

CD8⁺ T Cell-Mediated Airway Hyperresponsiveness and Inflammation Is Dependent on CD4⁺IL-4⁺ T Cells¹

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CD4⁺ T cells, particularly Th2 cells, play a pivotal role in allergic airway inflammation. However, the requirements for interactions between CD4⁺ and CD8⁺ T cells in airway allergic inflammation have not been delineated. Sensitized and challenged OT-1 mice in which CD8⁺ T cells expressing the transgene for the OVA_{257–264} peptide (SIINFEKL) failed to develop airway hyperresponsiveness (AHR), airway eosinophilia, Th2 cytokine elevation, or goblet cell metaplasia. OT-1 mice that received naive CD4⁺IL-4⁺ T cells but not CD4⁺IL-4[–] T cells before sensitization developed all of these responses to the same degree as wild-type mice. Moreover, recipients of CD4⁺IL-4⁺ T cells developed significant increases in the number of CD8⁺IL-13⁺ T cells in the lung, whereas sensitized OT-1 mice that received primed CD4⁺ T cells just before challenge failed to develop these responses. Sensitized CD8-deficient mice that received CD8⁺ T cells from OT-1 mice that received naive CD4⁺ T cells before sensitization increased AHR and eosinophil numbers in bronchoalveolar lavage fluid when challenged with allergen. In contrast, sensitized CD8-deficient mice receiving CD8⁺ T cells from OT-1 mice without CD4⁺ T cells developed reduced AHR and eosinophil numbers in bronchoalveolar lavage fluid when challenged. These data suggest that interactions between CD4⁺ and CD8⁺ T cells, in part through IL-4 during the sensitization phase, are essential to the development of CD8⁺IL-13⁺ T cell-dependent AHR and airway allergic inflammation. *The Journal of Immunology*, 2007, 179: 2787–2796.

Bronchial asthma is characterized by chronic inflammation in the airways in response to a number of stimuli such as airborne allergens, infectious pathogens, and chemical agents (1). The hallmarks of the disease include infiltration of leukocytes in the airways, nonspecific airway hyperresponsiveness (AHR),³ subepithelial fibrosis, mucus hyperplasia, goblet cell metaplasia, and in most cases, elevation of serum IgE (2, 3). T cells play an important role in these processes through the release of various cytokines and chemokines (4). CD4⁺ T cells, especially Th2 cells that produce IL-4, IL-5, IL-9, and IL-13, are considered pivotal cells in the development of AHR and airway allergic inflammation (4–7). Patients with atopic asthma demonstrate increased production of Th2 cytokines from lymphocytes in bronchoalveolar lavage (BAL) fluid (8), and in animal models of acute allergic airway inflammation, adoptive transfer of Ag-primed Th2 cells can induce AHR and late airway responses in naive rats (9, 10). It has also been reported that the transfer of Th2 cells followed by airway allergen challenge in mice induces airway eosinophilia and AHR (11, 12).

CD8⁺ T cells, which are pivotal in tumor cell killing and protection during viral infection through secretion of IFN- γ and cytolytic factors, were considered to be much less important or even negative regulators

of the development of allergic inflammation (13, 14). Indeed, a number of studies reported protective effects of CD8⁺ T cells in allergic airway disease because of production of IFN- γ and the ability to suppress Th2 responses (15–17). However, several recent reports suggested that not only CD4⁺ T cells but also CD8⁺ T cells were essential to the development of AHR and allergic inflammation (18–20). Subsets of CD8⁺ T cells, which produce IL-4, IL-5, and IL-13 but not IFN- γ , labeled as Tc2 cells, are increased in BAL fluid of atopic asthma patients (21).

Among the phenotypes of memory CD8⁺ T cells, two types are distinguished based on functional and migratory aspects; central memory CD8⁺ T cells express high levels of CD62 ligand and CCR7, and preferentially reside in lymph nodes. In contrast, effector memory CD8⁺ T cells express lower levels of CD62 ligand and CCR7, migrate more readily to nonlymphoid tissues and sites of inflammation, and acquire effector cell function more rapidly (22–24). Ag-specific effector and central memory CD8⁺ T cells can be generated from CD8⁺ T cells from OT-1 mice, which are responsive to MHC class I-restricted OVA peptide (OVA_{257–264} SIINFEKL) by culturing Ag-pulsed cells with IL-2 or IL-15, respectively (24–27). Recently, effector memory CD8⁺ T cells but not central memory CD8⁺ T cells were shown to be essential for the development of AHR and airway allergic inflammation in adoptive transfer models (28, 29).

Although both CD4⁺ and CD8⁺ T cells appear capable of contributing to the development of AHR and allergic inflammation, the interactions between and requirements for CD4⁺ and CD8⁺ T cells in these responses have not been well defined. In some reports, the interactions between CD4⁺ and CD8⁺ T cells were essential for the establishment of immune responses. For example, the number of memory CD8⁺ T cell numbers and secondary responses to bacterial or viral challenge were decreased over time in CD4⁺ T cell-deficient animals, supporting a role for CD4⁺ T cells in the priming of CD8⁺ T cells against these pathogens (30–32). In light of these requirements and the suggested role for CD8⁺ T cells in the development of allergic airway responses, we examined the role of CD4⁺ T

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Received for publication June 19, 2007. Accepted for publication June 22, 2007.

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¹ This work was supported by Grants HL-36577 and HL-61005 from the National Institutes of Health and Grant R825702 from the Environmental Protection Agency.

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; MNC, mononuclear cell; PAS, Periodic acid-Schiff; WT, wild type.

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cells in the development of CD8⁺ T cell-mediated AHR and airway inflammation.

Materials and Methods

Animals

Age-matched (8- to 12-wk-old) C57BL/6 wild-type (WT) mice, OT-1 TCR transgenic (OT-1) mice, IL-4-deficient mice, and CD8-deficient mice were bred in the animal facility at the National Jewish Medical and Research Center (Denver, CO). The OT-1 mice, provided by Dr. T. Potter (National Jewish Medical and Research Center, Denver, CO), were backcrossed to C57BL/6 mice for over 10 generations. These mice express the OT-1 TCR (V α 2/V β 5) transgene, encoding a TCR specific for the OVA epitope (SIINFEKL-H2Kb) (33). Hemizygous OT-1 TCR transgenic mice were assessed for transgene status by flow cytometric analysis of peripheral blood with an Ab against the V α 2 and V β 5 subunit. C57BL/6 mice and IL-4-deficient mice (C57BL/6 background) were purchased from The Jackson Laboratory. Homozygous CD8-deficient mice, generated by targeting the CD8 α -chain gene in C57BL/6 mice (34), were obtained from Dr. P. Marrack (National Jewish Medical and Research Center, Denver, CO). Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

Sensitization and airway challenge

Mice were sensitized at 8 wk of age by i.p. injection of 20 μ g of OVA (grade V; Sigma-Aldrich) emulsified in 2.25 mg of Al(OH)₃ (Pierce) in a total volume of 100 μ l on days 0 and 14. After sensitization, animals were challenged with nebulized OVA (1% in saline) for 20 min on days 28, 29, and 30. Forty-eight hours after the last OVA challenge, AHR was assessed and BAL, serum, and tissues were obtained for further analysis. Controls were nonsensitized but challenged with OVA.

Determination of airway responsiveness

Airway function was assessed as previously described by measuring changes in lung resistance in response to increasing doses of inhaled methacholine (35). Data are expressed as a percentage of change from baseline resistance (lung resistance) values obtained after inhalation of saline.

