

Requirement for CD8⁺ T Cells in the Development of Airway Hyperresponsiveness in a Murine Model of Airway Sensitization

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Summary

To study the role of CD8⁺ T cells in allergic sensitization, we examined the effects of in vivo depletion of CD8⁺ T cells prior to sensitization on IgE production, immediate type cutaneous hypersensitivity and development of altered airway responsiveness. BALB/c mice were thymectomized and treated with anti-CD8 antibody resulting in depletion of CD8⁺ T cells (<1%) in spleen and lymphoid tissues. In these mice, sensitization to ovalbumin (OVA) via the airways still resulted in IgE anti-OVA responses and immediate cutaneous reactions to OVA, but the animals were unable to develop airway hyperresponsiveness, eosinophil infiltration of the lung parenchyma, or IL-5 production in the local lymph nodes of the airway. Transfer of CD8⁺ T cells from naive animals during sensitization (on day 8 of the 10-d protocol) fully restored the ability to develop airway hyperresponsiveness and this was accompanied by IL-5 production and eosinophil accumulation in the lung. These data indicate a critical role for CD8⁺ T cells in the production of IL-5 and the development of altered airway responsiveness after antigen sensitization through the airways.

Increased airway reactivity (AR)¹ or airway hyperresponsiveness (AHR) is a general feature of bronchial asthma (1). Whereas AHR may be provoked by many environmental stimuli, the underlying mechanisms resulting in the increased reactivity of the airway smooth muscles are still not fully understood. A major contributor appears to be inflammation of the airways, with concomitant increases in eosinophil and T cell infiltration of the airway mucosa (2–5).

There is increasing evidence for a pivotal role for T lymphocytes in the induction of AHR (6–10). In patients with allergic AHR, the production of allergen-specific IgE is under the control of T lymphocytes. IL-4 is released from activated T cells and stimulates B cells to proliferate and to undergo isotype switching to produce IgE (11). A second important cytokine, IL-5, produced by T lymphocytes and other cells, enhances the migration (12), differentiation and function (13) of eosinophils and mast cells in humans and animals (14, 15), key cells in the development of AHR. In-

creased numbers of eosinophils have been found in biopsies (16, 17) and broncho-alveolar lavage fluids (18) of asthmatic patients, and the degree of eosinophilic infiltration correlates with severity of the disease (4, 19). CD4⁺ T lymphocytes from broncho-alveolar lavage fluid (8) or mucosal biopsies (20, 21) express elevated levels of mRNA for IL-4 and IL-5, and serum IL-5 levels correlate with the numbers of eosinophils in peripheral blood (10).

In contrast to the well-defined role of the CD4⁺ T cell subset, a distinct role for CD8⁺ T cells in the regulation of IgE production and development of AHR is less clear. A T suppressor cell dysfunction with impaired concanavalin A-induced suppressor cell activity (22) may be critical for the development of allergic diseases and has been described in patients with atopic dermatitis, allergic rhinitis and bronchial asthma (23–25). A decrease in the number of peripheral T suppressor cells was observed in asthma and allergic rhinitis and proposed to be causally related to these diseases (26, 27). On the other hand, an increase of CD8⁺ T cells was observed in the peripheral blood (28) and in broncho-alveolar lavage fluid (29) of asthmatic patients, and CD8⁺ T cell infiltration of the bronchial mucosa after allergic sensitization has been described in animal models of increased AR (30–32). Moreover, CD8⁺ T cells, separated from the

¹Abbreviations used in this paper: AR, airway activity; AHR, airway hyperresponsiveness; EFS, electrical field stimulation; hpf, high power fields; ICH, immediate cutaneous hypersensitivity; MNC, mononuclear cells; OVA, anti-ovalbumin; PBLN, peribronchial lymph node; PLN, peripheral lymph nodes.

peripheral blood of asthmatic patients, were shown to produce IL-5 and enhance eosinophil survival in vitro (33). Recent studies support the presence of T_{H2} -like $CD8^+$ T cells that produce IL-4, IL-5, and IL-10 but not interferon-gamma (IFN- γ) or interleukin 2 (IL-2) (34–37).

To better delineate the role of $CD8^+$ T cells in IgE production and in the development of AHR, we studied the effects of in vivo depletion of $CD8^+$ T cells in a mouse model of airway sensitization. In this model, BALB/c mice are induced to develop elevated levels of anti-ovalbumin (OVA) IgE (38), allergen-specific immediate cutaneous hypersensitivity (ICH) (39) and increased AR to electrical field stimulation (EFS) (40) after a 10-d sensitization period to aerosolized OVA. These responses also occur after adoptive transfer of sensitized peribronchial lymph node (PBLN) cells (40) or OVA-specific IgE^+ B cells (41) from sensitized animals to naive recipients. However, the increase in AR also depends on local allergen challenge of the airways, suggesting that in addition to a requirement for allergen-specific IgE, other factors are necessary for the development of AHR.

Here, we show that depletion of $CD8^+$ T cells before sensitization prevents the development of AHR despite production of allergen-specific IgE and development of ICH. The requirement for $CD8^+$ T cells in the induction of AHR was confirmed in cell transfer experiments; transfer of nonprimed $CD8^+$ T cells restored the ability to develop AHR in $CD8$ T cell-depleted mice. The development of AHR in sensitized animals was associated with eosinophil infiltration of the lung tissue and increased IL-5 production by PBLN cells. The failure of $CD8$ T cell-depleted mice to develop AHR was associated with the inability to recruit eosinophils into the lung tissue and a decrease in IL-5 production by PBLN cells. These data suggest a direct, enhancing role of $CD8^+$ T cells in the development of AHR through regulation of IL-5 production and eosinophil inflammation.

Materials and Methods

Animals. Pathogen-free, female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) of 3–12 weeks of age, maintained under institutional approved guidelines, were used in all experiments.

In Vivo $CD8$ T Cell Depletion. Mice of 3–4 weeks of age were thymectomized and two weeks later were injected i.p. with 200 μ l of ascites fluid containing anti- $CD8$ antibodies from the clone YTS 169 (rat IgG anti-mouse $CD8$; American Type Culture Collection [ATCC], Rockville, MD). Antibody specificity and optimal dilution of the ascites was measured and titrated in vitro and in vivo. Injections were repeated three times at 3-d intervals. Control animals received i.p. injections of 0.2 mg rat IgG (Sigma Chem. Co., St. Louis, MO) at the same time points. Sham thymectomy without antibody injection had no effect on any of the responses measured.

