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# Contribution of Antigen-Primed CD8<sup>+</sup> T Cells to the Development of Airway Hyperresponsiveness and Inflammation Is Associated with IL-13<sup>1</sup>

Nobuaki Miyahara, Katsuyuki Takeda, Taku Kodama, Anthony Joetham, Christian Taube, Jung-Won Park, Satoko Miyahara, Annette Balhorn, Azzeddine Dakhama, and Erwin W. Gelfand<sup>2</sup>

The role of Th2/CD4 T cells, which secrete IL-4, IL-5, and IL-13, in allergic disease is well established; however, the role of CD8<sup>+</sup> T cells (allergen-induced airway hyperresponsiveness (AHR) and inflammation) is less clear. This study was conducted to define the role of Ag-primed CD8<sup>+</sup> T cells in the development of these allergen-induced responses. CD8-deficient (CD8<sup>-/-</sup>) mice and wild-type mice were sensitized to OVA by i.p. injection and then challenged with OVA via the airways. Compared with wild-type mice, CD8<sup>-/-</sup> mice developed significantly lower airway responsiveness to inhaled methacholine and lung eosinophilia, and exhibited decreased IL-13 production both in vivo, in the bronchoalveolar lavage (BAL) fluid, and in vitro, following Ag stimulation of peribronchial lymph node (PBLN) cells in culture. Reconstitution of sensitized and challenged CD8<sup>-/-</sup> mice with allergen-sensitized CD8<sup>+</sup> T cells fully restored the development of AHR, BAL eosinophilia, and IL-13 levels in BAL and in culture supernatants from PBLN cells. In contrast, transfer of naive CD8<sup>+</sup> T cells or allergen-sensitized CD8<sup>+</sup> T cells from IL-13-deficient donor mice failed to do so. Intracellular cytokine staining of lung as well as PBLN T cells revealed that CD8<sup>+</sup> T cells were a source of IL-13. These data suggest that Ag-primed CD8<sup>+</sup> T cells are required for the full development of AHR and airway inflammation, which appears to be associated with IL-13 production from these primed T cells. *The Journal of Immunology*, 2004, 172: 2549–2558.

Asthma is characterized by persistent airway inflammation and airway hyperresponsiveness (AHR)<sup>3</sup>; T cells play a key role in orchestrating the disease process through the release of various cytokines (1). CD4<sup>+</sup> T cells, especially Th2-type cells, which produce IL-4, IL-5, and IL-13, are considered pivotal cells in the development of AHR and eosinophilic inflammation (1–5). In atopic asthma, increased expression of Th2-type cytokines has been shown in lymphocytes in bronchoalveolar lavage (BAL) fluid (6). In animal studies, adoptive transfer of Ag-primed T cells can induce AHR and late airway responses in naive rats (7–9). It has also been reported that the transfer of Th2-type cells in mice induces airway eosinophilia and AHR (10–12).

In contrast, there are relatively few studies that have focused on the involvement of CD8<sup>+</sup> T cells in AHR and airway inflammation. Although CD8<sup>+</sup> T cells are important effectors of cell-mediated immunity, their precise role in the pathogenesis of asthma is

unclear. Viral infections characteristically elicit a CD8<sup>+</sup> T cell lymphocytosis, dominated by cytolytic cells secreting IFN- $\gamma$ , which in turn may lead to Th2 suppression (13, 14). A number of studies have reported that CD8<sup>+</sup> T cells play a protective role in allergic disease (15–19). In the studies using rat models, depletion of CD8<sup>+</sup> cells up-regulated the late airway responses, AHR, and airway inflammation (15, 16), and administration of Ag-primed CD8<sup>+</sup> T cells suppressed these responses (17). In contrast, we have previously shown that depletion of CD8<sup>+</sup> T cells abrogated AHR and airway inflammation in mice exposed to allergen exclusively via the airways and in the absence of adjuvant (20). These seemingly contradictory results may reflect the heterogeneity of CD8<sup>+</sup> T cells and the identification of at least two distinct subsets (21–23). The CD8/T cytotoxic (Tc)1 subset secretes Th1-type cytokines, and the Tc2 subset secretes Th2-type cytokines. Indeed, Cho et al. (24) demonstrated increased numbers of IL-4/CD8<sup>+</sup> T cells in the blood of atopic patients with mild asthma. Activated CD8<sup>+</sup> T cell infiltration into peribronchial tissues has been associated with asthma deaths (25). Together, these studies are in keeping with a role for CD8<sup>+</sup> T cells, at least a subset, in the development of AHR and airway inflammation.

Nonetheless, the role of CD8<sup>+</sup> T cells in the development of AHR and airway inflammation remains controversial, and often has been based on indirect evidence. To more directly address this issue, we investigated first the responses of CD8-deficient (CD8<sup>-/-</sup>) mice to OVA sensitization and challenge. When compared with wild-type (WT) mice, CD8<sup>-/-</sup> mice developed a lower degree of AHR and airway inflammation and lower levels of IL-13 production in the BAL fluid and supernatants from cultured peribronchial lymph node (PBLN) cells. We therefore conducted adoptive-transfer experiments and determined that OVA-primed CD8<sup>+</sup> T cells, but not naive CD8<sup>+</sup> T cells or OVA-primed CD8<sup>+</sup>

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<sup>3</sup> Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; Tc, T cytotoxic; WT, wild type; PBLN, peribronchial lymph node; RL, lung resistance; Cdyn, dynamic compliance; MCh, methacholine; MNC, mononuclear cell; PAS, periodic acid Schiff; MBP, major basic protein; PI, PMA/ionomycin.

T cells from IL-13-deficient mice, restored the levels of IL-13 production *in vivo* that may be necessary for the full development of AHR and airway inflammation.

## Materials and Methods

### Animals

Homozygous CD8<sup>-/-</sup> mice, generated by targeting the *CD8 $\alpha$*  chain gene in C57BL/6 mice (26), were obtained from Dr. P. Marrack (National Jewish Medical and Research Center). IL-13-deficient (IL-13<sup>-/-</sup>) mice were generated by targeting the *IL-13* gene in 129Sv  $\times$  C57BL/6 (27). These mice were housed under specific pathogen-free conditions and maintained on an OVA-free diet in the Biological Resources Center at the National Jewish Medical and Research Center. Control WT C57BL/6 mice and 129Sv  $\times$  C57BL/6 (IL-13<sup>+/+</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Both female and male mice, 6–12 wk of age, were used in these experiments. Controls were matched with the deficient mice with regard to both age and gender in each experimental group. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

### Sensitization and airway challenge

CD8<sup>-/-</sup> and WT mice were assigned to control (Neb) and treatment groups (ipNeb) based on the following treatments: 1) airway challenge with OVA nebulization alone (three times) (Neb group); and 2) *i.p.* sensitization with OVA and OVA airway challenge (ipNeb group). Mice were sensitized by *i.p.* injection of 20  $\mu$ g of OVA (grade V; Sigma-Aldrich, St. Louis, MO) emulsified in 2.25 mg of alum (AlumImject; Pierce, Rockford, IL) in a total volume of 100  $\mu$ l on days 1 and 14. Mice were subsequently challenged via the airways by inhalation exposure to aerosols of OVA (1% in saline) for 20 min on days 28–30. OVA aerosols were produced by an ultrasonic nebulizer (particle size, 1–5  $\mu$ m; De Vilbiss, Somerset, PA). On day 32, airway function was measured as described below, followed by collection of samples for further analyses.

### Assessment of airway function

Airway function was assessed as previously described by measuring changes in lung resistance (RL) and dynamic compliance (C<sub>dyn</sub>) in response to increasing doses of inhaled methacholine (MCh) (28). Data are expressed as percentage of changes from baseline RL and C<sub>dyn</sub> values obtained after inhalation of saline.

### Bronchoalveolar lavage

Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with HBSS. Total leukocyte numbers were counted by cell counter (Coulter Counter; Coulter, Hialeah, FL). Cytospin slides were stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and differentiated by standard hematological procedures.

### Measurement of total and OVA-specific Abs

Serum levels of total IgE, OVA-specific IgE, IgG1, and IgG2a were measured by ELISA as previously described (29). The anti-OVA Ab titers of samples were related to internal pooled standards that were generated in the laboratory and expressed as ELISA units. Total IgE level was calculated by comparison with known mouse IgE standard (BD PharMingen, San Diego, CA). The limit of detection was 100 pg/ml for IgE.

