

Vaccine-Induced CD8⁺ T Cell-Dependent Suppression of Airway Hyperresponsiveness and Inflammation^{1,2}

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Suppressing the abnormalities associated with asthma has been difficult to accomplish using immunotherapy or vaccination once the disease is established. The effector cells necessary for effective immunization/vaccination and immunotherapy of asthma are also not well understood. Therefore, we vaccinated allergen (OVA)-sensitized mice to determine whether therapeutic immunization could suppress airway hyperresponsiveness (AHR) and inflammation and to identify key immune effector cells and cytokines. Mice were immunized with a vaccine comprised of Ag and cationic liposome-DNA complexes (CLDC), a vaccine which has previously been shown to elicit strong CD4⁺ and CD8⁺ T cell responses and activation of Th1 immunity. We showed that immunization with the OVA-CLDC vaccine significantly suppressed AHR, eosinophilia, goblet cell metaplasia, and Th2 cytokine production. In contrast, immunization with CLDC alone suppressed eosinophilia and Th2 cytokine production, but failed to suppress AHR and goblet cell changes. Using adoptive transfer experiments, we found that suppression of AHR was mediated by Ag-specific CD8⁺ T cells and was dependent on IFN- γ production by the transferred T cells. Thus, we conclude that generation of strong, allergen-specific CD8⁺ T cell responses by immunization may be capable of suppressing AHR and allergic airway inflammation, even in previously sensitized and challenged mice. *The Journal of Immunology*, 2009, 183: 181–190.

The increasing incidence of morbidity and mortality associated with asthma is a major concern in this country, particularly among certain ethnic groups (1, 2). Despite improvements in the management of asthma and the wider use of drugs such as inhaled corticosteroids, there is still a major need for more effective approaches to the treatment of patients with asthma (3). Currently, treatment with available medications may still not prevent acute exacerbations or the long-term loss of lung function associated with asthma (4, 5). New therapeutic approaches have been evaluated, based on advances in our knowledge of the pathophysiology of the disease, but few have demonstrated consistent efficacy in clinical trials (6).

Immunotherapy with DNA enriched in CpG dinucleotide sequences as adjuvants, including CpG oligonucleotides and plasmid DNA, has been evaluated for the treatment of allergic diseases, including asthma. The rationale for this approach has been that unmethylated CpG oligodeoxynucleotides are known to be potent inducers of Th1 immune responses, which may suppress the Th2 dominant cytokine pattern associated with asthma (7, 8). For example, short unmethylated immunostimulatory sequences contain-

ing unmethylated CpG oligodeoxynucleotides have been evaluated in models of allergic disease and in small-scale clinical trials (9, 10) and studies have shown that vaccines prepared using immunostimulatory sequences and protein Ags have been of some benefit in the treatment of cancer, infection, and allergic disease (11).

Recent studies have demonstrated that combining cationic liposomes with DNA or RNA nucleic acids significantly increases the potency of activation of innate immunity (12). Moreover, cationic liposomes and CpG-containing nucleic acid complexes combined with Ags can function as effective vaccine adjuvants, particularly with respect to cross-priming CD8⁺ T cell responses to protein Ags (13). For example, Zaks et al. (14) demonstrated that immunization with cationic liposome-DNA complexes (CLDC)⁴ and protein Ags induced a marked expansion of Ag-specific T cells capable of controlling the growth of tumors and *Mycobacterium tuberculosis*.

The pathogenesis of asthma is complex and previous studies have emphasized the role of Th2 CD4⁺ T cells as the primary effector cells responsible for secreting pro-allergic cytokines such as IL-4, IL-5, IL-9, and IL-13 (15). However, it is now recognized that CD8⁺ T cells also play an important role in the pathogenesis of asthma and the development of airway hyperresponsiveness (AHR) and allergic airway inflammation, which is mediated in part by CD8⁺ T cell secretion of Th2-type cytokines (5, 16, 17). Yet, in other studies, CD8⁺ T cells have been shown to have a regulatory or suppressive effect on lung allergic responses through secretion of Th1 cytokines such as IL-12 and IFN- γ (18, 19). Thus, CD8⁺ T cells appear to exhibit functional plasticity in vivo and can alter their phenotype depending on local conditions (20). Therefore, we hypothesized that it might be possible to suppress

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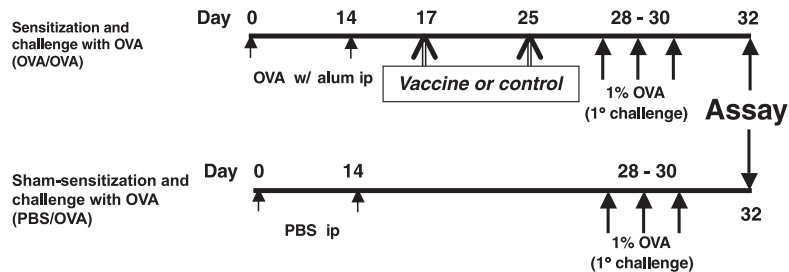
² The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

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⁴ Abbreviations used in this paper: CLDC, cationic liposome-DNA complex; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; MCh, methacholine; PAS, periodic acid-Schiff; RL, lung resistance; WT, wild type.