Cell Transfer. $CD8^+$ T cells were positively selected from spleens of naive, nonsensitized BALB/c donor mice using anti- $CD8$ conjugated magnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany). The selected fraction contained >95%

$CD8^+$ T cells, the depleted fraction contained less than 1% $CD8^+$ T cells as determined by FACS analysis. $CD8$ T cell-depleted mice were injected with 1×10^7 $CD8^+$ T cells i.v. on day 8 of the 10-d sensitization period. As a control, $CD8^+$ -depleted animals received 1×10^7 of $CD8$ -depleted spleen MNC (<1% $CD8^+$ T cells).

For in vitro depletion of $CD8^+$ PBLN T cells before culture, anti- $CD8$ beads were used.

Sensitization Procedure. Mice were exposed to OVA (Sigma) as described previously (40). Briefly, a solution of 1% OVA in PBS was delivered by ultrasonic nebulization for 20 min daily over a period of 10 consecutive days (day 1 to 10) in a closed chamber. Control mice were exposed to PBS following the same protocol.

Intradermal Skin Testing. All mice were tested for immediate cutaneous hypersensitivity on day 11 as previously described (39). Test solutions were OVA (500 μ g in 1 ml PBS) and PBS. Wheal reactions were read after 15–20 min in a blinded fashion and scored as positive if the diameter in any direction exceeded 3 mm.

Measurement of Airway Responsiveness. Airway responsiveness to electrical field stimulation was determined 2 d after completion of the sensitization protocol as previously described (40). Briefly, tracheas were removed and preparations of ~0.5-cm length were placed in Krebs-Henseleit solution suspended by triangular supports transducing the force of contractions. Electrical field stimulation with an increasing frequency from 0.5–40 Hz was applied and the contractions measured. Frequencies resulting in 50% of the maximal contraction (ES_{50}) were calculated from linear plots for each individual animal and were compared for the different groups.

Serum Collection. Venous blood was obtained from the tail vein on day 11 and clotted at room temperature. The serum was stored after centrifugation (5 min, 5,000 rpm) at -20°C .

Determination of Serum Antibody Titers by ELISA. Serum antibody levels were determined as previously described (38). Briefly, ELISA plates (Dynatech, Chantilly, VA) were freshly coated with OVA (20 μ g in 1 ml NaHCO_3 buffer, pH 9.6) or polyclonal goat anti-mouse IgE (The Binding Site, Ltd., San Diego, CA; 3 μ g of antibodies in 1 ml buffer) and incubated overnight at 4°C . Plates were blocked with 0.2% gelatin buffer, pH 8.2, for 2 h at 37°C . Serum was diluted 1:10 in gelatin. Standards containing OVA-specific IgE and IgG1 were generated as described (39). For total immunoglobulins, commercial standards were used (Pharmingen, San Diego, CA). ELISA data were analyzed with the Microplate Manager software for the Macintosh computer (Bio-Rad Labs., Richmond).

Cell Preparation. Spleens, peripheral lymph nodes (PLN) (axillary, brachial, cervical, inguinal, para-aortal, and intestinal lymph nodes) and PBLN were removed and placed in PBS. Tissue was dispersed into single cell suspensions and mononuclear cells (MNC) were purified by Ficoll-Hypaque gradient centrifugation (Lymphocyte Separation Medium; Organon Teknika, Durham, NC). Cells were washed, counted and resuspended to a final concentration of 4×10^6 cells per ml in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) tissue culture medium, containing heat-inactivated fetal calf serum (FCS 10%; Hyclone, Logan, UT), L-glutamine (2 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (5 mM), Hepes buffer (15 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml) (all from GIBCO BRL).

FACS Analysis. Spleen MNC and PLN cells from one animal in each experimental group were analyzed at day 1 before beginning the sensitization protocol; spleen MNC and PBLN were analyzed on day 12 after completion of the sensitization period.

Lymph node cells were pooled from 3–4 animals and incubated in staining buffer (PBS, 2% FCS, 0.2% sodium azide) with anti-CD4 (FITC-RM4-4; PharMingen, San Diego, CA) or anti-CD8 (biotin-53.6.7; Pharmingen) antibodies at 4°C. Stained cells were analyzed using an Epics cytofluorograph (Coulter Electronics, Hialeah, FL). Fluorescence intensity was compared with negative controls and cells incubated with phycoerythrin-coupled streptavidin alone.

Histology. Lung tissue was removed and fixed in 4% paraformaldehyde. 5- μ m-thick sections were cut around the main bronchus in a standardized way for all lungs. Eosinophils were stained in a combined method using astra blue and vital new red as described (42). Numbers of eosinophils were counted in a blind fashion under light microscopy using 400 \times magnification. A minimum of 10 high power fields (hpf) were examined and the results are expressed as mean \pm SD of numbers of eosinophils per hpf.

Cytokine Production. Spleen MNC and PBLN cells were plated at 4×10^6 cells/ml in 96-well round-bottom tissue culture plates and incubated with PMA (10 nM) and ionomycin (0.5 μ M) for 48 h at 37°C. Cell-free supernatants were harvested and cytokine production measured using a mouse IL-5 ELISA kit (Endogen, Cambridge, MA). Serum levels of IL-5 were determined similarly. The limits of detection of IL-5 in this kit were 2 ng/ml.

Cytoplasmic Staining of Cytokine Protein. Spleen MNC and PBLN cells were prepared for cytoplasmic staining of cytokine protein as described (43). Briefly, cells were stimulated for 4 h with PDB (10 ng/ml) and ionomycin (0.5 μ M), permeabilized with saponin (Sigma, 0.1% in HBSS), blocked with goat and donkey Ig, and then stained with biotinylated anti-IL-5 or anti-IFN- γ antibodies (PharMingen), followed by PE-conjugated streptavidin. In a second step, FITC-conjugated anti-CD4 and anti-CD8 antibodies (PharMingen) were added and the frequencies analyzed using an Epics cytofluorograph (Coulter Electronics). Fluorescence intensity was compared to isotype controls matching the anti-cytokine antibodies or non-stimulated cells.