### Cell preparation and culture

Spleen 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 (Organon Teknika, Durham, NC). PBLN cells were washed, counted, and resuspended to a final concentration of  $4 \times 10^6$  cells/ml in RPMI 1640 (Life Technologies, Gaithersburg, MD) tissue culture medium, containing heat-inactivated 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 5 mM glutamine, 15 mM HEPES buffer, and 50  $\mu$ M 2-ME. Cells ( $4 \times 10^5$ ) were cultured for 24 h in 96-well round-bottom plates at 37°C in a 5% CO<sub>2</sub> atmosphere, in the presence or absence of OVA (10  $\mu$ g/ml), or PMA (10 nM) and ionomycin (0.5  $\mu$ M).

### Proliferation assay

Spleen and PBLN MNC were cultured in 96-well round-bottom plates at  $2 \times 10^5$  cells/well in the presence or absence of OVA for 5 days and pulsed

with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well) for the last 6 h of culture. Proliferative responses were determined by scintillation counting of incorporated [<sup>3</sup>H]thymidine.

### Measurement of cytokines

Cytokine levels in the BAL fluid and cell culture supernatants were measured by ELISA as previously described (29). IFN- $\gamma$ , IL-4, IL-5, IL-10, IL-12 (BD PharMingen, San Diego, CA), and IL-13 (R&D Systems, Minneapolis, MN) ELISAs were performed according to the manufacturer's directions. The limits of detection were 4 pg/ml for IL-4, IL-5, and IL-13, and 10 pg/ml for IL-10, IL-12, and IFN- $\gamma$ .

### Histologic studies

After lavage, the lungs were fixed in 10% formalin and processed into paraffin blocks. Tissue sections, 5- $\mu$ m thick, were stained with H&E and periodic acid Schiff (PAS) for identification of mucus-containing cells (29). The number of mucus-containing cells per millimeter of basement membrane was determined as described (29). Eosinophils were identified by immunostaining of major basic protein (MBP) in lung sections using a rabbit polyclonal anti-mouse MBP Ab (kindly provided by Dr. J. Lee (Mayo Clinic, Scottsdale, AZ)) as previously described (28). The slides were examined in a blinded fashion using a Nikon (Melville, NY) microscope equipped with a fluorescein filter system. The number of eosinophils and goblet cells in the tissues were evaluated using IPLAB2 software (Signal Analytics, Vienna, VA) for the Macintosh computer, counting six to eight different fields per animal.

### Purification of CD8<sup>+</sup> T cells—donor cells

Spleens of WT mice, which were sensitized twice (days 1 and 14) with OVA plus alum, were removed 14 days after the last sensitization (day 28). Cells were harvested by mincing the tissues and subsequently passing them through a stainless steel sieve. Cells were then washed and suspended in PBS/BSA (0.5%). MNC were first isolated by Ficoll-Hypaque gradient centrifugation (Organon Teknika). Purification of CD8<sup>+</sup> T cells was conducted by negative selection using immunomagnetic cell sorting (MACS; Miltenyi Biotec, Auburn, CA). After washing, cells were incubated with a mixture of biotin-conjugated Abs against CD4 (L3T4), CD45R (B220), DX5, CD11b (Mac1), and Ter-119 for 30 min on ice, followed by washing. The cells were incubated with anti-biotin MicroBeads, for an additional 30 min on ice, and passed through a MACS column, and the cells that were not retained on the column were collected. The cells that were retained on the column were also collected for the cell transfer and used as Ag-primed non-CD8 T cells. Spleens of naive WT mice, OVA-sensitized IL-13<sup>-/-</sup> mice and IL-13<sup>+/+</sup> mice were also removed and purified in the same way. To assess purification, cells were incubated with CyChrome 5-conjugated anti-CD3 (Caltag Laboratories, Burlingame, CA), FITC-conjugated anti-CD4, anti-CD8, or anti-B220 Abs (BD PharMingen), and PE-conjugated anti-CD11c (BD PharMingen), PE-conjugated anti-mouse NK cells, anti- $\gamma\delta$  TCR, anti- $\alpha\beta$  TCR (Caltag Laboratories), or anti-CD1d (BD PharMingen), and then analyzed by flow cytometry (FACSCalibur; BD Immunocytometry Systems, San Jose, CA).

### Adoptive transfer—recipient mice

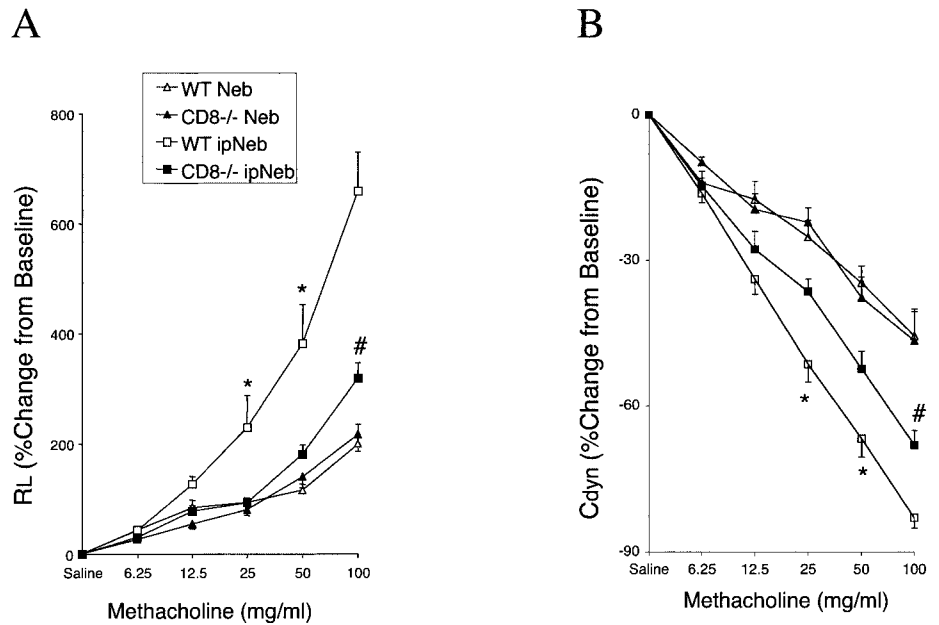
Recipient CD8<sup>-/-</sup> mice were sensitized twice with OVA plus alum on days 1 and 14. OVA-primed CD8<sup>+</sup> T cells ( $5 \times 10^6$ ) were administered *i.v.* via the tail vein to OVA-sensitized CD8<sup>-/-</sup> mice, 14 days after the last sensitization (day 28). Nonsensitized recipient CD8<sup>-/-</sup> mice that had not been sensitized with OVA plus alum also received  $5 \times 10^6$  primed CD8<sup>+</sup> T cells. Following transfer, the mice were exposed to three allergen challenges via the airways on days 28–30. Assays were conducted on day 32.

### Lung cell isolation

Lung cells were isolated as previously described (30) using collagenase digestion. Cells were resuspended in HBSS, and MNC were purified by Ficoll-Hypaque gradient centrifugation (Organon Teknika).

### Intracytoplasmic cytokine staining

Three-color analysis was performed using FACSCalibur. PBLN and lung MNC were stimulated for 4 h with PMA (5 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10  $\mu$ g/ml). Cells were stained for cell surface markers with CyChrome 5-conjugated anti-CD3 (Caltag Laboratories) and FITC-conjugated anti-CD4 or anti-CD8 Abs (BD PharMingen). After washing, cells were fixed in 4% paraformaldehyde and permeabilized with saponin (0.1% saponin in PBS). The permeabilized cells were blocked with normal goat serum followed by incubation with PE- or biotin-conjugated anti-cytokine Abs or similarly labeled isotype-matched control



**FIGURE 1.** RL (A) and Cdyn (B) in CD8<sup>-/-</sup> mice and WT mice. RL and Cdyn values were obtained in response to increasing concentrations of inhaled MCh, as described in *Materials and Methods*. Data represent the mean ± SEM (*n* = 12 in each group). The CD8<sup>-/-</sup> mice showed lower responsiveness to inhaled MCh compared with WT mice. #, Significant difference (*p* < 0.001) between CD8-deficient mice (CD8<sup>-/-</sup> ipNeb) and CD8-sufficient mice (WT ipNeb); and #, significant difference (*p* < 0.05) between CD8<sup>-/-</sup> ipNeb vs CD8<sup>-/-</sup> Neb and WT Neb. \*, Significant difference (*p* < 0.05) between CD8<sup>-/-</sup> ipNeb and WT ipNeb.

Abs for 30 min. Abs against IL-4, IL-5, and IFN-γ were purchased from BD PharMingen; biotinylated goat anti-mouse IL-13 and control Ab were purchased from R&D Systems. The cells incubated with biotin-conjugated Abs were washed twice and stained with PE-conjugated streptavidin for an additional 20 min. The stained cells were washed with saponin buffer twice and resuspended in PBS. Staining was analyzed by flow cytometry on FACSCalibur using CellQuest software (BD Biosciences, Mountain View, CA).