BAL analysis

Immediately after assessment of airway function, lungs were lavaged via the tracheal tube with 1 ml of HBSS at room temperature. Total leukocyte numbers were measured (Coulter Counter; Beckman Coulter). Cytospin slides were stained with Leukostat (Fisher Diagnostics) and differentiated by standard hematological procedures in a blinded fashion.

Lung histology

Lungs were fixed in 10% formalin and processed into paraffin. Mucus-containing goblet cells were detected by staining of paraffin sections (5- μ m thick) with periodic acid-Schiff (PAS). Histology analysis was done in a blinded manner by light microscopy linked to an image system. The number of PAS-positive goblet cells was determined in cross-sectional areas of the airway wall. Six to 10 different sections were evaluated per animal. The obtained measurements were averaged for each animal and the mean values and SE were determined for each group.

Measurement of BAL fluid cytokines

Levels of cytokines were determined using commercially available ELISAs following the manufacturer's instructions. ELISA kits for detection of IL-4, IL-5, and IFN- γ in supernatants were obtained from BD Pharmingen. The IL-13 ELISA kit was purchased from R&D Systems. The limits of detection for each assay were as follows: 4 pg/ml for IL-4 and IL-5, 10 pg/ml for IFN- γ , and 1.5 pg/ml for IL-13.

Purification of CD4⁺ and CD8⁺ T cells

Purification of CD4⁺ T cells from C57BL/6 (WT) mice or IL-4-deficient mice or CD8⁺ T cells from OT-1 mice was conducted as previously described (20). Briefly, spleen cells were harvested by mincing the tissues and subsequently passing them through a stainless steel sieve. After washing with PBS, mononuclear cells (MNC) were isolated by Histopaque gradient centrifugation (Sigma-Aldrich). Purification of CD4⁺ or CD8⁺ T cells was conducted by negative selection using the CD4 isolation kit or CD8 isolation kits (Miltenyi Biotec) in accordance with the manufacturer's instructions. Purity of both cell populations after purification exceeded 95% as assessed by flow cytometry.

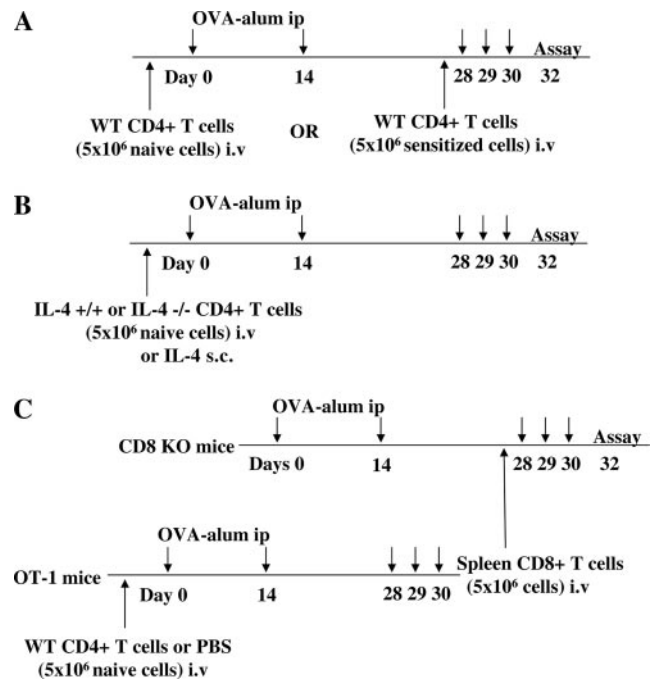


FIGURE 1. Experimental protocol for Ag sensitization and challenge with cell transfer or IL-4 administration. **A**, CD4⁺ T cells from WT mice were transferred into OT-1 mice either before sensitization (naive cells) or before challenge (primed T cells). **B**, CD4⁺ T cells from IL-4-deficient mice or WT mice or IL-4 were given to OT-1 mice before sensitization. **C**, CD8⁺ T cells were isolated from OT-1 mice that received CD4⁺ T cells or PBS before sensitization, and injected into sensitized CD8^{-/-} mice just before challenge.

Transfer of CD4⁺ and CD8⁺ T Cells

Naive CD4⁺ T cells (5×10^6) from WT mice or IL-4-deficient mice were transferred to OT-1 mice i.v. just before the first OVA (sensitization) injection. After transfer of naive CD4⁺ T cells, the mice were sensitized and challenged to OVA as described. OVA-primed CD4⁺ T cells (5×10^6) from spleens of WT mice that were sensitized to OVA were transferred to OVA-sensitized OT-1 mice followed by OVA challenge. OVA-primed CD8⁺ T cells (5×10^6) from OT-1 mice that received naive CD4⁺ T cells or no cells just before sensitization were transferred to sensitized CD8-deficient recipient mice just before allergen challenge (Fig. 1).

IL-4 administration

To determine whether IL-4 secreted from transferred CD4⁺ T cells played an essential role in the functional activation of CD8⁺ T cells in OT-1 mice, recombinant murine IL-4 (1 μ g/injection; PeproTech) was administered by s.c. injection to OT-1 mice on days 1 to 3 and 14 to 16. As a control, mice

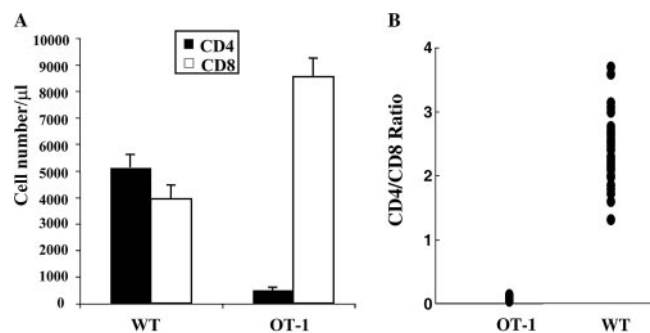


FIGURE 2. Flow cytometric analysis of CD4⁺ and CD8⁺ T cells in peripheral blood of WT ($n = 16$) and OT-1 mice ($n = 4$ mice per group). **A**, Absolute number of CD4⁺ and CD8⁺ T cells in peripheral blood. **B**, Percentage of CD4⁺ to CD8⁺ T cells.