Statistical Analysis. Results of antibody and cytokine levels are expressed as the mean \pm SD. The results of the *in vitro* assessment of airway responsiveness are reported as the mean \pm SEM. The Tukey-Kramer HSD test was used for multiple comparisons of the different groups; levels of differences between the individual groups were determined by Student's two-tailed unpaired *t* test with a *P* value of <0.05 for significance.

Results

Depletion of CD8⁺ T Cells *In Vivo*

The aim of these experiments was to investigate the effects of CD8 T cell depletion before airway sensitization to OVA on IgE production, cutaneous reactivity and AR. The status of depletion was determined by FACS analysis of spleen MNC and PLN cells before sensitization. The PLN from nonsensitized control animals contained $64 \pm 2.9\%$ CD4⁺ and $11.3 \pm 2.2\%$ CD8⁺ T cells (Fig. 1). Following thymectomy and injection of anti-CD8 antibody, the PLN contained less than 1% CD8⁺ T cells. Similar results were observed for spleen MNC. After depletion, the frequency of CD8⁺ T cells decreased from $7.5 \pm 1.2\%$ to $0.9 \pm 0.3\%$, whereas CD4⁺ T cells remained at similar levels ($35 \pm 2.5\%$ before vs. $33.2 \pm 2.9\%$ after depletion) (Fig.

T cell distribution of spleen MNC and PLN after CD8 T cell depletion

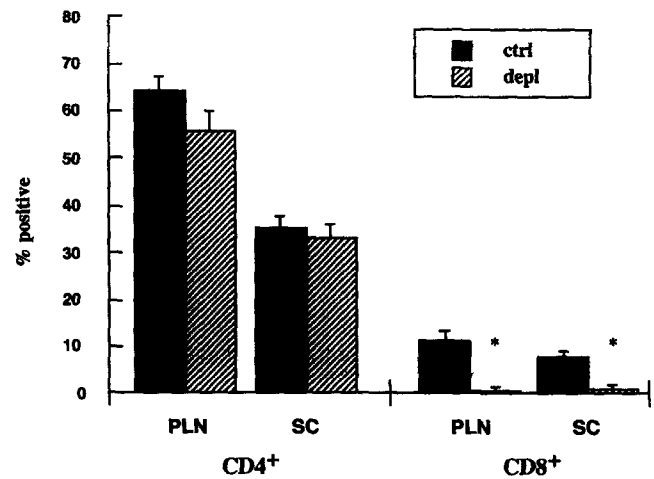


Figure 1. Thymectomy and anti-CD8 antibody treatment deplete CD8⁺ T cells. Mice of 3–4 wk of age were thymectomized. 10 d later, 200 μ l of mouse ascites containing anti-CD8 antibodies were injected *i.p.* Injections were repeated three times. Control animals received *i.p.* injections of 0.2 mg rat IgG at the same time points. FACS[®] staining of PLN cells and spleen MNC for CD4⁺ and CD8⁺ T cells was performed on day 1 of the sensitization protocol to OVA. Expressed are the frequencies as a percent of total MNC for the two T cell subsets of control (*n* = 20) and depleted animals (*n* = 16) from four independent experiments **P* < 0.001 compared to nondepleted controls.

1). The slight decrease in the number of CD4⁺ T cells following the depletion protocol is explainable by depletion of CD4⁺/CD8⁺ double-positive T cells. The total number of spleen MNC per mouse was reduced by 10.5% from $38 \pm 4 \times 10^6$ in nondepleted animals to $34 \pm 3.8 \times 10^6$ after CD8⁺ T cell depletion. To exclude the possibility that the anti-CD8⁺ antibodies injected *in vivo* only blocked CD8⁺ binding rather than depleting CD8⁺ T cells, the cell populations were double-stained with anti-CD3 and either anti-CD4 or anti-CD8. In the CD8⁺ depleted mice, all CD3⁺ cells were also CD4⁺.

Sensitization to OVA Increases Total Cell Number and CD8⁺ T Cells in PBLN

Mice were sensitized to OVA via the airways by ultrasonic nebulization on 10 consecutive days. To investigate possible changes in total cell number and T cell distribution at the site of local allergen challenge, we analyzed PBLN of nonsensitized and sensitized animals for the distribution of CD4⁺ and CD8⁺ T-cells. After completion of sensitization, the PBLN increased in total cell number by fivefold ($3.9 \pm 0.8 \times 10^6$ PBLN cells per mouse after sensitization vs. $0.82 \pm 0.08 \times 10^6$ PBLN cells per nonsensitized mouse, *P* < 0.001). This enlargement of the PBLN was accompanied by a relative increase in the CD8⁺ T-cell subset (Fig. 2) with a shift in the CD4/CD8 ratio from 5.4 ± 0.3 in nonsensitized mice to 3.96 ± 0.7 in sensitized animals

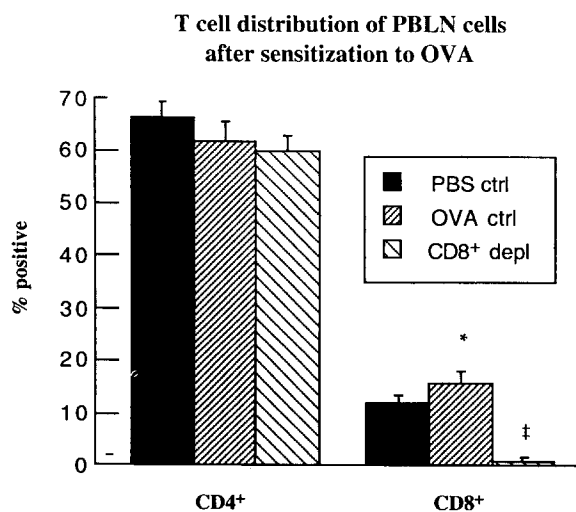


Figure 2. Sensitization to OVA increases the frequency of CD8⁺ T cells in the PBLN. BALB/c mice were sensitized to OVA on days 1–10. PBLN were harvested on day 12 and stained for CD4⁺ and CD8⁺ T cells. Compared are nonsensitized (PBS ctrl, *n* = 20), sensitized nondepleted (OVA ctrl, *n* = 16) and sensitized CD8⁺-depleted animals (CD8 depl, *n* = 20). Expressed are the frequencies as a percent of total PBLN cells for the two T cell subsets from four independent experiments **P* < 0.05 compared to PBS ctrl †*P* < 0.001 compared to OVA ctrl.