*Statistical analysis*

Values for all measurements are expressed as mean ± SEM. ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by unpaired two-tailed Student's *t* test. Significance levels were set at a *p* value of 0.05.

**Results**

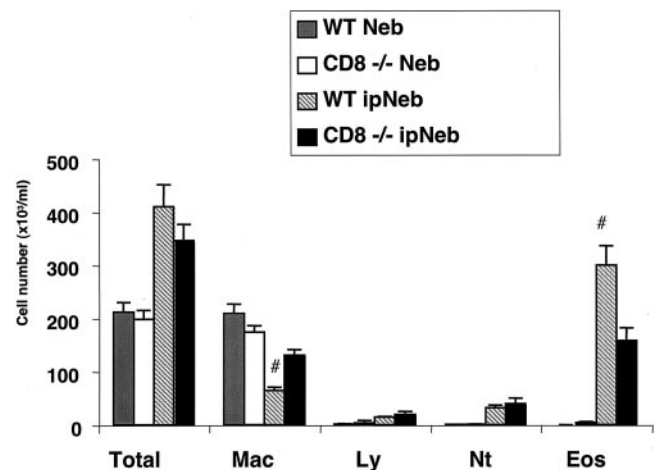
*CD8<sup>-/-</sup> mice develop significantly lower AHR compared with WT mice after sensitization and challenge*

Fig. 1 shows the changes in RL and Cdyn in response to increasing doses of inhaled MCh for both WT and CD8<sup>-/-</sup> mice. Intraperitoneal OVA sensitization and airway challenge led to the development of increased AHR in WT mice, illustrated by significant increases in RL and decreases in Cdyn, as previously described in this model (28). In contrast, OVA-sensitized and -challenged CD8-deficient mice developed only small increases in RL above non-sensitized and OVA-challenged control mice (*p* < 0.05), but the levels were significantly lower than in OVA-sensitized and -challenged WT mice (*p* < 0.001). When Cdyn was monitored, a similar pattern of differences between the groups was observed.

*Lung inflammation in CD8<sup>-/-</sup> mice and WT mice*

In parallel to the assessment of lung function, the inflammatory cell populations in the BAL fluid were examined (Fig. 2). Following sensitization and allergen challenge, the proportion of eosinophils reached up to 70% of total BAL cells in WT mice but was significantly lower (~45% of total cells) in the CD8<sup>-/-</sup> mice (*p* < 0.01). In contrast, macrophages comprised 40% of the total cell population in the CD8<sup>-/-</sup> mice and ~20% in the WT mice (*p* < 0.01). There were no statistically significant differences between the two groups in the numbers of total cells, neutrophils, or lymphocytes that were recovered in the BAL fluid.

Lung histology showed an intense infiltration of inflammatory cells in the perivascular tissue area and, to a slightly lesser extent, in the peribronchial spaces in the lungs of OVA-sensitized and -challenged WT mice. In CD8<sup>-/-</sup> mice, fewer eosinophils and lymphocytes were detected in these sites compared with WT mice after sensitization and challenge with OVA. No signs of lung tissue inflammation were seen in both strains of mice after exposure to only 3 days of nebulization with OVA, without prior sensitization. Immunostaining with anti-MBP revealed significantly higher numbers of eosinophils in the peribronchial tissue of WT mice (mean ± SEM, 138.8 ± 16.6 cells/mm<sup>2</sup>) when compared with CD8<sup>-/-</sup> mice (mean ± SEM, 83.8 ± 13.1 cells/mm<sup>2</sup>; *p* < 0.05) following sensitization and challenge. In nonsensitized but challenged animals, only a few eosinophils were detected in the airway tissue (mean ± SEM, 13.1 ± 2.8 cells/mm<sup>2</sup> for WT mice, and 8.3 ± 3.1 cells/mm<sup>2</sup> for CD8<sup>-/-</sup> mice).



**FIGURE 2.** Cellular composition of BAL fluid. CD8-deficient (CD8<sup>-/-</sup>) and WT mice were sensitized and challenged as described in *Materials and Methods*. BAL fluid was obtained from the same groups as described in Fig. 1. The results for each group are expressed as means ± SEM. #, Significant differences (*p* < 0.01) between CD8<sup>-/-</sup> ipNeb and WT ipNeb.

To assess the degree of goblet cell hyperplasia, tissue sections were stained with PAS. No PAS-positive cells were observed in the airways of nonsensitized but OVA-challenged mice from both strains. However, following OVA sensitization and challenge, WT mice developed significantly higher numbers of PAS-positive goblet cells in the airways as compared with CD8<sup>-/-</sup> mice (mean  $\pm$  SEM, 55.2  $\pm$  4.0 cells/mm of basement membrane in WT mice vs 31.4  $\pm$  5.4 cells/mm of basement membrane in CD8<sup>-/-</sup> mice;  $p < 0.01$ ).

#### OVA-specific Ab responses in CD8<sup>-/-</sup> mice and WT mice

Levels of OVA-specific Abs and total IgE levels in the serum were not significantly different between WT mice and CD8<sup>-/-</sup> mice after sensitization and challenge with OVA (Table I). Challenge alone on three consecutive days was insufficient to trigger Ab responses in either group of mice.

#### Cytokine levels in BAL fluid

OVA sensitization and challenge did not result in significant differences between the two strains of mice in the levels of IL-4, IL-5, IL-10, IL-12, or IFN- $\gamma$  in the BAL fluid (Fig. 3). However, following sensitization and challenge, the levels of IL-13 were significantly lower in the BAL fluids recovered from CD8<sup>-/-</sup> mice as compared with WT mice ( $p < 0.05$ ).

#### In vitro cytokine production

To determine whether the differences observed between the two strains of mice in their in vivo cytokine production were due to differences in Ag-specific T cell responsiveness, PBLN cells were isolated from sensitized and challenged mice, and were restimulated in culture for in vitro cytokine production with OVA or the combination of PMA/ionomycin (PI). Levels of IL-4, IL-5, IL-10, IL-12, IL-13, and IFN- $\gamma$  were measured in the culture supernatants by ELISA. Consistent with the in vivo observations, there were no significant differences between CD8<sup>-/-</sup> mice and WT mice in either OVA- or PI-stimulated, or spontaneous secretion of IL-4, IL-5, IL-10, IL-12, and IFN- $\gamma$  (data not shown). However, following restimulation in vitro with OVA or PI, CD8<sup>-/-</sup> PBLN cells secreted significantly lower amounts of IL-13 ( $p < 0.05$ ) than did their WT counterparts (Fig. 4A).

#### Proliferative responses to OVA

To further investigate possible differences at the level of Ag-induced T cell proliferation, MNC were prepared from spleen and PBLN from both strains of mice and cocultured with OVA. Proliferation responses were evaluated by measuring [<sup>3</sup>H]thymidine incorporation. OVA-induced proliferative responses in MNC from PBLN were virtually identical for both strains of mice (Fig. 4B). Similar results were seen when OVA-induced proliferative responses in spleen MNC were examined (data not shown). These

Table I. Concentrations of total IgE and OVA-specific Abs in the serum of CD8<sup>-/-</sup> and WT mice<sup>a</sup>

Group	Total IgE (ng/ml)	OVA-Specific (ELISA U/ml)		
		IgE	IgG1	IgG2a
WT/Neb	3.5 $\pm$ 2.2	0	0	24.4 $\pm$ 2.2
CD8 <sup>-/-</sup> Neb	2.4 $\pm$ 1.3	0	0	20.1 $\pm$ 8.3
WT/ipNeb	48.2 $\pm$ 5.4	29.5 $\pm$ 10.0	436.3 $\pm$ 61.9	39.4 $\pm$ 10.3
CD8 <sup>-/-</sup> ipNeb	49.6 $\pm$ 5.5	32.8 $\pm$ 3.9	476.2 $\pm$ 84.3	40.1 $\pm$ 12.5

<sup>a</sup> Data represent mean  $\pm$  SEM ( $n = 12$ ).

