sensitization and primary challenge, CD4+ mice developed a characteristic cytokine profile in the BAL fluid when compared with nonsensitized but challenged mice (or sensitized but nonchallenged mice, data not shown). Small increases in IL-4 levels were seen as well as larger increases in IL-5 and

IL-13 levels accompanied by decreases in IL-10 and IFN-γ (Figure 2). In the CD4−/− mice, sensitization and challenge failed to increase IL-4 and IL-13 levels or lead to a reduction in IFN-γ; IL-5 levels increased while IL-10 levels were reduced. Of note, in addition to the comparable increases in BAL IL-5 levels in both CD4+/+ and CD4−/− mice, following sensitization and challenge (compared with nonsensitized mice), the number of bone marrow eosinophils increased in both WT and CD4−/− mice to a similar extent (CD4+/+: from 0.48 ± 0.11 × 10^6 to 1.26 ± 0.34 × 10^6/femur; CD4−/− from 0.35 ± 0.08 to 0.92 ± 0.16 × 10^6/femur, $n = 4$).

As might be expected with the low IL-4 (and IL-13) response, OVA-specific IgE levels were much reduced in the CD4−/− mice (7.3 ± 2 EU/ml, $n = 8$) compared with the CD4+ mice (135.8 ± 5 EU/ml, $n = 8$, $P < 0.05$). Levels of OVA-specific IgG1, IgG2a, and IgG2b were similar in the WT and CD4−/− mice. Furthermore, in keeping with the low levels of IL-13, following sensitization and challenge few mucin-containing goblet cells were detected in lung sections compared with the large increase seen in the CD4+/+ animals (data not shown).

As shown in Figure 3, when allergen-induced proliferative responses were compared in lung mononuclear cell preparations, there were significant responses in both strains of sensitized and challenged mice, although the responses were higher in the CD4+/+ mice.

Development of AHR following Primary Challenge in CD4−/− Mice Is Allergen-Specific and Dependent on IL-5 and CD8+ T Cells

To demonstrate that the response in the CD4−/− mice was not restricted to OVA as well as being allergen-specific, mice were initially sensitized to OVA or RW and then challenged with either OVA or RW. As shown in Figure 4A, mice that were sensitized and challenged with the same allergen demonstrated MCh-dependent increases in lung resistance, whereas mice that were sensitized to one allergen but challenged with the other, failed to show a significant increase in RL. These data demonstrate the ability of the CD4−/− mice to develop AHR to more than one allergen and in an allergen-specific way.

In light of the comparable increases in BAL IL-5 levels in both WT and CD4−/− mice, we determined if IL-5 played a role in the development of AHR in the CD4−/− mice as has been described previously in normal mice (13, 14). In these experiments, the animals were treated with anti–IL-5 before challenge but after sensitization or before sensitization. The inhibitory effects of anti–IL-5 when given before challenge on the development of AHR and lung eosinophilia were confirmed in the CD4+/+ mice (data not shown). Despite the absence of BAL eosinophilia in the CD4−/− mice following sensitization and challenge (Figure 1C), anti–IL-5 treatment after sensitization and before challenge was nonetheless effective in preventing development of AHR in these mice (Figure 4B), and the degree of inhibition was to the same extent observed in the CD4+/+ mice. As expected, anti–IL-5 treatment did not change the previously observed absence of airway eosinophilia. These data suggested that IL-5 may play an important role in the development of AHR in the deficient mice, but independently of the accumulation of eosinophils in the airways or lung tissue. Anti–IL-5 treatment before sensitization was without effect (Figure 4B), similar to results previously shown in CD4+/+ mice (14).