(*P* < 0.04). This shift was due to an increase in the CD8⁺ T cell subset from 12.1 ± 1.5% to 16.0 ± 2.3% after sensitization, paralleled by a relative decrease in CD4⁺ T cells from 66.5 ± 2.5% to 62.0 ± 2.8% after sensitization.

Depleted Cell Fractions Do Not Recover after Sensitization with Allergen

To exclude the possibility that the CD8⁺ T cell subset depleted by thymectomy and antibody treatment recovers or expands during the sensitization period, we analyzed spleen MNC and PBLN cells 2 d after completion of the sensitization protocol. The frequency of CD8⁺ T cells remained less than 1% in PBLN cells and spleen MNC (0.6% ± 0.5 and 0.7 ± 0.4, respectively) (Fig. 2). This indicates that the depletion protocol was efficient in removing the specific T cell subset during the whole period of sensitization without recovery of the depleted cell fraction.

Effect of CD8⁺ T Cell Depletion on OVA Sensitization

Serum Anti-OVA and Total IgE Levels. To analyze the effect of CD8 T cell depletion before sensitization on IgE production, we measured total IgE and allergen-specific IgE in the sera of sensitized, nondepleted and sensitized, CD8 T cell-depleted animals. Serum was obtained immediately before determination of airway responsiveness, 2 d after completion of the 10-d OVA-nebulization period. Depletion of CD8⁺ T cells resulted in an increase in total IgE production (Fig. 3); total IgE levels increased from 26 ± 4 ng/ml for nondepleted mice to 43 ± 10 ng/ml after CD8⁺ depletion. Allergen-specific IgE levels of depleted animals remained essentially at the levels of nondepleted, sensitized animals (2650 ± 350 EU/ml vs 2500 ± 300 EU/ml in sensitized, nondepleted mice). Both of the

Levels of total [ng/ml] and anti-OVA IgE [EU/ml]

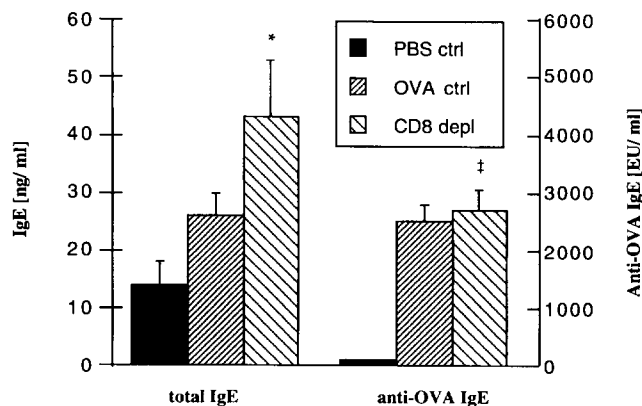


Figure 3. CD8⁺ T cell depletion increases total and OVA-specific IgE production after sensitization to OVA. Serum from nonsensitized (PBS ctrl, *n* = 20), sensitized, nondepleted (OVA ctrl, *n* = 16) and sensitized, CD8 T cell-depleted mice (CD8 depl, *n* = 20) were obtained 2 d after completion of OVA sensitization. Total IgE (ng/ml) and OVA-specific IgE levels (ELISA units, EU/ml) were measured by ELISA and compared to standards. Expressed are the mean ± SD from four independent experiments. **P* < 0.05 compared to PBS control, † not significant compared to OVA control; ‡ not significant compared to OVA control.

groups developed significantly elevated OVA-specific IgE levels compared to nondepleted, nonsensitized mice (<10 EU/ml; *P* < 0.01). This indicates that sensitization to OVA via the airways leads to the production of allergen-specific IgE and that the depletion of CD8⁺ T cells before sensitization does not interfere with the production of allergen-specific or total IgE.

Immediate Cutaneous Hypersensitivity. After intradermal injection of OVA, the majority of OVA-sensitized, nondepleted mice developed allergen-specific immediate wheal responses (Table 1). Depletion of CD8⁺ T cells did not af-

Table 1. Effect of CD8 T Cell Depletion on Immediate Cutaneous Reactivity to OVA

	Positive Responders	
	Numbers	%
PBS ctrl	0/20	0
OVA ctrl*	14/16	87
CD8 ⁺ depl‡	17/20	85

CD8⁺ T cell depletion does not alter immediate cutaneous hypersensitivity to OVA. Skin reactivity to OVA of nonsensitized (PBS ctrl, *n* = 20), sensitized, nondepleted (OVA ctrl, *n* = 16) and sensitized, CD8 T cell-depleted (CD8 depl, *n* = 20) mice was examined 1 d after completion of the sensitization protocol. Testing was by intradermal injection of OVA (500 µg/ml in PBS). Expressed are the percentages and numbers of positive responders to OVA from four independent experiments.

**P* < 0.001 compared with PBS ctrl.

‡*P* < 0.001 compared with PBS ctrl.

fect the frequency of positive responders, corresponding to the data for allergen-specific IgE production.

Development of Airway Hyperresponsiveness Is Inhibited after CD8 T Cell Depletion. To evaluate AR after sensitization to OVA via the airways, we stimulated segments of tracheas of individual mice and compared the electrical frequencies leading to half-maximal contraction (ES_{50}). Nonsensitized mice had ES_{50} values of 4.0 ± 0.2 Hz (Fig. 4). OVA-sensitized, nondepleted mice had ES_{50} values of 2.65 ± 0.28 , indicating AHR. In sensitized animals depleted of $CD8^+$ T cells, the response to EFS was similar to non-sensitized animals with a mean ES_{50} of 3.87 ± 0.20 ($P < 0.001$ compared to OVA-sensitized, nondepleted mice; Fig. 4). These data indicate that the development of hyperresponsiveness of tracheal smooth muscles to electrical field stimulation after sensitization to OVA was prevented by depletion of $CD8^+$ T cells before sensitization. This implies a pivotal role for this T cell subset in the induction or regulation of increased airway responsiveness that is unrelated to effects on IgE production.