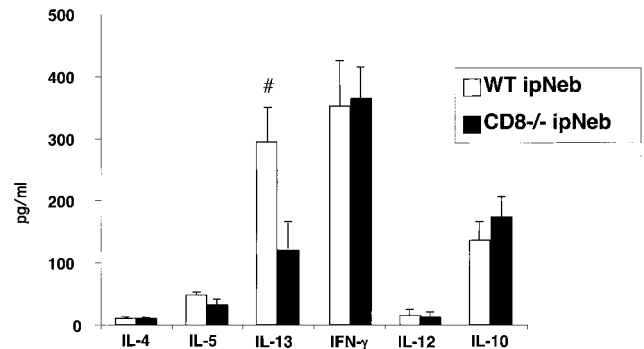


FIGURE 3. Cytokine levels in BAL fluid of CD8-deficient (CD8<sup>-/-</sup>) and WT mice. IL-4, IL-5, IL-13, IFN- $\gamma$ , IL-10, and IL-12 levels in BAL fluid from the ipNeb groups shown in Fig. 1 were measured in supernatants by ELISA, as described in *Materials and Methods*. CD8<sup>-/-</sup> mice had equivalent amounts of IL-4, IL-5, IL-10, IL-12, and IFN- $\gamma$  in their lavage fluid, but lower IL-13 after OVA sensitization and challenge. The results for each group are expressed as means  $\pm$  SEM. #, Significant differences ( $p < 0.05$ ) between CD8-deficient mice (CD8<sup>-/-</sup> ipNeb) and WT mice (WT ipNeb).

data indicate that Ag-specific T cell proliferation was not impaired in CD8<sup>-/-</sup> mice.

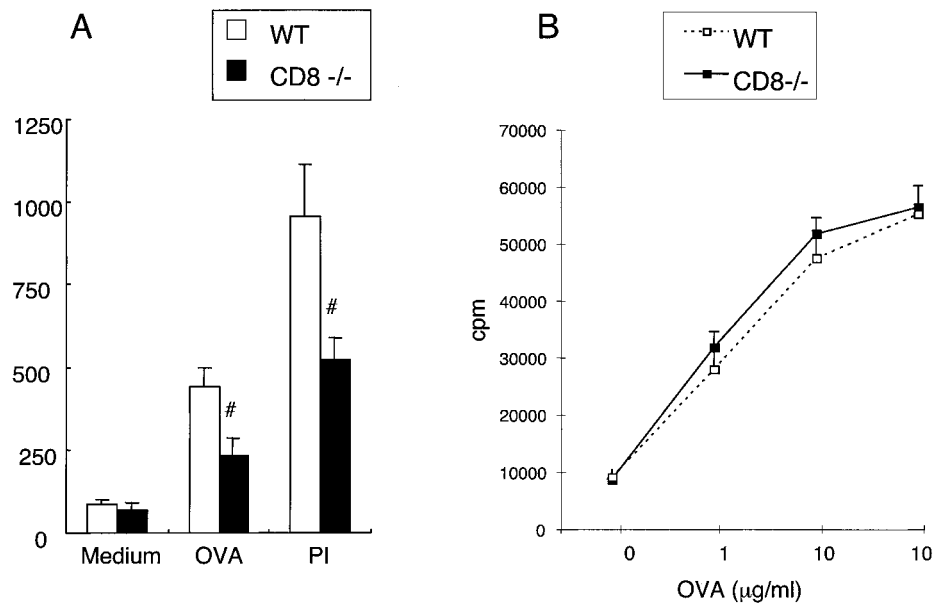
#### Reconstitution of CD8<sup>-/-</sup> mice

To address whether the absence of CD8<sup>+</sup> T cells is responsible for the decreases in airway, inflammatory, and cytokine responses, we reconstituted the CD8<sup>-/-</sup> mice with CD8<sup>+</sup> T cells. Recipient CD8<sup>-/-</sup> mice were sensitized with OVA on days 1 and 14. On day 28, 2 h before OVA challenge,  $5 \times 10^6$  CD8<sup>+</sup> T cells were transferred via i.v. injection into the tail vein. The donor CD8<sup>+</sup> T cells were negatively selected from the spleens of donor mice using magnetic beads as described in *Materials and Methods*. Donor WT mice consisted of two groups. In one group, mice were not sensitized, and in another group, mice were sensitized with OVA on days 1 and 14, and the cells were obtained on day 28. Non-CD8<sup>+</sup> T cells obtained from sensitized mice were also collected and transferred. IL-13<sup>+/+</sup> mice and IL-13<sup>-/-</sup> mice were also sensitized to OVA, and CD8<sup>+</sup> T cells were selected in the same way. After transfer of these cells into recipient mice, these mice were challenged with OVA on three consecutive days; 48 h after last challenge, AHR measurements and evaluation of BAL fluid were performed.

As assessed by flow cytometry, the proportion of the transferred CD3<sup>+</sup>CD8<sup>+</sup> cells that were from OVA-sensitized, naive, IL-13<sup>+/+</sup> or IL-13<sup>-/-</sup> mice exceeded 95%. They were >99%  $\alpha\beta$ /CD8<sup>+</sup> T cells with <0.5% of cells being  $\gamma\delta$ /CD8<sup>+</sup> T cells in preparations from both groups. In all of the transferred cell populations (sensitized/challenged, naive, IL-13<sup>+/+</sup>, or IL-13<sup>-/-</sup> mice), CD3<sup>+</sup>CD4<sup>+</sup> cells were <1%; CD3<sup>+</sup>CD8<sup>+</sup> cells or  $\gamma\delta$ <sup>+</sup> cells were <0.5%; and CD11c<sup>+</sup> cells, NK<sup>+</sup> cells, or CD1d<sup>+</sup> cells were <0.1%. OVA-sensitized non-CD8<sup>+</sup> T cells were comprised of 45.1  $\pm$  2.2% CD4<sup>+</sup> cells, 53.8  $\pm$  3.4% B220<sup>+</sup> cells (B cells), ~1% CD11c<sup>+</sup> cells, ~1% NK<sup>+</sup> cells, <1%  $\gamma\delta$ <sup>+</sup> cells, and <1% CD3<sup>+</sup>CD8<sup>+</sup> cells.

Fig. 5 shows the results of reconstitution with CD8<sup>+</sup> T cells on airway responsiveness. Reconstitution of the CD8<sup>-/-</sup> mice with Ag-primed CD8<sup>+</sup> T cells fully restored the development of AHR, to levels that were comparable with those seen in WT mice. In contrast, transfer of OVA-sensitized non-CD8<sup>+</sup> T cells and naive CD8<sup>+</sup> T cells failed to restore AHR. Recipient CD8<sup>-/-</sup> mice that had not been sensitized with OVA, also received  $5 \times 10^6$  CD8<sup>+</sup> T

**FIGURE 4.** A, IL-13 production by PBLN cells after OVA sensitization. Mice were sensitized and challenged with OVA. Two days after completion of OVA challenge, PBLN MNC were cultured ( $4 \times 10^5$  cells/well) in the absence or presence of OVA ( $10 \mu\text{g/ml}$ ) for 24 h. Cytokine levels in supernatants were determined by ELISA, expressed as mean  $\pm$  SEM (picograms per milliliter) ( $n = 12$  in each group). #, Significant differences ( $p < 0.05$ ) between CD8-deficient mice ( $\text{CD8}^{-/-}$ ) and WT mice. B, Proliferative responses of PBLN MNC. Mice were sensitized and challenged with OVA. Two days after completion of sensitization, PBLN cells ( $2 \times 10^5$  per well) were cultured in triplicate in the absence or presence of OVA for 5 days. Thymidine uptake was measured after pulsing the cells with  $1 \mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine for 6 h. Results for each group are expressed as mean  $\pm$  SEM ( $n = 8$  in each group).

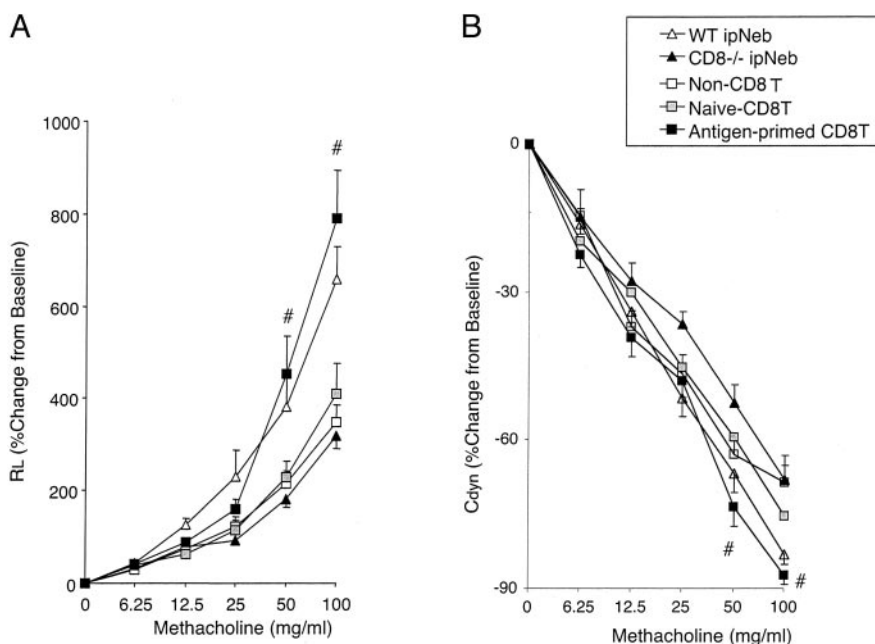


cells to determine whether transfer of Ag-primed  $\text{CD8}^+$  T cells by themselves can elicit AHR in the absence of sensitization of the recipient. Ag-primed  $\text{CD8}^+$  T cells, similar to naive  $\text{CD8}^+$  T cells, failed to elicit AHR in nonsensitized but challenged recipient  $\text{CD8}^{-/-}$  mice (data not shown).