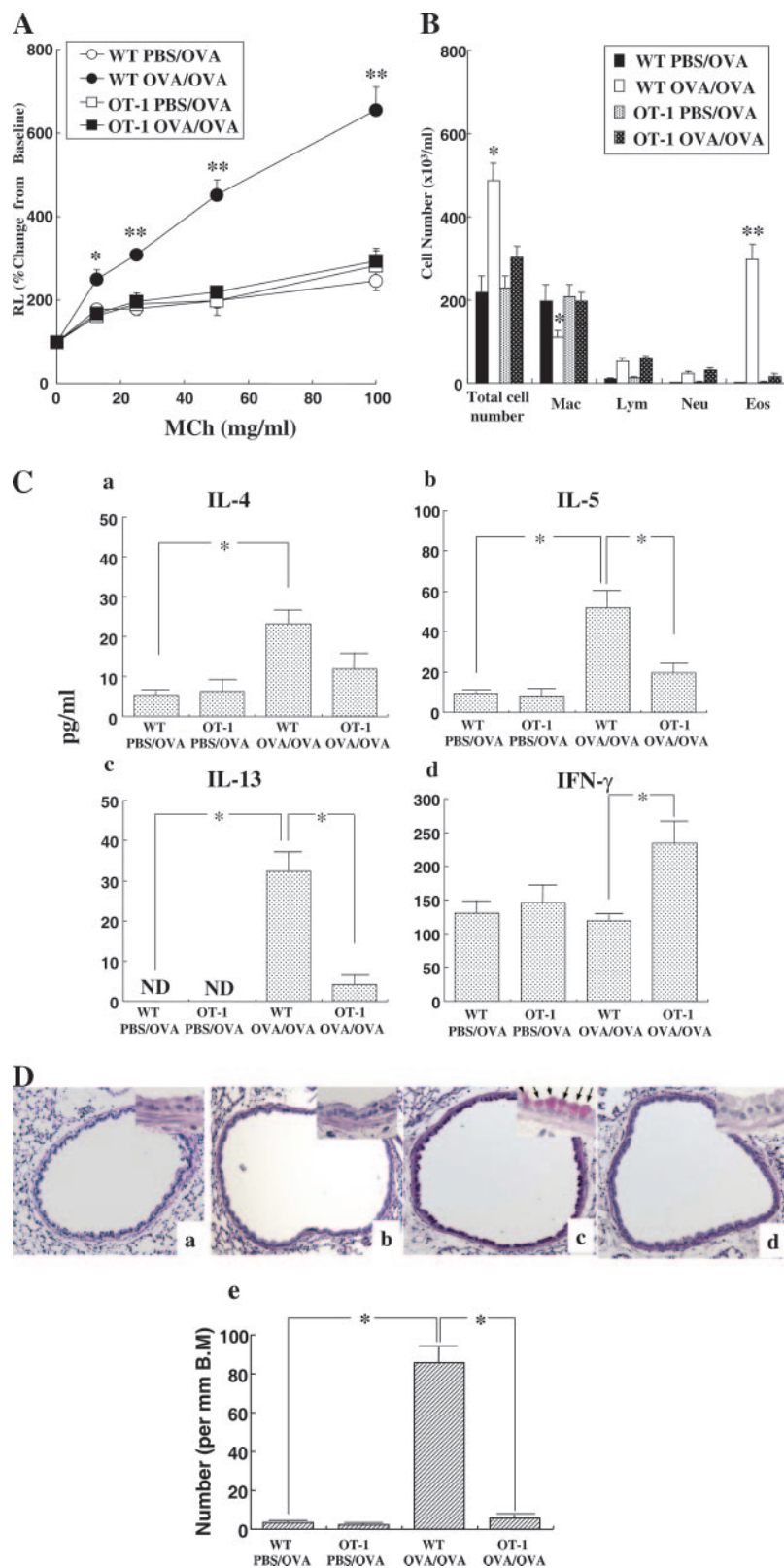


FIGURE 3. AHR and airway inflammation in challenged and sensitized mice. Changes in airway resistance (RL) (*A*) and cell composition (*B*) in BAL fluid are shown. Increasing concentrations of nebulized methacholine (MCh) were administered through the tracheal cannula 48 h after the last OVA challenge. Immediately after assessment of airway function, lungs were lavaged via the tracheal tube with 1 ml of HBSS at room temperature. *C*, Cytokine levels, IL-4 (*a*), IL-5 (*b*), IL-13 (*c*), and IFN- γ (*d*) in BAL fluid were determined by ELISA as described in *Materials and Methods*. *D*, Representative photomicrographs (*a*–*d*) and quantitative analysis of PAS-positive cells (arrows) (*e*) in lung tissue. The tissue sections were obtained 48 h after the last challenge. Shown are photomicrographs from challenged alone (PBS/OVA) littermate mice (*a*), challenged alone OT-1 mice (*b*), sensitized and challenged (OVA/OVA) WT mice (*c*), and sensitized and challenged OT-1 mice (*d*). Eos, eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils; ND, Not detectable. *, $p < 0.05$ compared with sensitized and challenged OT-1 mice and challenged alone WT mice (*A* and *B*), or between the groups indicated in *C* and *D*. Data represent mean $\pm$ SEM for $n = 12$ per group. **, $p < 0.01$ compared with sensitized and challenged OT-1 mice and challenged alone WT mice.

were injected with PBS in the same manner. These mice were also sensitized to OVA on days 1 and 14 followed by OVA challenges on days 28, 29, and 30.

CD4⁺ T cell depletion

One day before and 30 min before each OVA challenge, monoclonal anti-CD4 Ab (GK1.5; American Type Culture Collection) prepared as previously described (36) was administered i.v. (200 μ g in 100 μ l of PBS) to

the mice that received naive CD4⁺ T cells before sensitization. Control animals received the same amount of rat IgG (Sigma-Aldrich).

Lung cell isolation

Lung cells were isolated as previously described (37) using collagenase digestion. Cells were resuspended in HBSS, and MNC were purified by 35% Percoll (Sigma-Aldrich).