Transfer of Nonprimed $CD8^+$ T Cells into $CD8^+$ -depleted Mice Restores the Ability to Develop AHR

To exclude a role for non-specific effects of the anti- $CD8$ antibody treatment and confirm the role of $CD8^+$ T cells in the development of AHR, we transferred 10^7

$CD8^+$ T cells from spleens of naive mice intravenously into $CD8$ T cell-depleted recipients on day 8 of the 10-d sensitization protocol. A group of $CD8$ T-cell depleted animals received a similar number of $CD8$ -depleted splenic MNC ($<1\%$ $CD8^+$ T cells) from naive mice at the same time.

$CD8^+$ T Cell Transfer. We analyzed spleen MNC, PLN, and PBLN on day 12 (2 d after completion of sensitization) and compared $CD8$ T cell-depleted animals with $CD8$ T cell recipients. Following transfer, $CD8^+$ T cells were detected in recipient animals. The highest percentage of $CD8^+$ T cells was found in the PBLN; $9.6 \pm 0.7\%$ of PBLN cells after transfer were $CD8^+$ T cells, whereas only $3.4 \pm 0.4\%$ PLN and $3.2 \pm 0.3\%$ spleen MNC were $CD8^+$ after transfer (Fig. 5). In contrast, the number of $CD8^+$ T cells remained at $0.95 \pm 0.2\%$ of PBLN in depleted animals that received $CD8$ -depleted spleen MNC. Thus, transfer of $CD8^+$ T cells resulted in a 10-fold increase in the proportion of $CD8^+$ T cells in the PBLN of the depleted animals. This suggests some selective accumulation of $CD8^+$ T cells in the PBLN, the site of local challenge with allergen, as observed in nondepleted, sensitized animals (Fig. 2).

Airway Reactivity. Transfer of $CD8^+$ T cells from naive donor mice into $CD8$ T cell-depleted recipients on day 8 fully restored the ability to develop AHR when assayed on day 12. $CD8$ T cell recipients had ES_{50} values of 2.2 ± 0.3 Hz. Depleted control animals receiving $CD8$ T cell-depleted spleen MNC had ES_{50} values of 3.9 ± 0.2 (Fig. 4). These data indicate that transfer of $CD8^+$ T cells into $CD8$ T cell-depleted animals restores the ability to develop AHR.

CD8⁺ T cells are necessary for the development of AHR after sensitization to OVA

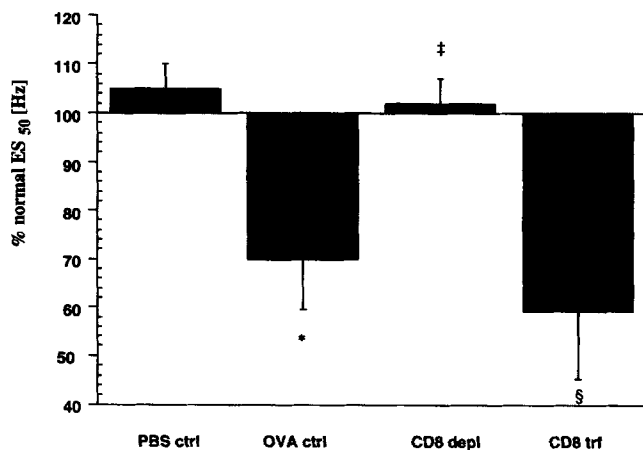


Figure 4. $CD8$ T cell depletion prevents the development of AHR after OVA sensitization. Nonsensitized (PBS ctrl, $n = 20$), sensitized, nondepleted (OVA ctrl, $n = 16$), sensitized, $CD8$ T cell depleted ($CD8^+$ depl, $n = 20$) and $CD8^+$ T cell-depleted recipients of $CD8^+$ T cells ($CD8^+$ trf, $n = 8$) are compared. 2 d after completion of the sensitization protocol to OVA, trachea smooth muscle segments were prepared as described in Material and Methods and exposed to increasing frequencies of electrical field stimulation. Expressed are the mean \pm SE of ES_{50} values (frequencies resulting in 50% maximal contraction) as a percent of normal, untreated control mice (ES_{50} 3.8 ± 0.2) for the different groups * $P < 0.01$ compared to nonsensitized mice; † $P < 0.01$ compared to sensitized, nondepleted mice; § $P < 0.03$ compared to sensitized, $CD8$ T cell-depleted animals.

CD8⁺ T cells in spleens, PLN and PBLN

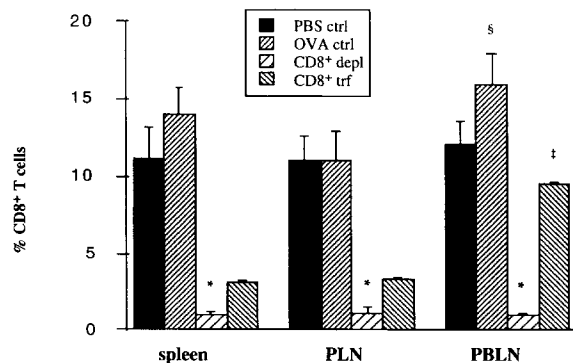


Figure 5. Passive transfer of $CD8^+$ T cells into $CD8^+$ T cell-depleted recipients. Mice were depleted of $CD8^+$ T cells before sensitization. 10^7 $CD8^+$ T cells from naive donor mice were transferred i.v. on day 8 of the 10-d sensitization protocol ($CD8$ trf, $n = 8$). $CD8$ T cell-depleted controls ($CD8$ depl, $n = 8$) received similar numbers of $CD8$ T cell-depleted spleen MNC. FACS[®] staining of spleen MNC, PLN, and PBLN was performed on day 12. Expressed are the frequencies of $CD8^+$ T cells in spleen, PLN and PBLN cells from two independent experiments. * $P < 0.001$ compared to spleen, PLN and PBLN of nondepleted, sensitized mice (OVA ctrl, $n = 8$); † $P < 0.04$ compared to spleen and PLN of $CD8$ T cell-depleted recipients of $CD8^+$ T cells ($CD8$ trf); § $P < 0.05$ compared to PBLN of nonsensitized animals (PBS ctrl, $n = 8$).