Analysis of the inflammatory cell populations in the BAL fluid of recipient mice showed that transfer of OVA-sensitized  $\text{CD8}^+$  T cells (but not naive  $\text{CD8}^+$  T cells or OVA-sensitized non- $\text{CD8}^+$  T cells) fully restored the number of eosinophils (Fig. 6A) and IL-13 levels in the BAL (B), in parallel to the development of AHR. The levels of the other cytokines were unaffected by either transfer (data not shown). Fig. 6C shows the results of *in vitro* IL-13 production from PBLN cells. After transfer of Ag-primed  $\text{CD8}^+$  T cells and challenge of the sensitized recipient  $\text{CD8}^{-/-}$  mice, *in vitro* IL-13 production from PBLN cells cocultured with OVA was similar to the levels seen in WT mice.

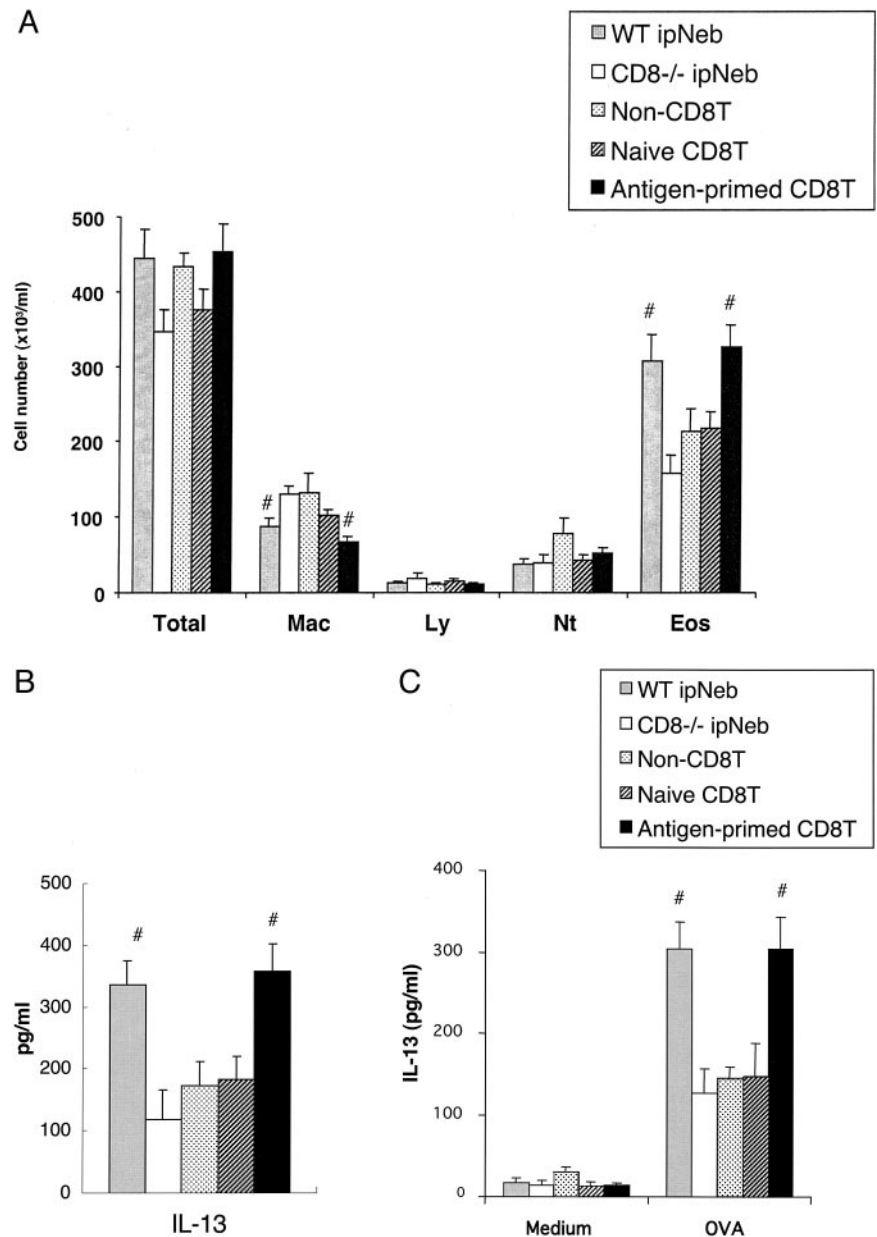
We previously showed that transfer of  $\text{CD8}^+$  T cells into sensitized  $\text{CD8}^{-/-}$  recipient mice resulted in a selective accumulation of  $\text{CD8}^+$  T cells in the PBLN following allergen challenge (20). To determine whether transferred  $\text{CD8}^+$  T cells accumulate in the PBLN in these experiments, analysis of the lymphocyte subsets of MNC preparations derived from PBLN of mice following transfer of Ag-primed  $\text{CD8}^+$  T cells was performed. In the reconstituted recipient  $\text{CD8}^{-/-}$  mice,  $\sim 2\%$  of the  $\text{CD3}^+$  cells were  $\text{CD8}^+$ . These data indicate that transferred Ag-primed  $\text{CD8}^+$  T cells migrated to the PBLN in these mice, which normally lack  $\text{CD8}^+$  cells ( $< 0.1\%$ ).

Fig. 7A shows the results of reconstitution with  $\text{CD8}^+$  T cells on airway responsiveness. Reconstitution of the  $\text{CD8}^{-/-}$  mice with  $\text{CD8}^+$  T cells from IL-13<sup>+/+</sup> mice fully restored the development of AHR (RL), to levels that were comparable with those seen in



**FIGURE 5.** Reconstitution of  $\text{CD8}^{-/-}$  mice with Ag-primed  $\text{CD8}^+$  T cells fully restores the development of AHR comparable with that of WT mice. OVA-sensitized  $\text{CD8}^{-/-}$  mice (recipient mice) received  $5 \times 10^6$   $\text{CD8}^+$  T cells *i.v.* via the tail vein, 2 h before the first airway challenge with aerosolized OVA. Recipient mice were comprised of three groups. In one group, mice received  $\text{CD8}^+$  T cells from spleens of naive WT mice (naive  $\text{CD8}^+$  T group;  $n = 10$ ). In another group, mice received  $\text{CD8}^+$  T cells from OVA-sensitized WT mice (Ag-primed  $\text{CD8}^+$  T group;  $n = 10$ ). In another group, mice received non- $\text{CD8}^+$  T cells from OVA-sensitized mice (non- $\text{CD8}^+$  T group;  $n = 10$ ). WT mice and  $\text{CD8}^{-/-}$  mice receiving no cells are also shown ( $n = 10$  in each group). AHR was monitored by measuring RL (A) and Cdyn (B) as described in *Materials and Methods*. The results for each group are expressed as means  $\pm$  SEM. #, Significant differences ( $p < 0.05$ ) are indicated by Ag-primed  $\text{CD8}^+$  T transferred group vs  $\text{CD8}^{-/-}$  ipNeb group.