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Primary Allergen Challenge Model



Secondary Allergen Challenge Model

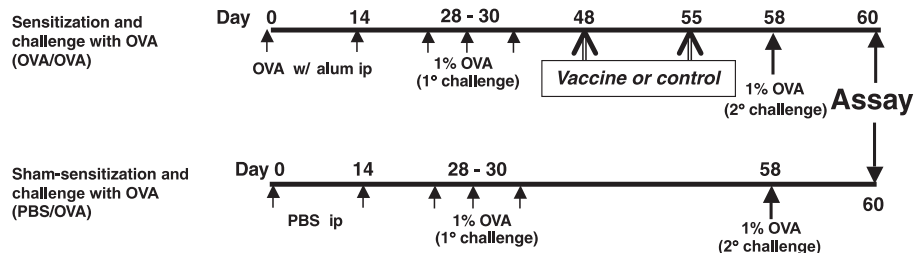


FIGURE 1. Experimental protocol. In the primary allergen challenge model, mice were sensitized by i.p. injections of OVA with alum on days 1 and 14. On days 28–30, mice were challenged via the airways with OVA (primary allergen challenge). In the secondary allergen challenge model, mice were sensitized and challenged with OVA (primary allergen challenge) followed by a single (secondary) allergen challenge on day 58. Control groups of mice were sham sensitized with PBS and all mice were assayed 48 h after the last allergen challenge. Vaccine or control treatments were administered 5 and 12 days before the last experimental day.

the functional and immunological abnormalities associated with asthma by using immunization/vaccination to induce high numbers of Th1-biased Ag-specific CD8⁺ T cells in the lungs and airways of allergen-sensitized and -challenged mice. In the studies described here, we immunized allergen-sensitized mice with a CLDC-based vaccine and assessed the effects on immunological responses in the lungs and airways on allergen challenge. To investigate the number and function of Ag-specific CD8⁺ T cells in vivo, we used Ag-conjugated K^b tetramer and C57BL/6 mice. The critical cellular and cytokine mediators of vaccine-induced responses were also investigated. The results of these studies suggest that allergen-CLDC vaccination to induce allergen-specific CD8⁺ T cells may have therapeutic effectiveness in patients with asthma.

Materials and Methods

Animals

Female C57BL/6 (wild-type (WT)) mice from 6 to 8 wk of age were obtained from Harlan. IFN- γ -deficient mice (IFN- γ ^{-/-}) were obtained from Dr. P. Marrack (National Jewish Health, Denver, CO) and were bred in the animal facility at National Jewish Health. The animals were maintained on an OVA-free diet. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Sensitization and airway challenge

To investigate the effects of vaccination, two distinct experimental protocols were followed to trigger allergen-induced airway inflammation and AHR. In the first protocol (primary allergen challenge; Fig. 1), mice were sensitized by i.p. injection of 20 μ g of OVA (grade V; Sigma-Aldrich) emulsified in 2.0 mg of alum (AlumImject; Pierce) in a total volume of 100 μ l on days 1 and 14. Mice were challenged via the airways with OVA (1% in saline) for 20 min on days 28–30 using an ultrasonic nebulizer (model NE-07; Omron Healthcare). In the secondary allergen challenge protocol, modified from a previous study (21), mice were sensitized and challenged (primary challenge) to OVA as above and then received a single (secondary) OVA challenge (1% in saline) for 20 min on day 58 following the return of all responses to the primary challenge to baseline; AHR to methacholine and eosinophilia in bronchoalveolar lavage (BAL) fluid were no longer detected. Control mice were sensitized with PBS followed by OVA challenge in the same way. In all groups, assays were conducted 2 days after the last allergen challenge.

DNA, liposome, and Ag protein preparation and administration

CLDC were prepared as previously described (14). Briefly, non-coding low-endotoxin content plasmid DNA was purchased from Althea Technol-

ogies (pMB75.6). In brief, 1,2-dioleoyl-3-trimethylammonium-propane (Avanti Polar Lipids) and cholesterol (Sigma-Aldrich) were mixed in a 1:1 molar ratio. Cationic lipids were mixed with DNA at a ratio of 32 nmol of total lipid to 1.0 μ g of DNA, to a final concentration of 100 μ g of DNA per ml in a sterile solution of 5% dextrose in water. Five micrograms of Ag protein were mixed with 20 μ g of CLDC immediately before immunization and administered by the i.p. route. Sensitized mice were immunized with a vaccine on days 20 and 27 before airway challenge with OVA. In the secondary allergen challenge protocol, mice received a vaccine on days 48 and 55 before allergen challenge. Cationic liposomes or CLDC without Ag were administered as a control.