CD8 T Cell Depletion Inhibits and CD8⁺ T Cell Transfer Restores Eosinophil Infiltration into the Lung Tissue

To investigate the histological changes that accompany sensitization to OVA, formalin-fixed lungs of sensitized mice were stained for eosinophils with vital new red. Sensitization to OVA via the airways was accompanied by significant eosinophilic infiltration into the lung parenchyma. In nonsensitized animals, an average of 0.3 ± 0.1 eosinophils/hpf was detected. After sensitization to OVA, the number increased by 15-fold to 5.2 ± 0.4 eosinophils/hpf ($P < 0.01$) (Fig. 6). Depletion of CD8⁺ T cells significantly reduced this eosinophilic infiltration to 1.7 ± 0.3 eosinophils/hpf ($P < 0.04$ compared to nondepleted, sensitized mice). Following transfer of CD8⁺ T cells, eosinophil infiltration into the lungs was observed with numbers increasing to 5.1 ± 0.2 eosinophils/hpf. These data indicate that depletion of CD8⁺ T cells is associated with reduced eosinophil infiltration of the lung tissues after airway sensitization to OVA, and that the transfer of CD8⁺ T cells restores this response.

In Vivo and In Vitro CD8 T Cell Depletion Decreases and CD8⁺ T Cell Transfer Restores IL-5 Production in PBLN Cells

To assess IL-5 production at the site of the local allergen challenge during sensitization to OVA, PBLN cells were stimulated for 48 h with PMA/ionomycin. In PBLN of sensitized, nondepleted animals, a sixfold increase in IL-5 production compared to nonsensitized mice was detected

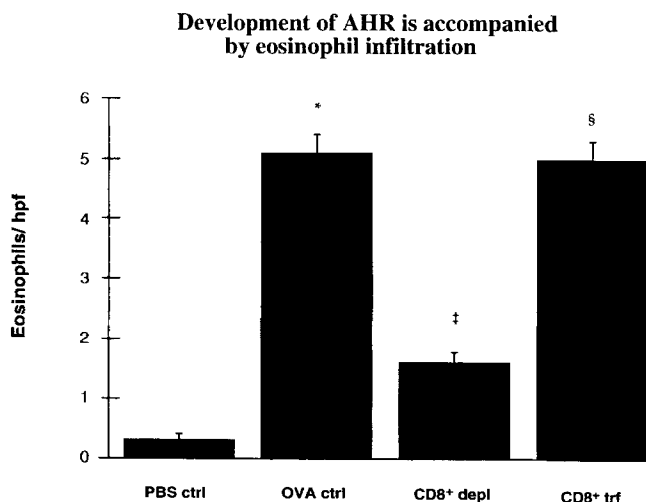


Figure 6. Development of AHR is accompanied by eosinophil infiltration of lung tissue. Formalin-fixed lungs were cut into 5- μ m-thick slices and stained for eosinophils with vital new red. Eosinophils were counted using 40 \times magnification. Compared are nonsensitized (PBS ctrl, $n = 8$), sensitized, nondepleted (OVA ctrl, $n = 8$), sensitized, CD8 T cell-depleted animals (CD8 depl, $n = 8$) and sensitized, CD8 T cell-depleted recipients of 10^7 unprimed CD8⁺ T cells (CD8 trf, $n = 8$) from two independent experiments. Expressed are the mean \pm SD of numbers of eosinophils per hpf. * $P < 0.01$ compared to nonsensitized animals; † $P < 0.04$ compared to nondepleted, sensitized mice; § $P < 0.04$ compared to sensitized, CD8⁺-depleted animals.

CD8 depletion decreases and CD8 transfer restores in vitro IL-5 production of PBLN after OVA sensitization

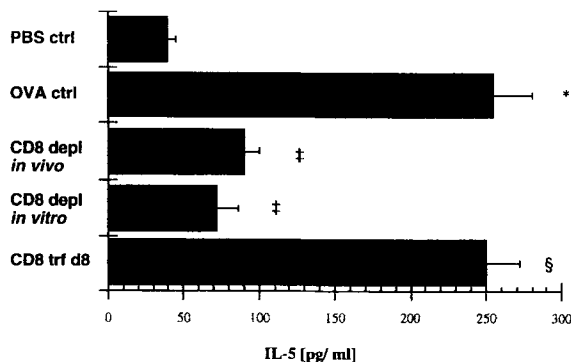


Figure 7. CD8 T cell depletion reduces and passive transfer of CD8⁺ T cells restores IL-5 production in PBLN after OVA sensitization. PBLN were harvested 2 d after completion of the OVA sensitization protocol and stimulated for 48 h in the presence of PMA (10 nM) and ionomycin (0.5 μ M). IL-5 concentrations in cell-free supernates were measured by ELISA. Compared are PBLN from nonsensitized (PBS ctrl, $n = 8$), sensitized, nondepleted (OVA ctrl, $n = 8$), sensitized, CD8 T cell-depleted animals (CD8 depl in vivo, $n = 8$), CD8-depleted PBLN from sensitized nondepleted mice (CD8 depl in vitro, $n = 8$) and sensitized, CD8 T cell-depleted recipients of 10^7 unprimed CD8⁺ T cells (CD8 trf, $n = 8$) from two independent experiments. Expressed are the mean \pm SD concentrations of IL-5 (pg/ml). * $P < 0.01$ compared to nonsensitized animals; † $P < 0.03$ compared to nondepleted, sensitized mice; § $P < 0.03$ compared to sensitized, CD8 T cell-depleted animals.

(255 ± 25 ng/ml after sensitization versus 40 ± 5 ng/ml in nonsensitized controls, $P < 0.03$) (Fig. 7). This indicates that airway sensitization is accompanied by an augmentation of in vitro IL-5 production by cells at the site of allergen challenge. CD8⁺ T cell depletion before sensitization resulted in a significant decrease in the production of IL-5 by an equivalent number of sensitized PBLN cells. In parallel experiments, in vitro CD8 T-cell depletion of PBLN cells from OVA sensitized mice prior to culture also significantly reduced IL-5 production by an equivalent number of cells. After transfer of CD8⁺ T cells into CD8-depleted recipients on day 8, the in vitro production of IL-5 from PBLN was restored to similar levels as observed in nondepleted, sensitized animals.