**FIGURE 6.** A, Cell composition of BAL fluid after transfer of CD8<sup>+</sup> T cells. Groups are the same as in Fig. 5A. The results for each group are expressed as means  $\pm$  SEM. #, Significant differences ( $p < 0.05$ ) between Ag-primed CD8<sup>+</sup> T transferred mice (Ag-primed CD8<sup>+</sup> T) and WT ipNeb vs CD8<sup>-/-</sup> ipNeb group. B, Cytokine levels in BAL fluid after transfer of CD8<sup>+</sup> T cells. Groups are the same as in Fig. 5A. Cytokine levels were measured in supernatants by ELISA, as described in *Materials and Methods*. The results for each group are expressed as means  $\pm$  SEM. #, Significant differences ( $p < 0.05$ ) between Ag-primed CD8<sup>+</sup> T transferred mice (Ag-primed CD8<sup>+</sup> T) and WT ipNeb vs non-CD8<sup>+</sup> transferred group (non-CD8<sup>+</sup> T), naive CD8<sup>+</sup> T transferred group (naive CD8<sup>+</sup> T), and CD8<sup>-/-</sup> ipNeb group. C, IL-13 levels in culture supernatants from PBLN after transfer of CD8<sup>+</sup> T cells. Cytokine levels after stimulation of cells with OVA were measured in supernatants with ELISA, as described in *Materials and Methods*. The results for each group are expressed as means  $\pm$  SEM ( $n = 8$  in each group). #, Significant differences ( $p < 0.05$ ) between Ag-primed CD8<sup>+</sup> T transferred mice (Ag-primed CD8<sup>+</sup> T) and WT ipNeb vs non-CD8<sup>+</sup> T transferred group (non-CD8<sup>+</sup> T), naive CD8<sup>+</sup> T transferred group (naive CD8<sup>+</sup> T), and CD8<sup>-/-</sup> ipNeb group.



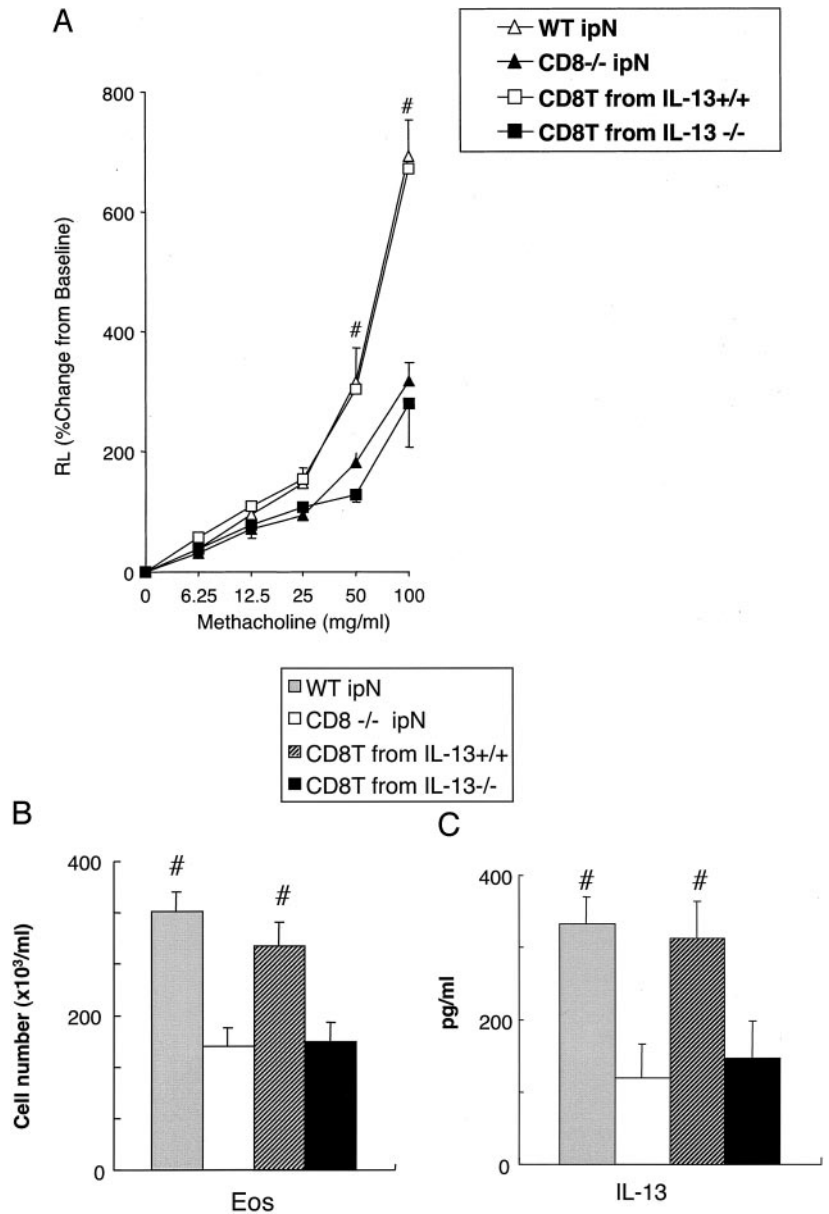
WT mice. In contrast, transfer of IL-13<sup>-/-</sup> CD8<sup>+</sup> T cells on a similar background (129Sv  $\times$  C57BL/6) failed to restore AHR. When Cdyn was monitored, a similar pattern of differences between the groups was observed (data not shown). Transfer of IL-13<sup>-/-</sup> CD8<sup>+</sup> T cells also failed to restore the number of eosinophils and IL-13 levels in the BAL, although transfer of IL-13<sup>+/+</sup> CD8<sup>+</sup> T cells fully restored these responses (Fig. 7, B and C).

#### CD8<sup>+</sup> T cells are a source of IL-13

The two major findings in the CD8<sup>-/-</sup> mice were attenuation of the AHR response and substantially reduced *in vivo* and *in vitro* production of IL-13. To determine whether CD8<sup>+</sup> T cells can serve as a source of IL-13 or indirectly augment IL-13 production via another cell type, we assessed IL-13 production at the single-cell level. MNC from both lung tissue and PBLN were obtained from WT mice following challenge alone or following sensitization and challenge. The cells were stimulated with PI incubated with Abs for intracellular staining of IL-4, IL-5, IFN- $\gamma$ , and IL-13, as described in *Materials and Methods*. The relative proportion of

different T cell subsets staining for the specific cytokines was calculated. In the PBLN from WT mice, intracytoplasmic IL-13 was detected in  $4.0 \pm 0.3\%$  of total CD4 T cells ( $2.4 \pm 0.2\%$  of total CD3 cells) and  $5.1 \pm 0.3\%$  of total CD8<sup>+</sup> T cells ( $2.1 \pm 0.1\%$  of total CD3 cells) following OVA sensitization and challenge. Few, if any, positive cells were identified in the challenged-only mice. IL-13 was detected in  $17.3 \pm 0.7\%$  of total CD8<sup>+</sup> T cells in the lungs of WT mice following OVA sensitization and challenge, with few cells detected following challenge alone (Fig. 8). Fig. 9 summarizes the numbers of cytokine-producing cells in the lungs from WT mice. After sensitization and challenge with OVA, the number of IL-13-producing T cells significantly increased in both CD4 T cells and CD8<sup>+</sup> T cells, when compared with mice challenged alone. These results demonstrate that not only CD4 T cells but also CD8<sup>+</sup> T cells are a source of IL-13, as well as IL-4 and IL-5 following sensitization and challenge with OVA. Similar results were seen when the frequency of cytokine-producing cells in PBLN was examined (data not shown).

**FIGURE 7.** Reconstitution of  $CD8^{-/-}$  mice with Ag-primed  $CD8^{+}$  T cells from IL-13-deficient mice fails to restore the development of AHR. OVA-sensitized  $CD8^{-/-}$  mice (recipient mice) received  $5 \times 10^6$   $CD8^{+}$  T cells i.v. via the tail vein, 2 h before the first airway challenge with aerosolized OVA. Recipient mice were comprised of two groups. In one group, mice received  $CD8^{+}$  T cells from spleens of OVA-sensitized IL-13-sufficient mice ( $CD8^{+}$  T from IL-13 $^{+/+}$  group;  $n = 6$ ). In another group, mice received  $CD8^{+}$  T cells from OVA-sensitized IL-13-deficient mice ( $CD8^{+}$  T from IL-13 $^{-/-}$  group;  $n = 6$ ). WT mice and  $CD8^{-/-}$  mice receiving no cells are also shown ( $n = 8$  in each group). AHR was monitored by measuring RL (A) as described in *Materials and Methods*. The results for each group are expressed as means  $\pm$  SEM. #, Significant differences ( $p < 0.05$ ) are indicated by  $CD8^{+}$  T from IL-13 $^{-/-}$  group and  $CD8^{-/-}$  ipNeb group vs WT ipNeb group and  $CD8^{+}$  T from IL-13 $^{+/+}$  group. B, Number of eosinophils in BAL fluid after transfer of  $CD8^{+}$  T cells. Groups are the same as in Fig. 7A. The results for each group are expressed as means  $\pm$  SEM. #, Significant differences ( $p < 0.05$ ) are indicated by  $CD8^{+}$  T from IL-13 $^{-/-}$  group and  $CD8^{-/-}$  ipNeb group vs WT ipNeb group and  $CD8^{+}$  T from IL-13 $^{+/+}$  group. C, Levels of IL-13 in BAL fluid after transfer of IL-13 $^{-/-}$   $CD8^{+}$  T cells. Groups are the same as in Fig. 7A. Cytokine levels were measured in supernatants by ELISA, as described in *Materials and Methods*. The results for each group are expressed as means  $\pm$  SEM. #, Significant differences ( $p < 0.05$ ) are indicated by  $CD8^{+}$  T from IL-13 $^{-/-}$  group and  $CD8^{-/-}$  ipNeb group vs WT ipNeb group and  $CD8^{+}$  T from IL-13 $^{+/+}$  group.