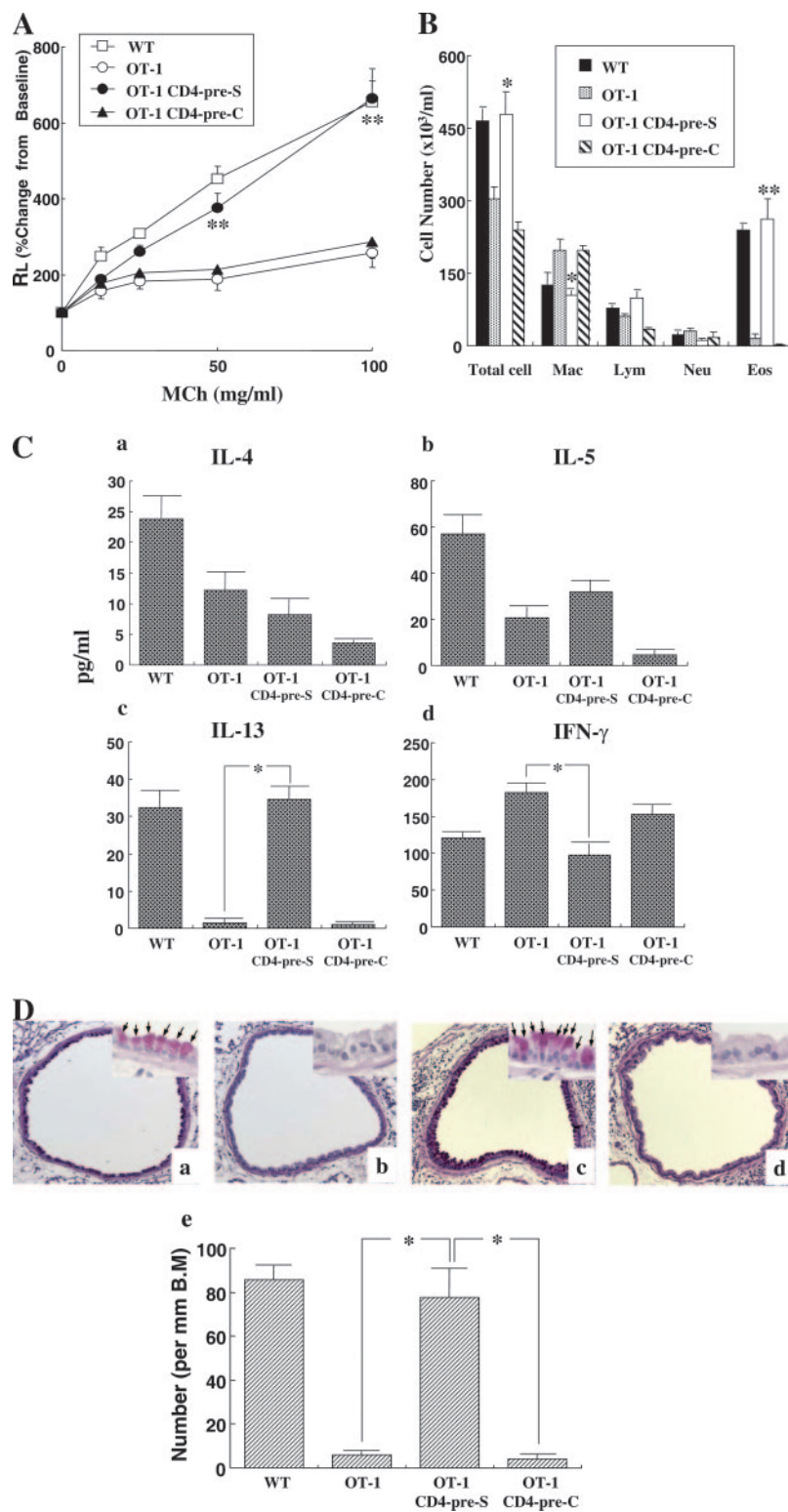


FIGURE 4. Transfer of CD4⁺ T cells in the development of AHR and allergic inflammation. Changes in airway resistance (RL) (A) and (B) cell composition in BAL fluid in the mice that received CD4⁺ T cells. OT-1 recipients received naive CD4⁺ T cells before sensitization (OT-1 CD4-pre-S) or before challenge (CD4-pre-C). C, Cytokine levels, IL-4 (a), IL-5 (b), IL-13 (c), IFN- γ (d) in BAL fluid were determined by ELISA as described in *Materials and Methods*. D, Representative photomicrographs (a–d) and quantitative analysis of PAS-positive cells (arrows) (e) in lung tissue. Photomicrographs are from sensitized and challenged WT mice (a), sensitized and challenged OT-1 mice (b), sensitized and challenged OT-1 mice that received naive CD4⁺ T cells before sensitization (CD4-pre-S) (c), and sensitized and challenged OT-1 mice that received primed CD4⁺ T cells before challenge (CD4-pre-C) (d). *, $p < 0.05$ compared with sensitized and challenged OT-1 mice (A and B), or between the groups indicated in C and D. Data represent mean $\pm$ SEM of $n = 12$ per group. **, $p < 0.01$ compared with sensitized and challenged OT-1 mice.

Intracellular cytokine staining

Intracellular cytokine staining was performed as previously described (20). Briefly lung MNCs were stimulated for 5 h with PMA (10 ng/ml) and ionomycin (500 μ g/ml) in the presence of brefeldin A (10 μ g/ml). After washing, cells were stained for cell surface markers with mAbs against CD3 (145-2C11, hamster IgG), CD4 (RM4-5, rat IgG2a), and CD8 (53-6.7, rat IgG2a). All fluorochrome-labeled mAbs and isotype control IgGs were purchased from BD Pharmingen. After fixation and permeabilization, cells were stained with PE- or biotin-conjugated anti-cytokine Abs or similarly labeled isotype-matched control Abs against IFN- γ , which was purchased from BD Pharm-

ingen, and biotinylated goat anti-mouse IL-13 and control Ab, which were purchased from R&D Systems. After washing, staining was analyzed by flow cytometry on FACSCalibur using CellQuest software (BD Biosciences).

Statistical analysis

The Mann-Whitney U test was used to determine the levels of difference between all groups. The data were pooled from three independent experiments with four mice per group in each experiment ($n = 12$). Significance was assumed at values of $p < 0.05$. Values for all measurements were expressed as mean $\pm$ SEM.

Results

AHR and airway inflammation in OT-1 mice

To first determine the phenotypes within the T cell compartment, flow cytometric analysis of peripheral blood from WT or OT-1 mice was performed. As shown in Fig. 2, the majority of the T cells in OT-1 mice were CD8⁺ compared with WT mice (Fig. 2). The majority of spleen and lung T cells were also CD8⁺ T cells (data not shown).

WT mice and OT-1 mice received injections of OVA (plus alum) sensitization on days 1 and 14, followed by three allergen challenges on days 28, 29, and 30. As shown in Fig. 3A, sensitized and challenged (OVA/OVA) WT mice developed significant increases in lung resistance in response to increasing doses of inhaled methacholine. However, sensitized and challenged OT-1 mice did not develop increases in response to methacholine over the response seen in control mice that were challenged alone (PBS/OVA). Analyses of the cell composition of BAL fluid demonstrated that airway eosinophilia developed in the sensitized and challenged WT mice, but not in the sensitized and challenged OT-1 mice (Fig. 3B).

Examination of cytokine levels in the BAL fluid of these mice revealed that levels of IL-4, IL-5, and IL-13 were elevated in sensitized and challenged WT groups, whereas IL-5 and IL-13 in BAL fluid from sensitized and challenged OT-1 mice were significantly lower. In contrast, IFN- γ levels in the BAL fluid from OT-1 mice after sensitization and challenge were higher than in the sensitized and challenged WT mice (Fig. 3C).

Similarly, evidence for goblet cell metaplasia and mucus hyperproduction could be detected in the sensitized and challenged WT mice, but few goblet cells were detected in sensitized and challenged OT-1 mice (Fig. 3D).

Transfer of CD4⁺ T cell to OT-1 mice

To define whether CD4⁺ T cells were essential to the development of AHR and allergic inflammation in the OT-1 mice, CD4⁺ T cells were transferred into OT-1 mice either before sensitization (pre-S naive cells) or before challenge (pre-C primed T cells) (Fig. 1A). The transfer of naive CD4⁺ T cells before sensitization followed by three airway challenges led to the development of AHR in the OT-1 mice to the same level as demonstrated in WT mice (Fig. 4A). In contrast, transfer of primed CD4⁺ T cells just before the first of the three challenges in sensitized OT-1 mice did not induce AHR (Fig. 4A). In parallel, the development of AHR and BAL eosinophilia was only observed in mice recipient of naive CD4⁺ T cells just before sensitization and not in OT-1 recipients of primed CD4⁺ T cells at the time of challenge (Fig. 4B).

Analysis of cytokine levels in the BAL fluid indicated that transfer of naive CD4⁺ T cells into OT-1 mice before sensitization induced an increase in IL-13 levels and a reduction in IFN- γ levels in BAL fluid. However, transfer of primed CD4⁺ T cells before Ag challenge did not alter the cytokine profiles in BAL fluid, and profiles were similar to sensitized and challenged OT-1 mice that received no CD4⁺ cells (Fig. 4C).

In a similar fashion, goblet cell metaplasia only developed in the mice that received naive CD4⁺ T cells before sensitization; numbers were similar to those in sensitized and challenged WT mice. In contrast, transfer of primed CD4⁺ T cells to sensitized OT-1 mice just before challenge did not induce goblet cell metaplasia (Fig. 4D).