The CD4−/− mice, in the absence of CD4+ T cells, had an increase in the relative numbers of CD8+ T cells as previously reported (12); in lung digests, comparing challenge alone to sensitization and challenge, CD8+ T cells increased from 0.26 ± 0.04 × 10^6 to 1.46 ± 0.9 × 10^6 in the CD4+/+ mice but from 2.98 ± 0.7 × 10^6 to 4.55 ± 1.1 × 10^6 in the CD4−/− mice ($n = 8$). In the CD4+/+ mice, the number of CD4+ T cells increased from 1.54 × 10^6 in challenged only mice to 4.85 ± 0.01 × 10^6 in sensitized and challenged mice. Under these conditions, NK cells, which were low in challenged-only mice, increased slightly in both WT and CD4−/− sensitized and challenged mice (0.15–0.18 × 10^6 cells). With these differences in mind, we determined the role of CD8+ T cells in the development of AHR by depleting them using an anti-CD8β antibody before allergen challenge but after sensitization. Treatment with this antibody fully depleted CD8+ T cells from the circulation and in lung digests in both WT and CD4−/− mice, with less than 0.1 × 10^6 CD8+ T cells detected in the blood or lung digests of either group of mice. As illustrated in Figure 4C, depletion of CD8+ T cells in the CD4−/− mice resulted in a significant reduction in AHR, to levels seen in control (challenged but not sensitized) mice. Of interest, similar treatment of the CD4+/+ mice also resulted in a reduction in AHR (Figure 4C) and BAL eosinophilia (from 316 ± 47 × 10^3 to 181 ± 20 × 10^3 cells [$n = 8$]) after treatment with anti-CD8β. In addition, CD8+ T cell depletion in these CD4−/− mice resulted in a significant decrease in BAL levels of

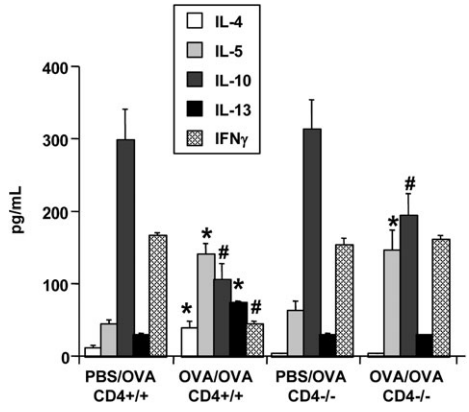

Figure 2. BAL cytokine levels. BAL cytokine levels were determined by ELISA. The same animals as in Figure 1 were used. $n = 12$. *$P < 0.05$ increased compared with corresponding challenged alone; #$P < 0.05$ decreased compared with corresponding challenged alone.

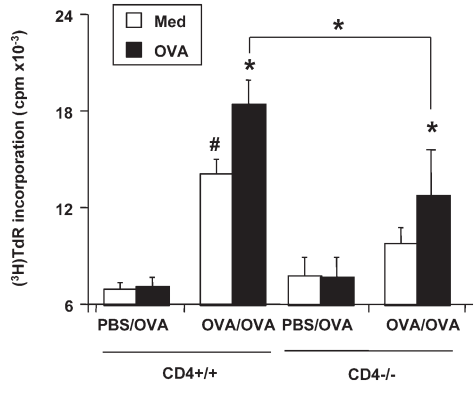

Figure 3. Allergen-specific T cell proliferation. Lung mononuclear cells were cultured with medium or 10 μg/ml OVA for 5 d and [^{3}H]TdR incorporation determined. Mice are the same as in Figure 1. $n = 12$. *$P < 0.05$ between sensitized and challenged groups and between sensitized and challenged CD4+/+ and CD4−/− mice; #$P < 0.05$ for cells from sensitized and challenged mice cultured with medium alone.