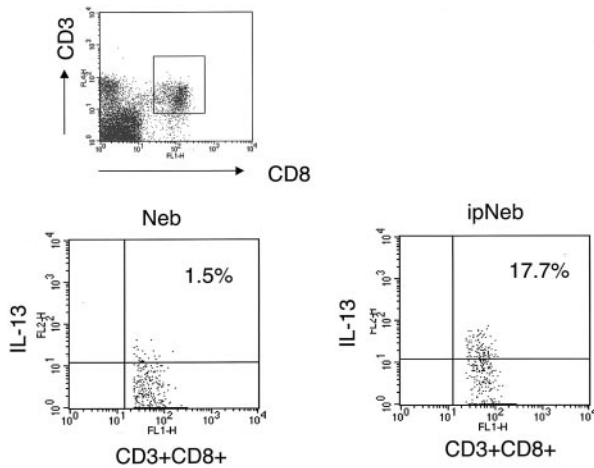
## Discussion

In some previous studies of allergic inflammation and AHR, the mobilization of  $CD8^{+}$  T cells into the lungs of human subjects and in animal models has led to the assumptions that  $CD8^{+}$  T cells might play a suppressive role (15–19). In the present study, however, we arrived at a somewhat different conclusion. We implicated a critical role for  $CD8^{+}$  T cells in the full development of AHR and airway inflammation. Using  $CD8^{-/-}$  mice, targeted for *CD8 $\alpha$* , allergen-induced AHR and airway inflammation were significantly reduced compared with WT mice in the absence of  $CD8^{+}$  T cells. The failure to fully develop AHR and lung inflammation in these mice was not apparently due to abnormalities in pulmonary structure or to consequences of deletion of *CD8 $\alpha$*  on other cell types, because AHR was reconstituted in the deficient mice following adoptive transfer of primed  $CD8^{+}$  T cells.

One of the notable findings in the present study is that  $CD8^{+}$  T cells contribute to AHR and eosinophilic inflammation, perhaps through IL-13. There is now increasing evidence of Th2-like  $CD8^{+}$  T cells in humans and mice (20–23, 31–33). In a different model, Hamelmann et al. (20) previously showed that  $CD8^{+}$  T

cells produce IL-5 after exposure to OVA exclusively via the airways, in the absence of adjuvant. In that study, depletion of  $CD8^{+}$  T cells prevented the development of AHR, detected by electrical field stimulation. AHR was fully restored by reconstitution of  $CD8^{+}$  T cells, and  $CD8^{+}$  T cells were shown to be a source of IL-5. In the present study, using sensitized (allergen with adjuvant) and challenged mice, no differences in IL-5 levels were found in the BAL fluid of  $CD8^{-/-}$  and WT mice, but a significant reduction in IL-13 levels in the BAL fluid and culture supernatants from stimulated PBLN cells in the  $CD8^{-/-}$  mice was seen. When  $CD8^{-/-}$  mice were reconstituted with primed (but not naive)  $CD8^{+}$  T cells, the transferred  $CD8^{+}$  T cells were detected in the PBLN of recipient mice, and the levels of IL-13 from stimulated PBLN cells and in the BAL fluid were fully restored. This accumulation of functional  $CD8^{+}$  T cells in the PBLN of recipient mice confirms previous results and emphasizes that challenge of the sensitized mice is a necessary prerequisite for this response to occur (20). The ability of activated  $CD8^{+}$  T cells from the lung as well as PBLN cells to synthesize IL-13 was confirmed at the single-cell level by intracellular cytoplasmic staining. This ability of



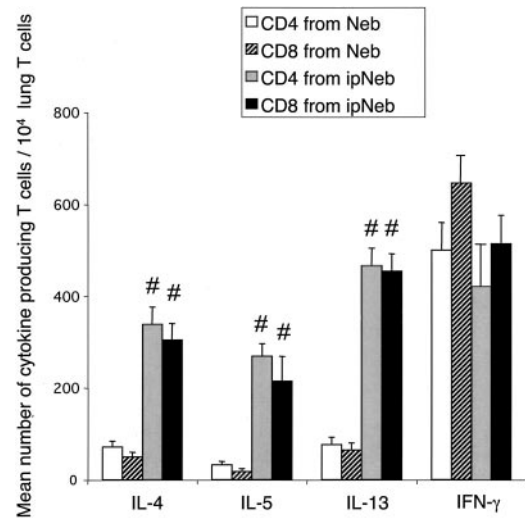


**FIGURE 8.** Intracellular detection of IL-13 protein in lung CD8<sup>+</sup> T cells from challenged-only WT mice and WT mice following sensitization and challenge with OVA. Lung MNC were isolated and stimulated with PI, and then cells were fixed, permeabilized, and stained with anti-mouse IL-13 Ab, as described in *Materials and Methods*. CD3<sup>+</sup>CD8<sup>+</sup> cells were gated, and analyzed for intracellular detection of IL-13 protein. Quadrants were set based on isotype control staining. The data shown are representative of three independent experiments.

CD8<sup>+</sup> T cells to produce IL-13 has been previously reported in human T cell clones that were specific for EBV (31) and recently in patients with atopic dermatitis (32). In atopic dermatitis patients, CD8<sup>+</sup> T cells responded to a superantigen and produced IL-13 as well as IL-5; in fact, the CD8<sup>+</sup> T cells were the major source of IL-13 in this in vitro culture system. As shown in this study, lung CD8<sup>+</sup> T cells were also capable of synthesizing IL-4, IL-5, and IFN- $\gamma$ . Furthermore, reconstitution of CD8<sup>-/-</sup> mice with IL-13<sup>-/-</sup> CD8<sup>+</sup> T cells failed to restore AHR and airway inflammation, demonstrating that CD8<sup>+</sup> T cells can contribute to the development of AHR and airway inflammation, at least in part through IL-13 production.

Because CD8<sup>-/-</sup> mice were developed by targeting *CD8 $\alpha$* , and dendritic cells are one of the specialized migratory APCs (34), the function of dendritic cells that express CD8 $\alpha$  could be impaired in these deficient mice. Similarly,  $\gamma\delta$  T cells, which may contribute to the development of allergen-induced AHR and inflammation (35), may also express CD8 $\alpha$ . When CD8<sup>+</sup> T cells were purified before transfer into CD8<sup>-/-</sup> recipients, the numbers of CD3<sup>-</sup>CD8<sup>+</sup> or CD11c<sup>+</sup> cells were negligible. These data indicate that the effects of contaminating non-CD8<sup>+</sup> T cells, such as NK or dendritic cells, should not have been a factor in the reconstitution experiments. However, it is virtually impossible to totally exclude such a possibility. A similar argument holds for potential contamination by  $\gamma\delta$  T or NK T cells, which were not detected after CD8<sup>+</sup> T cell purification. Based on these assumptions, it appears that the reduced AHR and airway inflammation in the CD8<sup>-/-</sup> mice was the consequence of the lack of  $\alpha\beta$ /CD8<sup>+</sup> T cells. Following sensitization and challenge with OVA, Ag-specific MNC proliferative responses, as well as cytokine responses, other than IL-13, in CD8<sup>-/-</sup> mice were similar to those seen in the WT mice. These results indicate similar T cell responsiveness to specific Ag with or without CD8<sup>+</sup> T cells. Furthermore, these results indicate that the function of APCs remained intact, even without expression of CD8 $\alpha$ .

CD8<sup>-/-</sup> mice showed lower eosinophil numbers compared with WT mice, although the levels of IL-5 in the BAL fluid were no different than in the WT mice. IL-13 levels in the BAL fluid of the



**FIGURE 9.** Numbers of cytokine-producing CD4 T cells and CD8<sup>+</sup> T cells per 10<sup>4</sup> lung T cells from challenged-only WT mice and WT mice following sensitization and challenge with OVA. Lung MNC were isolated and stimulated with PI, and then cells were fixed, permeabilized, and stained with PE-conjugated rat anti-mouse IL-4, IL-5, and IFN- $\gamma$ , and biotin-conjugated goat anti-mouse IL-13 Ab, as described in *Materials and Methods*. Expressed are means  $\pm$  SEM from three independent experiments ( $n = 8$ ). #, Significant differences ( $p < 0.05$ ) between CD4 from Neb group and CD8<sup>+</sup> from Neb group vs CD4 from ipNeb group and CD8<sup>+</sup> from ipNeb group.