Intracellular cytokine staining for IL-13 in lung CD8⁺ T cells

To determine whether transfer of CD4⁺ T cells to OT-1 mice before sensitization induced the functional activation of lung

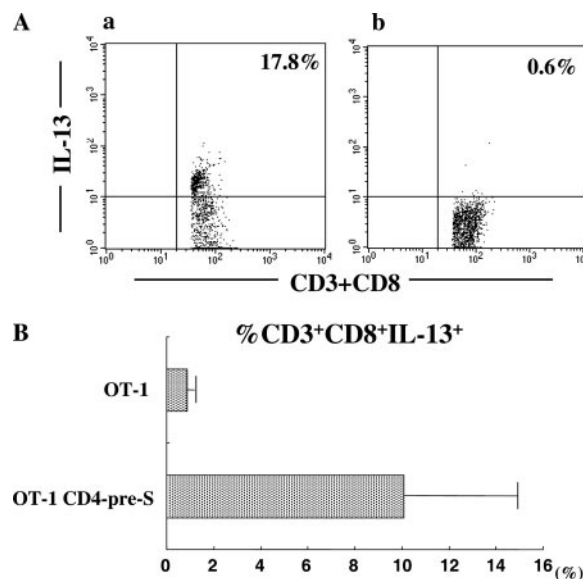


FIGURE 5. Transfer of CD4⁺ T cells prior to sensitization is required for the functional activation of lung CD8⁺ T cells. Intracellular detection of IL-13 protein in lung CD3⁺CD8⁺ T cells (A) from sensitized and challenged OT-1 mice that received naive CD4⁺ T cells before sensitization (CD4-pre-S) (a) or sensitized and challenged OT-1 mice that did not receive any cells (b). Lung MNC were isolated and stimulated with phorbol/ ionomycin, fixed, permeabilized, and stained with anti-mouse CD3, CD8, and IL-13 Ab and quantitated as described in *Materials and Methods*. CD3⁺CD8⁺ T cells were gated on and analyzed for intracellular detection for IL-13. B, The percentage of CD3⁺CD8⁺IL-13⁺ cells within the CD3⁺CD8⁺ gated population is shown. Mean $\pm$ SEM from three independent experiments ($n = 12$) is shown. *, $p < 0.05$.

CD8⁺ T cells, we assessed the number of CD8⁺IL-13⁺ T cells in the lung by intracellular cytokine staining. Lung MNCs were isolated and stimulated for 5 h with PMA/ionomycin. After washing, cells were stained with CD3, CD8, and IL-13 Abs. The number of CD8⁺IL-13⁺ T cells in OT-1 mice that received naive CD4⁺ T cells before sensitization were markedly increased when compared with the number in the lungs of OT-1 mice that received no CD4⁺ T cells (Fig. 5). The number of CD8⁺IFN- γ ⁺ T cells in the lung were not different between the two groups (data not shown).

Transfer of CD4⁺ T cell from IL-4-deficient mice to OT-1 mice

Based on these data, it appeared that a population of lung CD8⁺IL-13⁺ T cells expanded in OT-1 mice that received naive CD4⁺ T cells just before sensitization. IL-4 is known to be important in the in vitro development of CD8⁺ T cells with a Tc2 phenotype (38, 39). To determine the role of CD4⁺-derived IL-4 in vivo, transfer of CD4⁺ T cells from IL-4-deficient mice or WT mice into OT-1 mice was compared. As shown in Fig. 6, A and B, the transfer of CD4⁺ T cells from IL-4-deficient mice into OT-1 mice before sensitization elicited significantly lower AHR and airway eosinophilia compared with the transfer of CD4⁺ T cells from WT mice.

The cytokine levels in BAL fluid indicated that transfer of WT CD4⁺ T cells into OT-1 mice before sensitization induced increased levels of IL-5 and IL-13, whereas transfer of CD4⁺ T cells from IL-4-deficient mice did not alter the cytokine profiles in BAL fluid compared with sensitized and challenged OT-1 mice that received no cell transfer (Fig. 6C).

Analyses of PAS staining of the airways showed that goblet cell metaplasia and mucus hyperproduction was poorly developed in

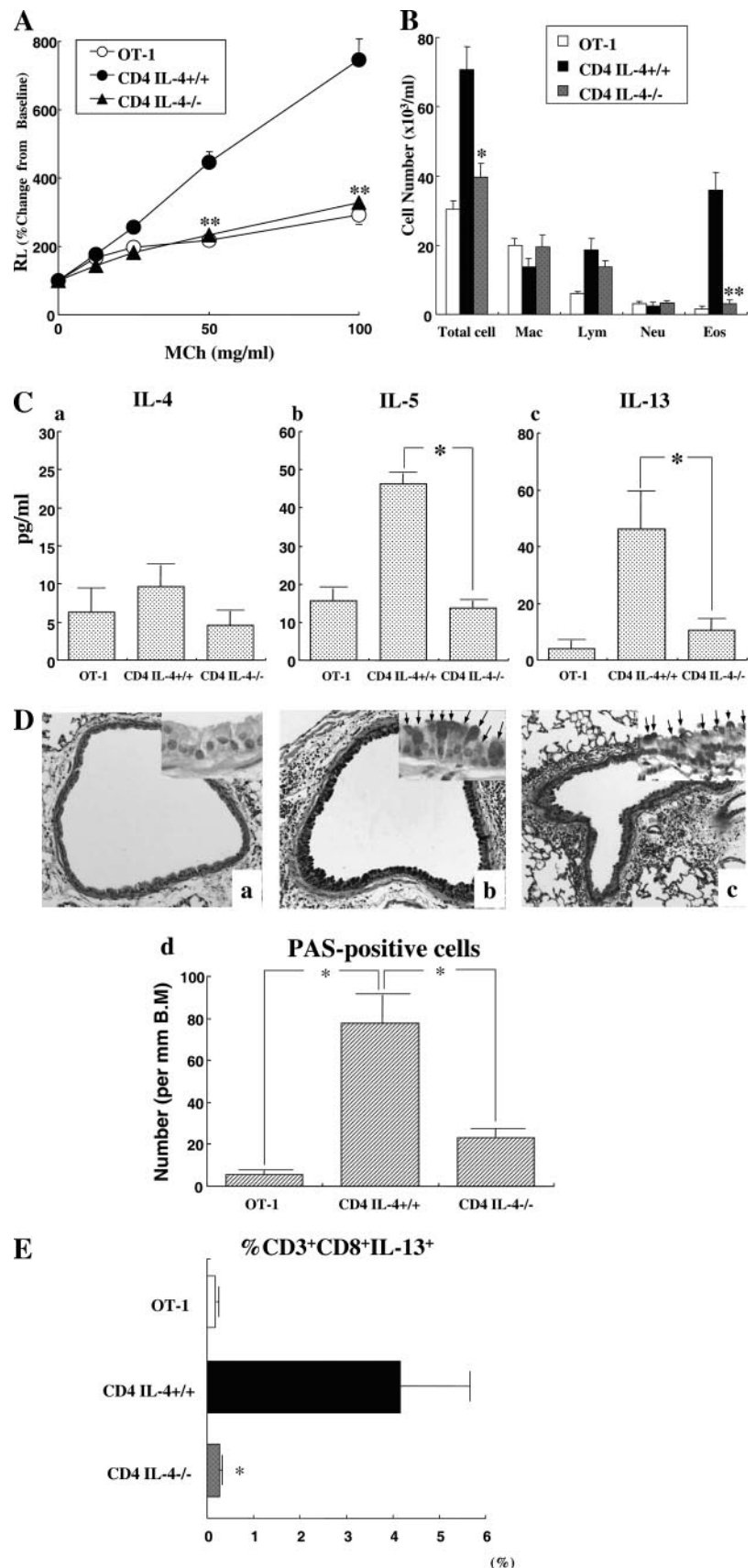
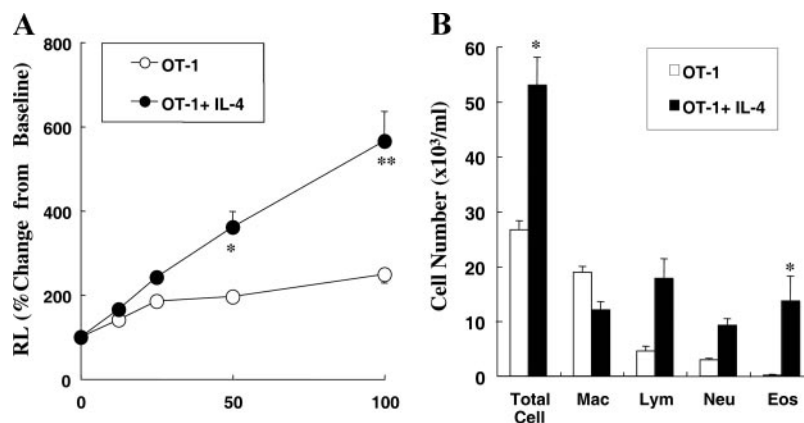