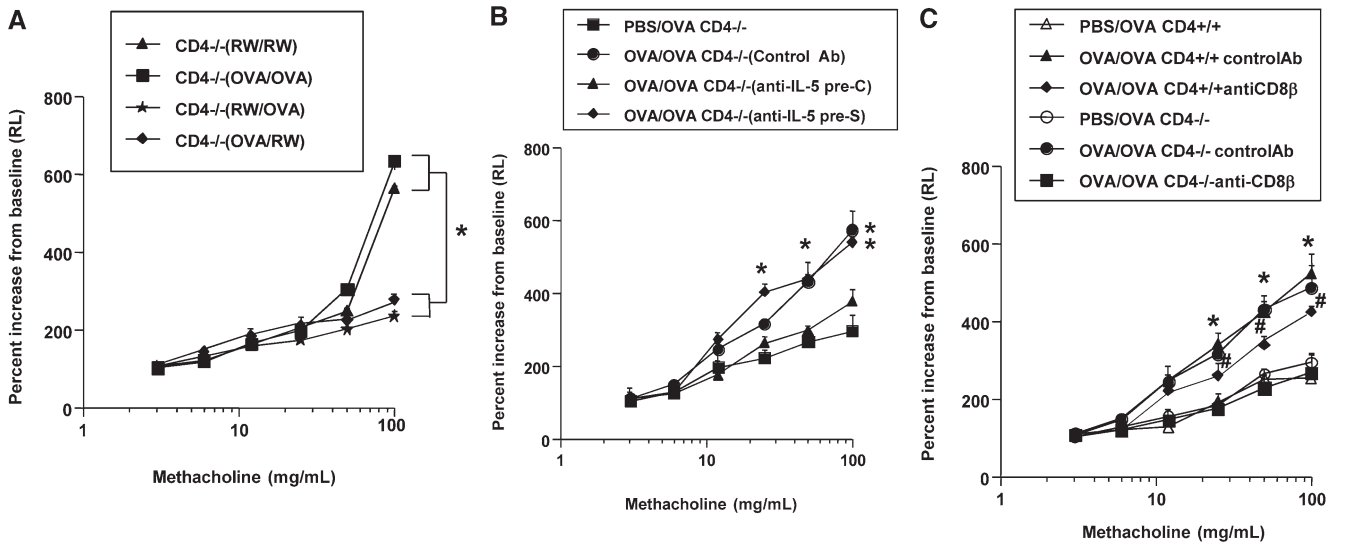

Figure 4. (*A*) Allergen-specific development of AHR. Mice were sensitized to either OVA or RW and then challenged with either OVA or RW (n = 8 in each group). *$P < 0.05$ compared with discordant sensitization and challenge cohorts. Baseline R_L values were the same in all groups. (*B*) Effect of anti–IL-5 on development of AHR. Mice were sensitized and challenged (primary) with OVA. Anti–IL-5 was administered intravenously either before challenge or before sensitization (n = 8 in each group). *$P < 0.05$ compared with control (PBS/OVA) or anti–IL-5 before challenge. (*C*) Effect of anti-CD8β on development of AHR. Anti-CD8β or control antibody was administered before the first of the primary challenges (n = 8 in each group). *CD4−/− control antibody treated compared with anti-CD8 treated mice; #$P < 0.05$ CD4+/+ comparing CD4+/+ anti-CD8 versus control antibody–treated animals.

IL-5 in sensitized and challenged mice (from 162 ± 14 pg/ml after control antibody treatment to 51 ± 8 pg/ml in anti-CD8–treated mice). In CD4+/+ mice, anti-CD8 treatment decreased IL-5 levels from 134 ± 16 pg/ml to 94 ± 11 pg/ml. These results confirm the results seen in other systems using CD8 depletion (10) or in CD8−/− mice (18).

Despite the increases in BAL IL-5 levels and bone marrow eosinophils, CD4−/− mice failed to develop BAL eosinophila after sensitization and challenge, suggesting additional factors may play a role in airway eosinophil accumulation in these mice. Eotaxin, RANTES, and GM-CSF have all been implicated in the recruitment (and survival) of eosinophils to the airways (19–21). As shown in Figure 5, sensitized and challenged CD4−/− mice showed little or no increase in the levels of these three eosinophil chemoattractants when compared with sensitized and challenged CD4+/+ mice.

Functional Capacity of Lung T Cells from CD4−/− Mice

To further confirm the functional capacity of the lung T cells from CD4−/− mice to induce AHR, adoptive transfer experiments were performed. Nylon-wool isolated T cells from nonsensitized but challenged or sensitized and challenged donor mice were injected intravenously into naive recipient mice, which were then exposed to six consecutive days of airway allergen challenge (1% OVA, 20 min/d). After nylon-wool column purification of lung T cells from the CD4−/− mice, ~ 90% of the cells were CD3+ and 75% were CD3+/CD8+. Transfer of cells from sensitized/challenged CD4−/− mice into either naive