Determination of airway responsiveness

Airway responsiveness was measured as the change in lung resistance (RL) after exposure to increased concentrations of aerosolized methacholine (MCh; Sigma-Aldrich). Mice were anesthetized, tracheostomized, and mechanically ventilated, and lung function was assessed as described previously (22). RL was continuously monitored for up to 3 min after aerosolized MCh exposure. The data of RL were continuously collected. Maximum values of RL were taken to express changes in airway function. Baseline values (saline) for RL were not significantly different among the groups.

BAL and measurement of cytokines in BAL fluid

Immediately after assessment of airway responsiveness, lungs were lavaged via the tracheal tube with HBSS (1 $\times$ 1 ml, 37°C). The number of leukocytes was counted (Coulter Counter) and differential cell counts were performed by counting at least 200 cells on cytocentrifuged preparations in a blinded manner (Cytospin 3; Shandon). Slides were stained with Wright-Giemsa and differentiated by standard hematological procedures. Cytokine levels in the BAL fluid were measured by ELISA as previously described (23). Briefly, measurements of IL-10, IL-12, IFN- γ , IL-4, and IL-5 were performed using OptEIA sets (BD Pharmingen). IL-13 measurements were performed using an ELISA kit (QuantikineM; R&D Systems). All followed the manufacturers' protocol. The limits of detection were 1.5 pg/ml for IL-13, 4 pg/ml for IL-4 and IL-5, and 10 pg/ml for IL-10, IL-12, and IFN- γ .

Histological studies

After obtaining BAL fluid, the right lung was inflated with 1 ml of 10% formalin through the trachea and fixed in formalin by immersion. Tissue blocks of lung tissue from four to five mice in each group were cut from around the main bronchus and embedded in paraffin blocks; two to three tissue sections (5 μ m) per mouse were then affixed to microscope slides and deparaffinized. The slides were then stained with H&E or periodic acid-Schiff (PAS). The slide images were captured using a microscope (BX40; Olympus America) equipped with a digital camera (Q-color 3; Olympus America) and images were stored on a Macintosh computer.