To determine if sensitization to OVA leads to an increase in circulating levels of IL-5, we measured IL-5 concentrations in the serum of sensitized mice, one day after completion of sensitization. Measurable amounts of IL-5 were only detected in sensitized, nondepleted animals and in sensitized, CD8-depleted recipients of CD8⁺ T cells; IL-5 serum concentrations reached 5 ± 2 ng/ml and 3.8 ± 2.5 ng/ml, respectively. No IL-5 was detected in the serum of nonsensitized animals or sensitized, CD8-depleted animals.

CD8⁺ T Cells Are a Major Source of IL-5

To determine if CD8⁺ T cells are a major source of IL-5 production themselves or indirectly augment IL-5 production by another cell type, we assessed cytokine production

CD8⁺ T cells are a major source of IL-5 in the PBLN of OVA-sensitized mice

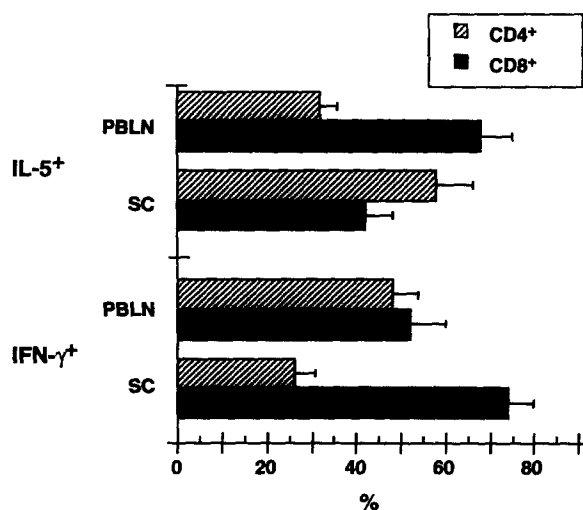


Figure 8. Distribution of IL-5⁺ and IFN- γ ⁺ T cells in PBLN and spleen. PBLN and spleen MNC from OVA-sensitized mice were stimulated (PDB 10 nM, ionomycin 0.5 μ M, 4 h) and stained for cytoplasmic protein using anti-IL-5 or anti-IFN- γ antibodies and for T cell surface markers with anti-CD4 and anti-CD8 antibodies. Total numbers of T cells stained for IL-5 were $4.5 \pm 0.5\%$ in PBLN and $3.4 \pm 0.8\%$ in spleen, total numbers of T cells stained for IFN- γ were $3.8 \pm 1.2\%$ in PBLN and $5.8 \pm 2.2\%$ in spleen. Expressed is the distribution of CD8⁺ and CD4⁺ T cell subsets producing IL-5 and IFN- γ in PBLN and spleen from two independent experiments ($n = 16$).

at the single cell level. PBLN and spleen MNC from OVA-sensitized mice were prepared, stimulated and stained with anti-cytokine antibodies as described (43). The distribution of different T cell subsets that stained for the specific cytokines was assessed. Among PBLN T cells, $4.5 \pm 0.5\%$ stained positively for IL-5 and $3.8 \pm 1.2\%$ were positive for IFN- γ . In spleen, the frequency of IL-5⁺ T cells was lower ($3.4 \pm 0.8\%$) and that of IFN- γ ⁺ cells higher ($7.8 \pm 2.2\%$) compared to PBLN. The majority of IL-5⁺ T cells in the PBLN was CD3⁺/CD8⁺, whereas less than one third of T cells that stained positive for IL-5 were CD4⁺ (Fig. 8). In the spleen, the majority of CD8⁺ T cells were positive for IFN- γ and not IL-5. These data indicate that CD8⁺ T cells are a major source of IL-5 in the PBLN of OVA-sensitized mice.

Discussion

The role of CD8⁺ T cells in allergic sensitization is obviously complex. In the present study, we have investigated this issue by analyzing the consequences of in vivo CD8 T-cell depletion before sensitization on IgE production, development of immediate cutaneous reactivity and AHR in a mouse model of airway sensitization. In contrast to other approaches (44–46), we chose the combination of thymectomy and injection of monoclonal anti-CD8 antibody to deplete CD8⁺ T cells. Analysis of the peripheral lymphoid tissues showed a virtually complete depletion of

the CD8⁺ T cell population after this treatment. Compared to studies using antibody injections alone (without thymectomy), this approach provides a more extensive and sustained depletion of the target T cell population (47). Even after 12 d of treatment and a 10-d sensitization protocol with daily allergen challenges, CD8⁺ T cells did not recover in the spleen or the local draining lymph nodes of the airways. To assess the consequences of CD8 depletion on allergic sensitization, we used a mouse model of local airway sensitization in the absence of adjuvant (38, 40). In this model, depletion of CD8⁺ T cells before sensitization of BALB/c mice to OVA via the airways resulted in increased total and anti-OVA IgE levels after sensitization. Predictably, in light of the allergen-specific IgE responses, sensitized, CD8 T cell-depleted mice developed allergen specific immediate cutaneous responses.

Despite elevated allergen-specific IgE levels, CD8 T cell depletion before sensitization with OVA via the airways prevented the development of AHR; responses of tracheal smooth muscle preparations to EFS were similar to those observed in nonsensitized animals. These results identify a critical role for CD8⁺ T cells in the development of altered AR, a finding that was confirmed in cell transfer experiments. Transfer of CD8⁺ T cells from naive donor mice into CD8 T cell-depleted recipients fully restored the development of AHR after sensitization; transfer of a similar number of T cells, depleted of CD8⁺ T cells, failed to restore the increases in AR. These data establish an essential role for CD8⁺ T cells in the development of AHR during sensitization in our model.

Transfer of unprimed CD8⁺ T cells on day 8 of the sensitization protocol appeared to be the optimal timepoint for reconstitution of the airway response. Transfer of cells at earlier time points was less effective (data not shown) and appeared to correlate with a reduced number of CD8⁺ T cells in the lymphoid organs, perhaps due to residual levels of anti-CD8 antibodies remaining from the last injection on day -3. On the other hand, cells transferred after completion of sensitization (day 10) were unable to affect the outcome of AR. Interestingly, when CD8⁺ T cells were transferred on day 8, the highest frequencies of CD8⁺ T cells were detected in the PBLN. This may indicate an enhanced homing ability of the transferred CD8⁺ T cells to the site of local allergen challenge. This is in concert with our findings of the increase in CD8⁺ T cell numbers in the PBLN following sensitization through the airways.