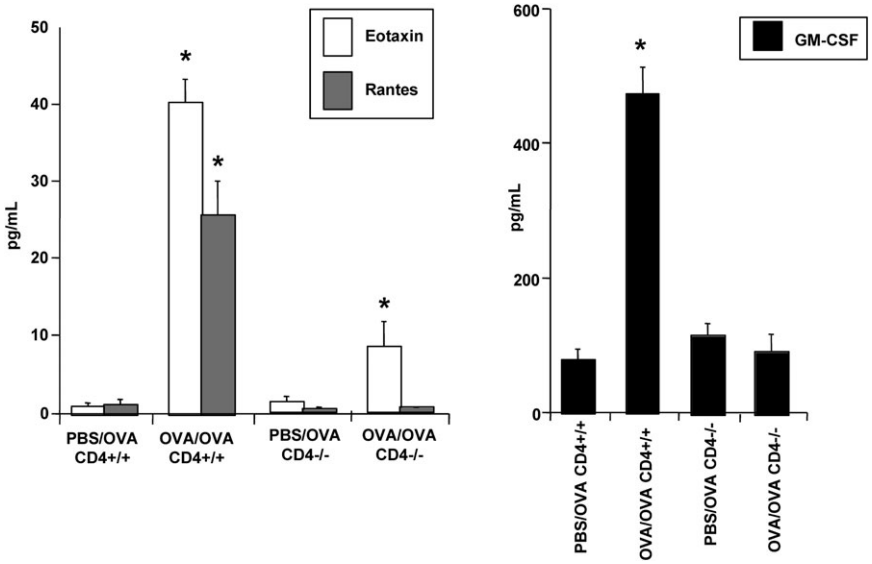

Figure 5. Effect of sensitization and challenge on BAL chemokine levels. Animals were the same as in Figure 1. n = 12. *$P < 0.01$ compared with animals challenged alone.

CD4+/+ or naive CD4−/− mice conferred the ability to develop AHR on subsequent airway allergen challenge; transfer of cells from non-sensitized but challenged CD4−/− mice failed to do so (Figure 6). These results were similar to the findings when lung T cells from CD4+/+ mice were adoptively transferred into naive recipients (Figure 6). In no case did the CD4−/− recipients develop BAL eosinophilia after cell transfer, despite the development of AHR, nor were levels of RANTES, eotaxin, or GM-CSF affected by cell transfer (data not shown).

Development of AHR following Secondary Challenge

To determine if the absence of CD4+ T cells affected the recall response to secondary airway allergen challenge, mice were sensitized and challenged (primary) to OVA in the usual way, and 6 wk later, when lung inflammation and AHR returned to baseline levels, a single provocative (secondary) airway challenge was performed and the animals were assessed 48 h later. After this secondary allergen provocation, CD4+/+ mice developed a significant increase in lung resistance to inhaled MCh when compared with the animals that were sensitized and challenged and then exposed to PBS (Figure 7A). This response to secondary allergen challenge was accompanied by a marked BAL eosinophilia, neutrophilia, and lymphocytosis (Figure 7B) and an increase in BAL IL-5 levels (from 49 ± 6 pg/ml in PBS challenged to 112 ± 21 pg/ml in OVA-challenged mice) and IL-13 levels (from 38 ± 3 pg/ml in PBS challenged to 78 ± 5 pg/ml in OVA-challenged mice). In contrast, CD4−/− mice failed to develop AHR when exposed to secondary challenge (Figure 7A). In these deficient mice, an increase in BAL lymphocytes, but no eosinophilia or neutrophilia, was observed after secondary challenge (Figure 7B) and IL-5 and IL-13 levels were not altered after secondary allergen challenge. Among these BAL lymphocytes, 82% (mean level) expressed CD3/CD8, 94% CD3, and < 2% stained with the pan-NK antibody.

When lung histopathology was examined following primary and secondary challenge, CD4+/+ mice demonstrated an increase in cellular infiltrate (H/E-staining), goblet cell metaplasia (PAS-staining), and eosinophilia (MBP-staining). In CD4−/− mice, after primary challenge some increase in infiltrating cell numbers was seen but no goblet cell metaplasia or lung eosinophilia. Following secondary challenge, the cellular infiltrate in the CD4−/− mice was lower with a similar absence of goblet cell metaplasia or lung eosinophilia as observed after primary challenge (data not shown).

DISCUSSION

Allergic asthma is a complex syndrome characterized by airway inflammation, AHR, and reversible airflow obstruction (22). However, the features of the disease, the cells involved and the mediators leading to the clinical manifestations can vary markedly between individuals and in the same individual at different stages of the disease. Central to the pathogenesis of the disease are T lymphocytes and a potentially important, although somewhat controversial, Th2/Th1 imbalance (3, 23). Lung eosinophilia is a fundamental characteristic of allergic asthma, but