FIGURE 6. CD4⁺IL-4⁺ T cells are required for development of lung allergic responses in OT-1 mice. Changes in airway resistance (RL) (A) and cell composition (B) in BAL fluid in sensitized and challenged OT-1 mice that received CD4⁺IL-4⁺ or CD4⁺IL-4⁻ T cells before sensitization. Eos, eosinophils; Lym, lymphocytes; Mac, macrophages; MCh, methacholine; Neu, neutrophils. C, Cytokine levels, IL-4 (a), IL-5 (b), and IL-13 (c) in BAL fluid were determined by ELISA as described in *Materials and Methods*. D, Representative photomicrographs (a–c) and the number of PAS-positive cells (arrows) in the lung tissues (d) are shown. Photomicrographs are from sensitized and challenged OT-1 mice (a), sensitized and challenged OT-1 mice that received naive CD4⁺ T cells from WT mice (CD4 IL-4^{+/+}) (b), and sensitized and challenged OT-1 mice that received naive CD4⁺ T cells from IL-4-deficient mice (CD4 IL-4^{-/-}) (c). E, The percentage of IL-13-producing (IL-13⁺) CD8⁺ T cells in the CD3⁺CD8⁺ gated population was determined by intracellular cytokine staining as described in *Materials and Methods*. Data represent mean $\pm$ SEM of $n = 12$ per group. *, $p < 0.05$ compared with CD4 IL-4^{+/+} group (A, B, and E), or between the groups shown (C and D). **, $p < 0.01$ compared with CD4⁺IL-4^{+/+} group.

mice that received CD4⁺ T cells from IL-4-deficient mice before sensitization compared with recipients of CD4⁺ T cells from WT mice (Fig. 6D).

Intracellular cytokine staining to detect CD8⁺IL-13⁺ T cells in the lung showed that OT-1 mice that received naive CD4⁺ T cells from IL-4-deficient mice before sensitization developed a significantly

FIGURE 7. IL-4 administration during sensitization reconstitutes AHR and airway inflammation. Changes in airway resistance (RL) (A) and cell composition (B) in BAL fluid in sensitized and challenged OT-1 mice that received IL-4 during the sensitization phase (OT-1+IL-4) and sensitized and challenged OT-1 mice following PBS treatment (OT-1). Eos, eosinophils; Lym, lymphocytes; Mac, macrophages; Neu, neutrophils. Data represent mean + SEM for $n = 8$ experiments. *, $p < 0.05$ or **, $p < 0.01$ compared with sensitized and challenged OT-1 mice.



lower number of lung CD8⁺IL-13⁺ T cells than recipients of CD4⁺IL-4⁺ T cells (Fig. 6E).

Administration of IL-4 to OT-1 mice in sensitization phase

Our data demonstrated the role of CD4⁺IL-4⁺ T cells to be essential for the activation of CD8⁺ T cells but did not eliminate a role for the CD4⁺ T cells themselves in the development of the lung allergic responses and AHR. To determine whether IL-4 alone could substitute for the transfer of CD4⁺IL-4⁺ T cells in the functional activation of CD8⁺ T cells in OT-1 mice, recombinant mouse IL-4 was administered during the sensitization phase to OVA. As shown in Fig. 7, IL-4 administration alone during sensitization augmented both AHR and airway eosinophilia compared with control (PBS) mice. Similarly, IL-4 administration to sensitized only or challenged only mice failed to alter airway responsiveness or airway inflammation (data not shown).

CD4⁺ T cell depletion before challenge of OT-1 mice that received CD4⁺ T cells before sensitization

To further elucidate the role of OT-1 CD8⁺ T cells in the development of AHR and airway inflammation more clearly, CD4⁺ T cells were depleted by administering anti-CD4 Ab before challenge of OT-1 mice recipients of naive CD4⁺ T cells before sensitization with OVA. The depletion of CD4⁺ T cells before and during challenge to OVA did reduce the development of AHR, airway eosinophilia, BAL IL-13 levels, and goblet cell metaplasia, but all parameters remained significantly higher than in OT-1 mice that did not receive CD4⁺ T cells (Fig. 8). The effectiveness of the depletion of CD4⁺ T cells in this manner was confirmed by administering the Ab to OT-1 mice during the sensitization phase, just after transfer of CD4⁺ T cells. This prevented the development of all lung allergic responses (data not shown). Although some activity may be attributed to the transferred CD4⁺ T cells, these data confirm the effective induction of OT-1 CD8⁺ T cells in the development of the full spectrum of allergic responses following their activation by donor CD4⁺ T cells during the sensitization phase.

Transfer of CD8⁺ T cells from OT-1 mice to CD8-deficient mice

To further establish the functional capacity of the OT-1 CD8⁺ T cells in the development of the full spectrum of lung allergic responses, CD8⁺ T cells were isolated from the different groups of OT-1 mice and injected into sensitized CD8^{-/-} mice just before challenge (Fig. 1C). As shown in Fig. 9, CD8^{-/-} recipients of OT-1 cells from donors that did not receive CD4⁺ T cells resulted in significantly lower AHR, airway eosinophilia, BAL IL-13 levels, goblet cell metaplasia, and mucus hyperproduction than the CD8^{-/-} mice that received no cells. However, transfer of OT-1

cells from mice that received CD4⁺ T cells before sensitization, reconstituted AHR, airway eosinophilia, IL-13 levels, and goblet cell metaplasia in the CD8^{-/-} recipients (Fig. 9).

Discussion

A number of recent studies describe a role for CD8⁺ T cells in the development of AHR and airway allergic inflammation (18–20). In asthma patients, airway infiltrating CD8⁺ T cells have the capacity to produce Th2 cytokines and modulate the disease (21), increased numbers of CD8⁺ T cells in the airways have been found in the lungs of patients after a fatal asthma attack (40), and the number of bronchial wall CD8⁺ T cells but not the number of eosinophils or thickness of the basement membrane correlated with decline in lung function (41). Based on these data, CD8⁺ T cells likely contribute to the pathophysiology of altered airway function and allergic inflammation. However, it is not known whether there is a critical interaction between CD4⁺ T cells and CD8⁺ T cells in the development of CD8⁺ T cell-mediated airway allergic inflammation.