Initial studies by other investigators and ourselves emphasized a suppressive function for CD8⁺ T cells, associating IFN- γ -producing CD8⁺ T cells with the inhibition of IgE production, and consequent downregulation of allergic sensitization (48, 49). Nevertheless, expansion of CD8⁺ T cells has been detected in patients with bronchial asthma (28, 29) as well as in animal models of airway sensitization (30–32). Holt et al. established a model they termed “inhalative tolerance,” in rats (50) and in mice (51). Repeated sensitization, beginning in the newborn period, resulted in a progressive decrease in the production of allergen-specific IgE. Allergen-reactive CD8⁺ T cells were capable of sup-

pressing IgE production and adoptively transferred tolerance in naive animals exposed to subsequent allergen challenge. As described below, a comparison to our data is difficult because of the use of adjuvant for sensitization. Kemeny et al. studied a rat-model of sensitization using ricin as an adjuvant for allergen-specific IgE-production (52, 53). They reported a suppressive effect of early activated CD8⁺ T cells on IgE production that was transferable to naive animals (54). Interestingly, *in vivo* depletion of CD8⁺ T cells before sensitization with ricin and allergen did not lead to the predicted enhancement in IgE production, but instead, inhibited the development of IgE responses (55). From these experiments, they concluded that two distinct populations or different functional stages of CD8⁺ spleen T cells may exist, one which suppresses IgE responses through the production of IFN- γ and the other which augments IgE responses by an unknown mechanism.

As with the development of IgE responses, there are a number of conflicting findings concerning the function of CD8⁺ T cells in the development of AHR. In a study on mice, depletion of CD8⁺ T cells failed to influence eosinophil infiltration of the trachea (56). In rat models of allergic sensitization, depletion of CD8⁺ T cells enhanced late airway responses (57) and AHR was transferable into naive recipients only by sensitized CD4⁺, but not CD8⁺ spleen T cells (58). However, there are major differences in the design of the studies that may explain the contrast with our results. In our model, sensitization of "high-responder" BALB/c mice via the airways in the absence of adjuvant induced AHR and depletion of CD8⁺ T cells before sensitization prevented AHR. In the study of rats, "low-responder" SD rats that normally do not generate AHR were sensitized systemically in the presence of adjuvant, and CD8⁺ T cells were depleted only after completion of sensitization. This approach fails to account for the role of CD8⁺ T cells during sensitization. In addition, the different route for sensitization and especially the use of adjuvant may also limit the effect of CD8 T cell depletion after sensitization. This is supported by a study investigating the effects of CD8 T cell depletion in which a change from subcutaneous to intraperitoneal sensitization or sensitization in the presence of adjuvant restored the formerly inhibited development of delayed type hypersensitivity in CD8 T cell-depleted mice (59).

Mechanistically, the contribution of CD8⁺ T cells in the development of AHR requires delineation. Following sensitization to OVA, we observed peribronchial infiltration of eosinophils in the lung parenchyma and increased IL-5 production by PBLN cells from sensitized animals. Depletion of CD8⁺ T cells before sensitization not only prevented the development of AHR but was associated with markedly reduced eosinophil numbers and a decrease in IL-5 production by the PBLN cells. Similarly, *in vitro*

CD8 T cell depletion of OVA-sensitized PBLN cells before culture significantly reduced IL-5 production. Transfer of CD8⁺ T cells into the CD8 T cell-depleted animals not only restored the development of altered AR but also fully restored the eosinophil response and IL-5 production. This supports the central role of eosinophils in the development of AHR (60) and of interleukin 5 (IL-5) as the major enhancing factor for the activation and function of eosinophils in humans (14) and animals (15).

Our data identify a critical role for CD8⁺ T cells in the production of IL-5 during allergic sensitization. This role may be an indirect one, through regulation of CD4⁺ T cell cytokine production (61), or may reflect the production of IL-5 from sensitized CD8⁺ T cells themselves. Our data examining production of IL-5 directly at the single cell level demonstrated that in sensitized PBLN, CD8⁺ T cells are the major source of IL-5 production. The differences in the cytokine profile of T cells in spleen and PBLN imply that the local environment during sensitization plays a major regulatory role in dictating the phenotype or functional development of CD8⁺ T cells. CD8⁺ T cells developing in the PBLN of animals sensitized via the airways are exposed to IL-4 produced by CD4⁺ T cells in this environment and develop a T_H2-like phenotype. In an environment where IFN- γ -production is higher, these lymphocytes may develop into cells with a T_H1-like phenotype. Our demonstration of the effects of IFN- γ treatment support the importance of the local environmental influences: systemic injection of IFN- γ only reduced total IgE levels, without affecting antigen-specific IgE production in mice sensitized exclusively through the airways. Only local administration of IFN- γ via the airways was effective in inhibiting allergen-specific IgE production and preventing AHR (62).

The ability of CD8⁺ T cells to produce IL-4 and IL-5 in even larger amounts than CD4⁺ T cells has already been demonstrated in human T cell clones and recently in rat splenic CD8⁺ T cells (37, reviewed in reference 63). There now is increasing evidence for the presence of T_H2-like CD8⁺ T cells in humans, rats and mice (64–67). When stimulated with IL-4, CD8⁺ T cells increase IL-4 and IL-5 production and lose the ability to produce IFN- γ (37, 68, 69). In transgenic mice expressing a virus-specific $\alpha\beta$ T cell receptor, challenge with the specific peptide after sensitization to a different antigen induced a T_H2-type immune response that triggered CD8⁺ T cells to produce high amounts of IL-5, resulting in eosinophil accumulation in the airways (70). In our model, allergic sensitization of the airways triggers a T_H2-response leading to IL-4 production and OVA-specific IgE production in this environment (71). It is in this environment that we propose the CD8⁺ T cells become committed to IL-5 production, leading to eosinophil infiltration, activation and altered airway responsiveness.

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