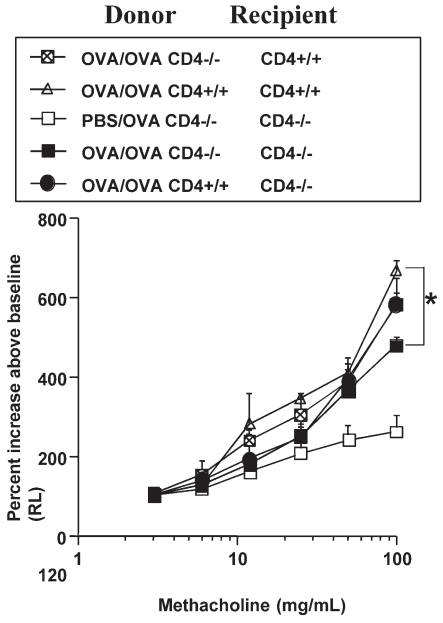

Figure 6. Adoptive transfer of lung T cells from sensitized and challenged CD4−/− mice reconstitutes AHR. Donor CD4+/+ or CD4−/− mice were sensitized and challenged or challenged alone. Lung T cells were isolated and injected intravenously into naive CD4+/+ or CD4−/− recipients who then received 6 d of airway allergen challenge ($n = 8$ for each group). *$P < 0.05$ sensitized and challenged versus challenged alone donor CD4−/− mice into recipient CD4−/− mice; *$P < 0.05$ for all other combinations of sensitized and donor mice versus challenged alone (data not shown).

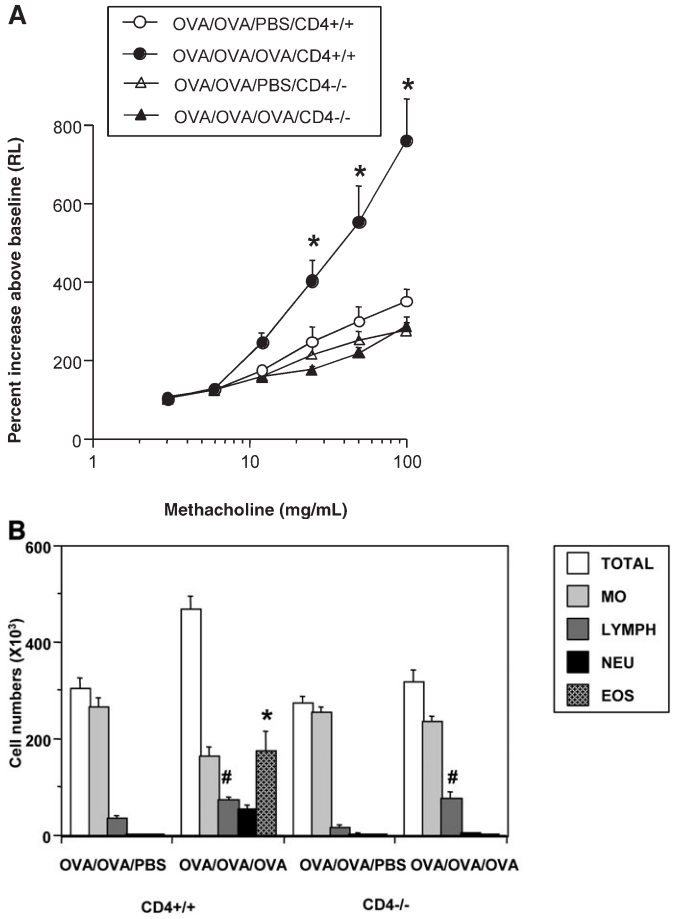

Figure 7. Failure of CD4−/− mice to develop AHR or airway inflammation following secondary allergen challenge. Mice received sensitization and primary challenge, followed 6 wk later by a single secondary challenge (OVA/OVA/OVA) or PBS (OVA/OVA/PBS). (*A*) Changes in RL. (*B*) Changes in lung inflammation ($n = 12$ in each group). *$P < 0.01$; #$P < 0.05$ compared with PBS secondary exposure.

increasingly, the role of eosinophils has been questioned given the absence of eosinophils (or other inflammatory cells) in lung biopsies of some individuals with asthma or the failure of anti–IL-5 to improve airway responsiveness despite elimination of eosinophils from the sputum and blood (24–26). Although some of the discrepancies in the data may be explained away by the extent of eosinophil depletion and their persistence in certain lung tissue sites (27, 28), the cumulative results have not yet identified a single or exclusive pathway underlying allergic asthma or development of AHR.