In the present study, OT-1 mice, MHC class I-restricted OVA peptide (OVA_{257–264} SIINFEKL) specific TCR transgenic mice, did not develop AHR or airway allergic inflammation following sensitization and challenge to OVA. The absence of functional CD4⁺ T cells in these mice may underlie this failure to respond to allergen. Transfer of CD4⁺ T cells into these mice before sensitization, but not before challenge, induced AHR, airway allergic inflammation, goblet cell metaplasia, and Th2 cytokine production accompanied by the emergence of an increased number of lung CD8⁺IL-13⁺ T cells. These data suggest that transferred CD4⁺ T cells together with recipient CD8⁺ T cells (OT-1 cells) participated in the development of AHR and allergic airway inflammation and that the interaction between CD4⁺ and CD8⁺ T cells during the sensitization phase was required for the functional activation of CD8⁺ T cells to become IL-13 producers.

Although CD4⁺ T cells are not required for primary CD8⁺ T cell responses against some pathogens, memory CD8⁺ T cell numbers and secondary responses to bacterial or viral challenges are decreased over time in CD4⁺ T cell-deficient animals (30–32). In these studies, it was not apparent at what stage CD4⁺ T cells were required to enhance the survival of Ag-specific memory CD8⁺ T cells. Sun et al. (42) reported that in the context of acute infection, CD4⁺ T cells were required during the maintenance phase of long-lived memory CD8⁺ T cell development. In some reports, APCs appeared to be crucial for CD4⁺ T cell help of effective CTL responses of CD8⁺ T cells through a number of mechanisms (43–46).

Several reports demonstrated that IL-4 is a critical cytokine for generating cells expressing a Th2 and Tc2 phenotype in vitro (38, 39, 47). IL-4 was also shown to be essential for development of AHR and

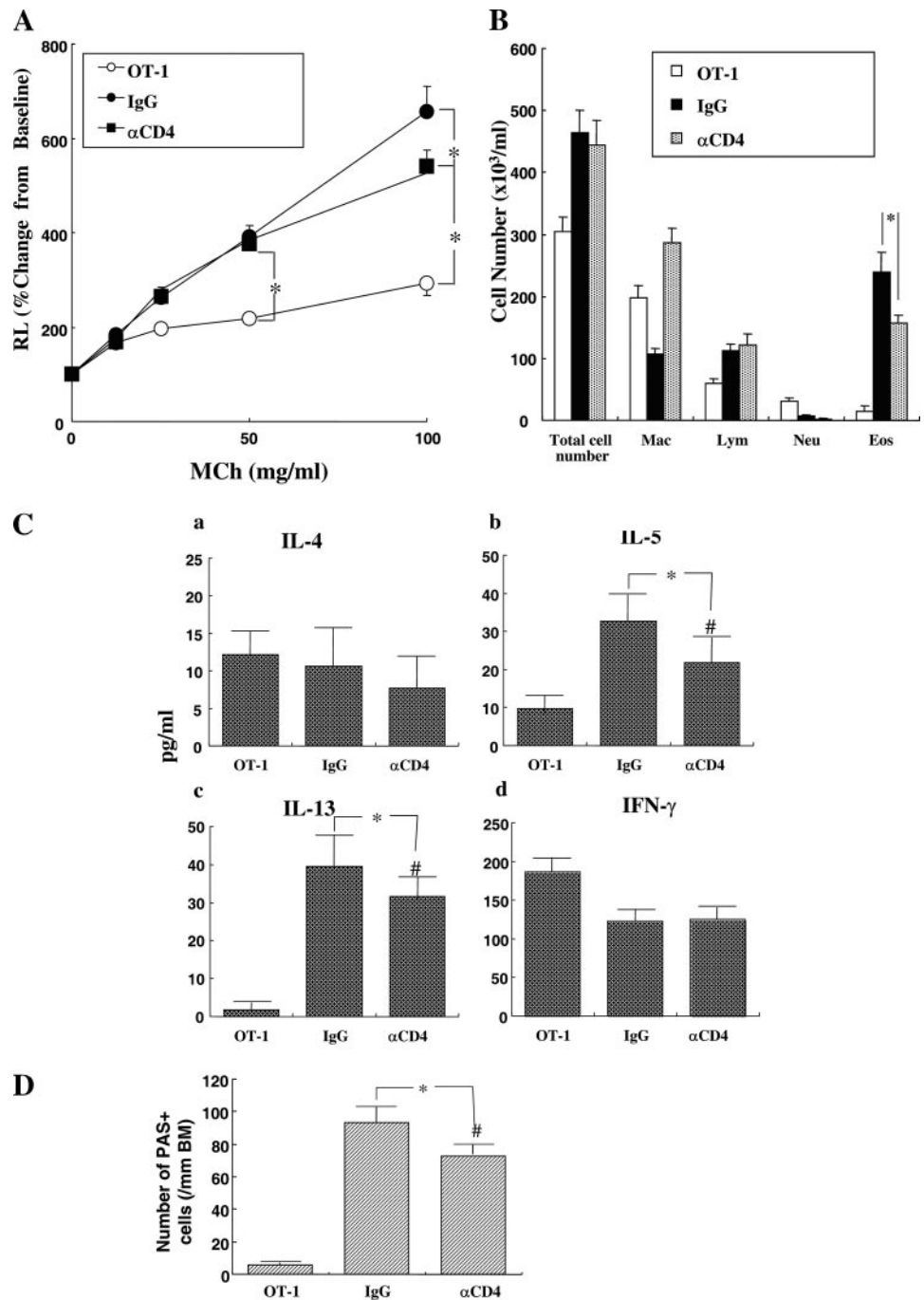


FIGURE 8. Depletion of CD4⁺ T cells before challenge of OT-1 recipients of CD4⁺ T cells during sensitization reduces but does not eliminate lung allergic responses. Sensitized OT-1 mice or sensitized OT-1 mice that received CD4⁺ T cells during the sensitization phase were treated with control Ab (IgG) or anti-CD4 (α CD4) during the challenge phase. Mice were studied for airway resistance (RL) (A), cell composition in BAL fluid (B), cytokine levels (C), including IL-4 (a), IL-5 (b), IL-13 (c), IFN- γ (d) in BAL fluid. D, Quantitative analysis of PAS-positive cells in the lung tissue is shown. Data represent mean $\pm$ SEM of $n = 12$ per group. *, $p < 0.05$ between the groups indicated; #, $p < 0.01$ comparing OT-1 mice to anti-CD4-treated mice.

allergic airway inflammation in some murine models (48), especially in the sensitization but not the challenge phase (49, 50). To define the mechanism underlying the interaction observed, we examined the effects of IL-4. Transfer of naive CD4⁺ T cells from IL-4-deficient mice before sensitization failed to trigger the development of AHR, airway inflammation, or goblet cell metaplasia in the challenged OT-1 mice and did not lead to any increase in the number of CD8⁺IL-13⁺ T cells in the lung. Taken together, these results implied that IL-4 was an essential cytokine for the functional activation of CD8⁺ T cells in the sensitization phase for the subsequent development of AHR and other allergic responses.

To further define that IL-4 was essential to the functional activation of the CD8⁺ T cells and to eliminate any direct role for the CD4⁺ T cells in the development of AHR and eosinophilic in-

flammation in the challenge phase, recombinant IL-4 was administered to OT-1 mice during the sensitization phase. Administration of IL-4 in this way reconstituted both AHR and airway eosinophilia, indicating the critical role of IL-4 during the sensitization phase in activating CD8⁺ T cells to become effectors of AHR and airway inflammation.