CD8<sup>-/-</sup> mice were significantly reduced compared with WT mice. It has been reported that administration of IL-13, or overexpression of IL-13 in the airways, induces airway eosinophilia (4, 36, 37). In contrast, Wills-Karp et al. (36) have reported that IL-13 antagonists have little inhibitory effect on eosinophilia. Thus, the relation of eosinophilic inflammation and IL-13 is controversial. In the present study, transfer of IL-13<sup>+/+</sup> but not IL-13<sup>-/-</sup> CD8<sup>+</sup> T cells restored eosinophilic inflammation, suggesting that the absence of IL-13 production from CD8<sup>+</sup> T cells might reduce eosinophilic inflammation in CD8<sup>-/-</sup> mice. IL-13 also has been shown to be essential to mucus hyperproduction and goblet cell hyperplasia (29, 37, 38). In view of the significantly reduced levels of IL-13 in the CD8<sup>-/-</sup> mice, it was initially surprising that the numbers of goblet cells were reduced by only 50% compared with WT mice. As suggested by Whittaker et al. (39), the lower levels of IL-13 in BAL fluid from CD8<sup>-/-</sup> mice may have been sufficient to trigger mucus production, but not other IL-13-dependent processes.

The results of the present study do contrast with some previous reports demonstrating the ability of CD8<sup>+</sup> T cells to inhibit Th2-type cytokine production, eosinophilic inflammation, and AHR. In rat models of allergic sensitization, depletion of CD8<sup>+</sup> T cells enhanced late airway responses (15) and AHR (16), and transfer of Ag-primed CD4 but not CD8<sup>+</sup> T cells into naive recipients elicited AHR (8). In the CD8<sup>+</sup> depletion studies in rats, depletion was performed only after completion of sensitization, and the role of CD8<sup>+</sup> T cells during sensitization was not addressed (15, 16). In addition, the depleting Ab, anti-CD8 (OX-8), may have depleted not only CD8<sup>+</sup> T cells but also CD8<sup>+</sup>/NK cells, which are one of the major sources of IFN- $\gamma$  and potentially IL-13 (40–42). Depletion of non-T CD8<sup>+</sup> cells such as  $\gamma\delta$  or NK cells after sensitization may have influenced their results. In their transfer experiments of CD8<sup>+</sup> T cells, the recipient mice were naive (7–9); AHR was not affected. This is similar to our results, because nonsensitized recipient CD8<sup>-/-</sup> mice, which received Ag-primed CD8<sup>+</sup> T cells and were subsequently challenged with OVA, did not develop

AHR. These data suggest that Ag-primed CD8<sup>+</sup> T cells on their own have limited effectiveness, but perhaps following interactions with other Ag-primed cells (e.g., CD4 T cells) can elicit AHR, and increase IL-13 synthesis. CD4 T cells may be required for CD8<sup>+</sup> T cell activation following viral infection (43–45), and the same mechanism may be operative in allergen-induced AHR through CD8<sup>+</sup> T cells.

Suzuki et al. (17) reported that transfer of Ag-primed CD8<sup>+</sup> T cells suppressed late airway responses and mRNA expression of Th2-type cytokines in the BAL fluid of rats. In their model, recipient rats had CD8<sup>+</sup> T cells that were primed with Ag, and then mice received additional CD8<sup>+</sup> T cells. It has also been shown that in vivo depletion of CD8<sup>+</sup> T cells by mAb, enhanced OVA-specific IgE in rats (15). In the absence of CD8<sup>+</sup> T cells (CD8<sup>-/-</sup> mice), there was no effect on total IgE or OVA-specific Ig levels after sensitization and challenge with OVA. Direct comparison of the results of these studies is difficult because of the different protocols used. The different routes of sensitization, different adjuvants, species differences, as well as differences in recipient status may all play a role in dictating the effect of transferred Ag-primed CD8<sup>+</sup> T cells. It is also possible that distinct populations or different functional stages of  $\alpha\beta^+$ /CD8<sup>+</sup> T cells may exist, some of which suppress Th2 responses and others of which enhance Th2 responses by an as-yet-unknown mechanism. Two memory CD8<sup>+</sup> T cell subsets, central and effector, have also been recently described and may show polarized responses (46). The different approaches and experimental designs may affect the polarization of distinct CD8<sup>+</sup> T subpopulations and, as a result, affect IgE or cytokine production, airway responsiveness, and airway inflammation.

IL-13 plays a central role in the development of AHR (4, 5, 29, 36, 47). Several cell types produce IL-13, including Th2-type cells, mast cells, airway smooth muscle cells, NK cells, and NK T cells; some have been implicated in the development of AHR (41, 42, 48, 49). Although we have shown that CD8<sup>+</sup> T cells produce IL-13, it is also possible that CD8<sup>+</sup> T cells may not only produce IL-13 but also induce another cell type to release IL-13 for the full development of AHR. Under such circumstances, perhaps small numbers of activated Ag-specific CD8<sup>+</sup> T cells can amplify IL-13 release through other cell types.

The pathway by which CD8<sup>+</sup> T cells are activated after allergen exposure is not clear. Exogenous proteins such as OVA are usually presented in association with MHC class II molecules and CD4 T cells, not MHC class I molecules, which are usually required for activation of CD8<sup>+</sup> T cells. Hence, CD8<sup>+</sup> T cells are not expected to be activated by OVA challenge in allergic airway inflammation. However, there are studies suggesting a leak between class I and class II pathways, at least in APC (50, 51). It has also been reported that OVA-specific, MHC I-restricted CD8<sup>+</sup> T cells are inducible in vivo by OVA sensitization in conjunction with certain adjuvants (51, 52).

It has been reported that  $\beta_2$ -microglobulin<sup>-/-</sup> mice that have no class I-restricted cells, including NK T cells (NK1.1<sup>+</sup>CD4<sup>+</sup> T cells) and CD8<sup>+</sup> T cells, develop AHR and eosinophilic inflammation (53, 54), suggesting that NK T cells or CD8<sup>+</sup> T cells are not required for these responses. Recently, however, it has been shown that NK T cells are essential for the development of AHR and eosinophilic inflammation (42, 55). This suggests the possibility that CD8<sup>+</sup> T cells or NK T cells might be activated, in part, independent of the class I pathway. It has been shown that CD8 $\alpha\alpha^+$  T cells do not need the class I molecule for Ag presentation (56–58). This might account for the differences between  $\beta_2$ -microglobulin-deficient mice and CD8-deficient mice. These CD8 $\alpha\alpha^+$  T cells, which do not exist in CD8-deficient mice, might

play some role in the  $\beta_2$ -microglobulin-deficient mice in allergen-related phenomena. In the present study, transfer of CD8<sup>+</sup> T cells from OVA-sensitized (but not nonsensitized) mice could restore AHR, suggesting that CD8<sup>+</sup> T cells could be activated by exogenous Ag and contribute to allergen-induced AHR and eosinophilic inflammation. The mechanism(s) underlying how CD8<sup>+</sup> T cells recognizes exogenous allergen leading to a polarized Tc2-like response remains to be defined.

It has been reported that CD4-deficient mice have abundant CD4<sup>+</sup>CD8<sup>-</sup> $\alpha\beta^+$  T cells, and these cells are able to control *Leishmania* infections (59). Therefore, it may be assumed that, in the CD8<sup>-/-</sup> mice, there are non-CD4/nonCD-8 T cells that can exhibit cytotoxic/suppressor effects that could possibly affect Th2 responses in the CD8<sup>-/-</sup> mice. However, it has been shown that these CD8<sup>-/-</sup> mice have no such T cell population, no abnormal CD4<sup>+</sup>CD8<sup>-</sup> $\alpha\beta^+$  T cell population, and no differences in T cell subsets in spleen and PBLN cells (26, 60), which was confirmed in our experiments (data not shown).

In summary, we have identified a critical role for Ag-primed CD8<sup>+</sup> T cells in allergen-induced AHR and airway inflammation, and have shown that CD8<sup>+</sup> T cells may be required for the full development of these responses. Our studies indicate that Ag-primed CD8<sup>+</sup> T cells can produce IL-13 and actively regulate AHR and airway inflammation. These data support a pathogenic role for the increased number of CD8<sup>+</sup> T cells observed in asthma and suggest that CD8<sup>+</sup> T cell activity may be one of the factors governing the development and maintenance of AHR and allergic eosinophilic airway disease.

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