In an attempt to more clearly delineate relevant pathways, animal models of allergen-induced airway inflammation and AHR have been developed. Their relevance to human disease is much debated as well (29, 30). Regardless, these models have underscored the complexity of the cells and mediators that can result in AHR with or without airway inflammation. As an example, in the mouse various T cells including CD4, CD8, γ/δ, NKT, Th1, Th2, have all been demonstrated to play contributory roles (3). As in asthma, a similar controversy about the role of eosinophils is described for animal models of allergen-induced AHR. Although IL-5–deficient mice do not develop AHR (31), reducing the numbers of eosinophils, for example using anti–IL-5, may not always be associated with lower AHR (32). Even in newly described murine models where eosinphils are targeted, the role of eosinophils in the development of AHR remains controversial (33, 34). Thus, eosinophil-dependent and -independent pathways have been suspected to play a role in the development of AHR (35). Many, but not all of these differences, may be ascribed to differences in sensitization and challenge protocols, route, frequency, dose and nature of the allergen used, as well as the specific read-out for monitoring airway function (35).

The initiation and regulation of pulmonary inflammation and development of allergen-induced alterations in airway function appear absolutely dependent on T lymphocytes. In the lung, α/β+ T cells are the most prevalent (36), and many studies in humans and animals have shown that the CD4+ subtype expressing the α/β T cell receptor is the major source of Th2-cytokines, characteristic of allergen-driven responses in the lung (3, 37, 38). Depletion of T cells can attenuate both AHR and airway eosinophilia and adoptive transfer of allergen-primed CD4+ T cells can induce allergic responsiveness (6, 39, 40). However, the inhibition of AHR following the depletion of CD4+ T cells is incomplete (compared with CD3+ T cell depletion) (9), implying that under certain circumstances, their absence may be compensated for by other cell types.

In the present study, we have shown that CD4−/− mice, after sensitization and primary challenge, are capable of developing AHR to inhaled MCh to an extent similar to that of their CD4+/+ counterparts. Unlike the CD4+/+ mice, which develop airway and tissue eosinophilia, goblet cell hyperplasia and mucin hyperproduction, increases in BAL IL-4 and IL-13 levels, and elevated serum allergen-specific IgE levels, the CD4−/− mice were unable to do so. In fact, only the increases in AHR and BAL IL-5 levels (and bone marrow eosinophilia) following sensitization and primary challenge were similar. Importantly, the response in CD4−/− mice was seen after sensitization and primary challenge with either OVA or RW and in an allergen-specific manner, indicating that the response was not restricted to single, perhaps unique allergen epitopes.

A major difference was seen following secondary challenge of sensitized CD4−/− mice. Unlike CD4+/+ mice, the deficient animals failed to develop AHR on secondary challenge; the only response detected was an increase in BAL lymphocytes. We previously showed that the contributors to the development of AHR following primary versus secondary challenge may also differ, especially the more obvious requirement for eosinophils in the primary response (13).

In the original description of these CD4−/− mice, the development of CD8+ T cells was said to be unaltered, at least for cytotoxic T cell activity against viruses (12). Moreover, CD4 was shown not to be absolutely necessary for positive selection or effector function of class II MHC-restricted helper T cells (41). As described previously in the periphery of mice lacking CD4+ T cells (12, 41), CD8+ T cells in the lung digests expanded to occupy the compartment normally occupied by the CD4+ T cells. Relative numbers of CD3+/CD4−/CD8− double-negative T (α/β+) cell numbers increased as well, as previously reported (41). Depletion of CD8+ T cells before challenge (but after sensitization) using an anti-CD8 mAb directed to the β chain fully depleted CD8+ T cells from the circulation, lymph nodes, and lung digests. These CD8-depleted/CD4−/− mice failed to develop AHR, suggesting that the CD8+ T cells were essential to the responses observed in the CD4−/− mice. The exact role of CD8+ T cells in the development of AHR and airway inflammation in the CD4−/− mice is not entirely clear. Several studies have suggested that CD8+ T cells play no role in or even attenuate development of AHR and inflammation in normal mice (42, 43). However, a role for CD8 T cells has been shown under a number of different conditions, including limited allergen exposure through the airways (10), in CD8−/− mice (18) and in allergen-sensitized and -challenged mice exposed to virus (44, 45). In both mice and humans, CD8+ T cells may contribute to Th2-mediated inflammatory responses and to the development of AHR and airway inflammation through the release of specific cytokines. We have shown that CD8+ T cells may be an important source of IL-5 (10) and, as recently demonstrated, an important source of IL-13 in the lungs (18, 46). Of note, in the absence of CD4+ T cells, no IL-13 was detected in the BAL fluid despite the presence of CD8+ T cells. These findings may implicate CD4+ T cells in the conversion of CD8+ T cells to a "Tc2" phenotype.