In addition, depletion of CD4⁺ T cells prior to challenge of OT-1 recipients of CD4⁺ T cells during the sensitization phase had only modest effects on the development of lung allergic responses. These data further confirmed the requirement for CD4⁺ T cells in the sensitization phase and that the effective induction of OT-1 CD8⁺ T cells was responsible for the full development of all lung allergic responses.

To directly confirm the functional role of these CD4⁺-induced OT-1 CD8⁺ T cells in the development of AHR, CD8⁺ T cells

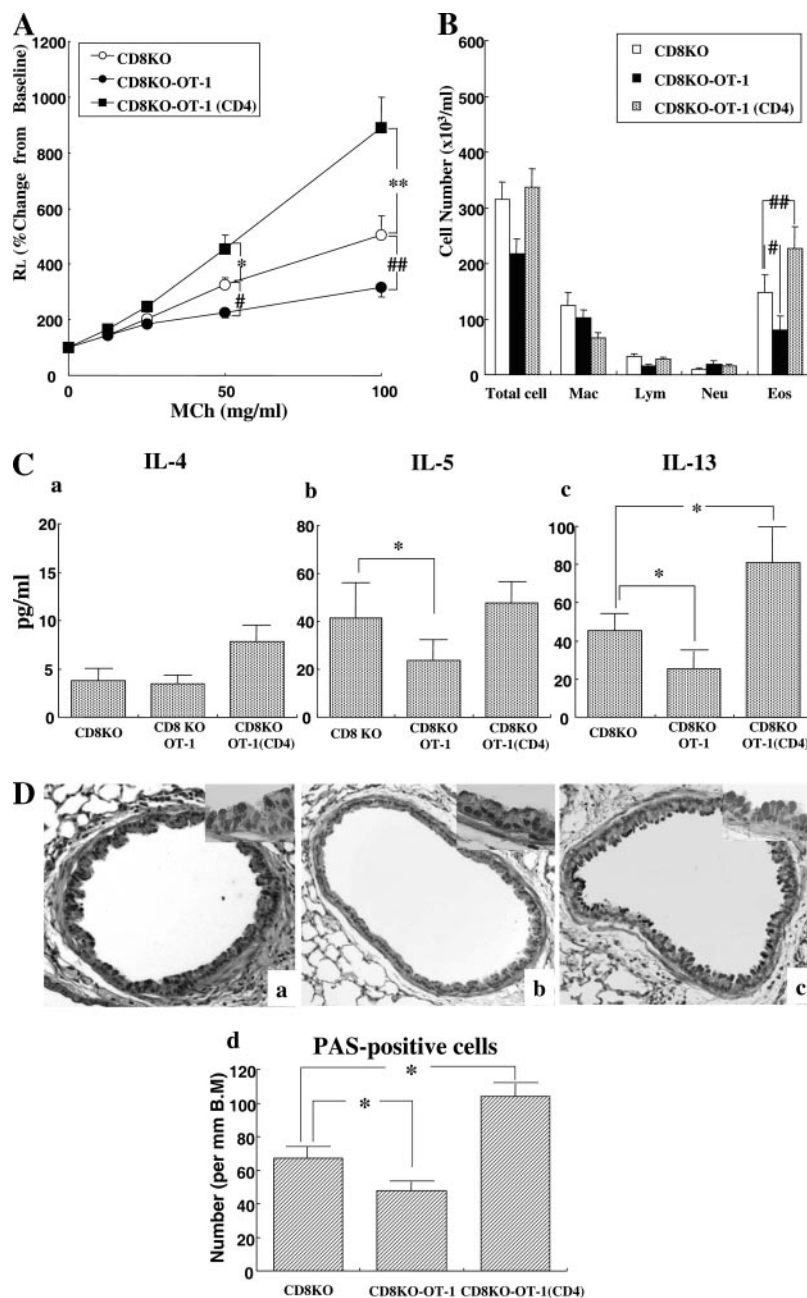


FIGURE 9. Functional capacity of the OT-1 CD8⁺ T cells in the spectrum of lung allergic responses. Changes in airway resistance (RL) (A) and cell composition (B) in BAL fluid in sensitized and challenged CD8-deficient (CD8KO) mice. C, Cytokine levels, including IL-4 (a), IL-5 (b), and IL-13 (c) in BAL fluid, were determined by ELISA as described in *Materials and Methods*. D, Representative photomicrographs (a–c) and quantitative analysis of PAS-positive cells (d) in the lung tissue are shown. Photomicrographs include sensitized and challenged CD8-deficient mice (a), sensitized and challenged CD8-deficient mice that received primed spleen CD8⁺ T cells from OT-1 mice given PBS before sensitization (CD8KO-OT-1) (b), sensitized and challenged CD8-deficient mice that received primed spleen CD8⁺ T cells from OT-1 mice given naive CD4⁺ T cells before sensitization (CD8KO-OT-1 (CD4)) (c). Data represent mean $\pm$ SEM for $n = 12$ per group. *, $p < 0.05$; **, $p < 0.01$; and ##, $p < 0.01$ between the groups indicated.

were isolated from OT-1 mice that did or did not receive CD4⁺ T cells during the sensitization phase, and were injected into CD8^{-/-} mice. These CD8^{-/-} mice have been shown to have a significantly reduced ability to develop AHR, airway eosinophilia, increased BAL IL-13 levels, or goblet cell metaplasia (20). CD8⁺ T cells from donor mice that did not receive CD4⁺ T cells in fact suppressed AHR and airway eosinophilia in the CD8-deficient recipients. This response contrasted with transfer of CD8⁺ T cells from OT-1 mice that did receive CD4⁺ T cells during the sensitization phase. In these CD8^{-/-} recipients, AHR, eosinophilic inflammation, Th2 cytokine production, and goblet cell metaplasia were fully restored.

IL-13 is known to be a critical regulator of AHR and airway inflammation and may be essential to the development of goblet cell metaplasia and mucus hyperproduction. Many cell types are potential sources of IL-13 (51), but as we have shown, in this and other studies where lung CD8⁺ T cells have been shown to play a critical role, that it is their ability to release IL-13 that is pivotal to

the development of AHR, airway inflammation, and goblet cell metaplasia (52, 53). Interference with IL-13 activity in these models abrogates lung allergic responses, including AHR (53, 54).

In summary, sensitized and challenged OT-1 mice did not develop AHR and airway allergic inflammation likely due to a reduced number and function in CD4⁺ T cells. Following transfer of WT CD4⁺IL-4⁺ T cells before sensitization but not before challenge and not following transfer of CD4⁺IL-4⁻ T cells, the OT-1 mice developed AHR and airway allergic inflammation. Furthermore, transfer of CD8⁺ T cells from OT-1 mice that received CD4⁺ T cells before sensitization augmented AHR and allergic airway inflammation in CD8-deficient recipient mice. In contrast, transfer of CD8⁺ T cells from OT-1 mice that did not receive CD4⁺ T cell before sensitization, suppressed AHR and allergic inflammation. These data suggest that CD4⁺ T cells and IL-4 during the allergen sensitization phase is central to the full functional activation of CD8⁺ T cells to become IL-13 producers and essential to the development of AHR, airway allergic inflammation, and goblet cell metaplasia.

Acknowledgments

We are grateful for the expert help of Diana Nabighian in preparing the manuscript and to Lynn Cunningham for performing the immunolabeling studies.

Disclosures

The authors have no financial conflict of interest.

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