In addition to the role for CD8+ T cells in the development of AHR, treatment of these mice with anti–IL-5 after sensitization but before challenge, abrogated the development of AHR. These results, similar to results in the CD4+/+ mice, identified a role for IL-5 in the development of AHR in the CD4−/− mice. However, as the CD4−/− mice developed AHR in the absence of airway eosinophilia, IL-5 likely contributed to the development of altered airway function via actions that are independent of eosinophils. A separation of airway eosinophilia and AHR is well described in several species, including humans (25, 26, 32). In guinea pigs, whereas a low dose of anti–IL-5 suppressed allergen-induced BAL eosinophilia without affecting AHR, higher antibody doses blocked AHR as well (47). Such data may provide support for actions of IL-5 on airway function which are not dependent on eosinophil activation. While IL-5 may be essential for eosinophil mobilization from the bone marrow, accumulation of eosinophils in the airways is also dependent on a number of eosinophil chemoattractants (19). In sensitized and challenged CD4−/− mice, eosinophil mobilization in the bone marrow appeared normal, supporting the physiologic consequences of the induced increases in IL-5 levels. However, these sensitized and challenged mice showed markedly reduced levels of eotaxin, RANTES, and GM-CSF, likely accounting for, at least in part, the reduced eosinophil numbers in the lung and airways (19–21). The failure of the CD4−/− mice to release these eosinophil chemoattractants in significant amounts identifies an important role for CD4+ T cells in this pathway, a role not provided by CD8+ T cells.

In the present study, we not only demonstrated the importance of CD8+ T cells by CD8 T cell depletion, but also by

adoptive transfer of nylon-wool isolated T cells from sensitized and challenged CD4−/− donor mice. In these transfer studies, ~ 75% of the cells expressed CD8; a significant percentage (15–20%) of the nylon-wool purified T cells were CD3+CD4−/CD8− double negative (DN) TCR αβ+. As a result, it is difficult to exclude a role for the DN cells as well as the CD8+ T cells in the response to allergen sensitization and challenge. Based on the results of the CD8 depletion studies as well as those of the adoptive transfer experiments, it may be that together, CD8β+ cells and DN play a role in this development of AHR. It is of interest that Bevan and colleagues have recently shown that the CD8+ T cell pool in CD4−/− mice is heavily "contaminated" with MHC-class II restricted cells, a population of cells not identified in acutely CD4-depeleted normal mice (41). This expansion of MHC class II restricted CD8+ T cells as well as the DN subset of cells in CD4−/− mice may explain their role in the development of AHR in mice lacking CD4+ T cells. As CD4 T cell depletion in normal mice shows no such expansion, this could also explain why CD4+ T cell depletion has such profound effects on the response to allergen sensitization and challenge, with little if any compensation by the remaining CD8+ T cells, at least in the short term.

In HLA-DQ transgenic mice lacking the CD4 gene, exposure to RW extract elicited AHR and a strong eosinophilic response, further confirming that CD4 and CD4+ T cells may not be necessary for the induction of a Th2-type response (48). Interestingly, in these CD4−/− mice no eosinophil peroxidase was detected, implying an absence of eosinophil activation.

However, based on these results it is clear that the CD8+ T cells which expand in the absence of CD4+ T cells are not fully functional, at least in the generation of a memory response, to secondary allergen challenge. Although the response to primary challenge of sensitized mice was accompanied by the development of AHR and increases in IL-5, the response to secondary challenge was not effective in eliciting either AHR or an IL-5 response. These findings are in keeping with the demonstrated role for CD4+ T cells in the development of CD8 T cell memory responses under a variety of conditions (49, 50).

In summary, using CD4−/− mice we show that CD4+ T cells are not absolutely necessary for the development of allergen-specific induction of AHR in a primary airway allergen challenge model. Whether this pathway contributes to AHR in the intact mouse is unclear at this time, but the results nonetheless reveal the complexity and diversity of the cellular pathways, which may contribute to the response following allergen exposure in the lungs of sensitized mice.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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