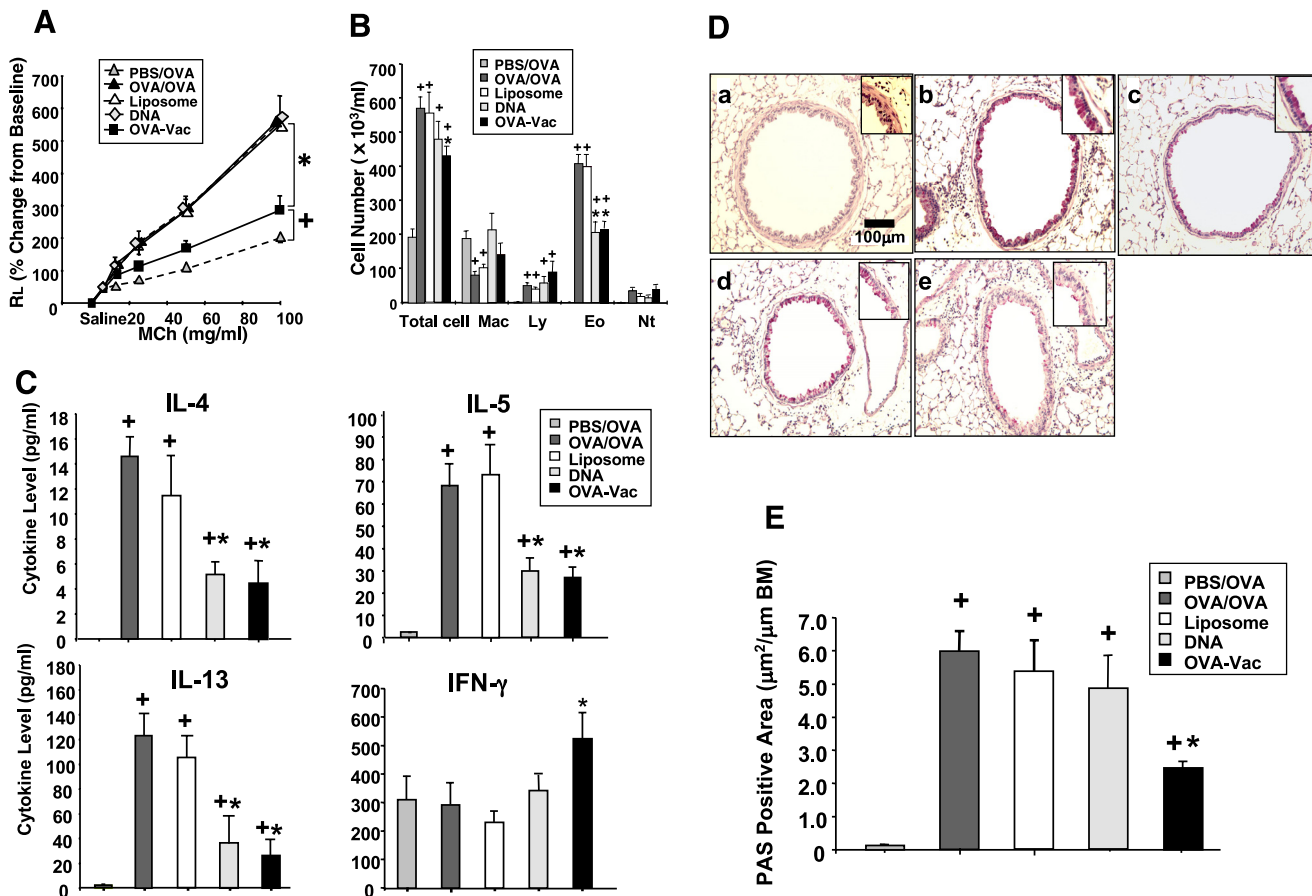


FIGURE 2. Effects of immunization on airway responses to allergen challenge in previously sensitized mice. Mice were sensitized and challenged with OVA (OVA/OVA) or received airway challenge with OVA without prior sensitization (PBS/OVA). Mice were immunized after they were sensitized but before airway challenge with OVA as described in *Materials and Methods*. Animals were immunized with the OVA-CLDC vaccine (OVA-Vac), CLDC (DNA), or liposomes alone (liposome). Following sensitization, AHR, cytokine, and cellular responses in the lungs of mice were assessed as described in *Materials and Methods*. **A**, The effects of immunization on RL in response to inhaled MCh (AHR) are shown. **B** and **C**, The effects of immunization on the cellular composition and cytokine concentrations in BAL fluid are illustrated, respectively. **D**, Histological responses in the lungs of immunized and control mice are presented, including a nonimmunized mouse that did not undergo allergen sensitization (**a**), a vaccinated mouse sensitized and challenged with OVA (**b**), a mouse immunized with liposomes alone (**c**), a mouse immunized with CLDC alone (**d**), and a mouse immunized with OVA-CLDC (**e**). **E**, Quantitative image analysis of PAS⁺ areas on lung histological sections are shown. Mac, Macrophages; Ly, lymphocytes; Eo, eosinophils; Nt, neutrophils. The results for each group are expressed as mean $\pm$ SEM ($n = 8$). Significant differences are shown as *, $p < 0.05$ compared with nonimmunized OVA/OVA mice or liposome-treated mice. +, $p < 0.05$ for PBS/OVA as assessed by ANOVA.

Goblet cell metaplasia was quantified as the number of pixels on the computer converted from PAS⁺ areas along the airway epithelium. The quantification was performed using NIH ImageJ software (version 1.38), available on the internet at <http://rsb.info.nih.gov/ij/download.html>. Six to eight different fields per slide in four to six samples from each group of mice were examined in a blinded manner.

Flow cytometric analysis

To determine the numbers of OVA-specific CD8⁺ T cells in the airways induced by the Ag-DNA-liposome vaccine, left lungs were taken after BAL was obtained. Lung cells were isolated on a 35% Percoll gradient (Sigma-Aldrich) after collagenase digestion as described previously (23) and stained with anti-CD8-allophycocyanin (BD Pharmingen) and PE-conjugated OVA-specific K^b tetramer. K^b tetramer production and staining were performed as previously described (24). To investigate IFN- γ production by OVA-specific CD8⁺ T cells, intracellular cytokine staining was performed. Lung cells were isolated as described above and stimulated for 4 h with the peptide SIINFEKL (10 μ M) or PMA (5 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (10 μ g/ml). Then cells were stained for anti-CD8-FITC and PE-OVA-K^b tetramer, followed by fixation and permeabilization with a Cytotfix/Cytoperm Plus Kit (BD Pharmingen). The permeabilized cells were incubated with anti-IFN- γ -allophycocyanin (BD Pharmingen) or a similarly labeled isotype-matched control Ab for 30 min. The stained cells were then washed twice and resuspended in PBS.

All flow cytometric analyses were performed using a FACSCaliber (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

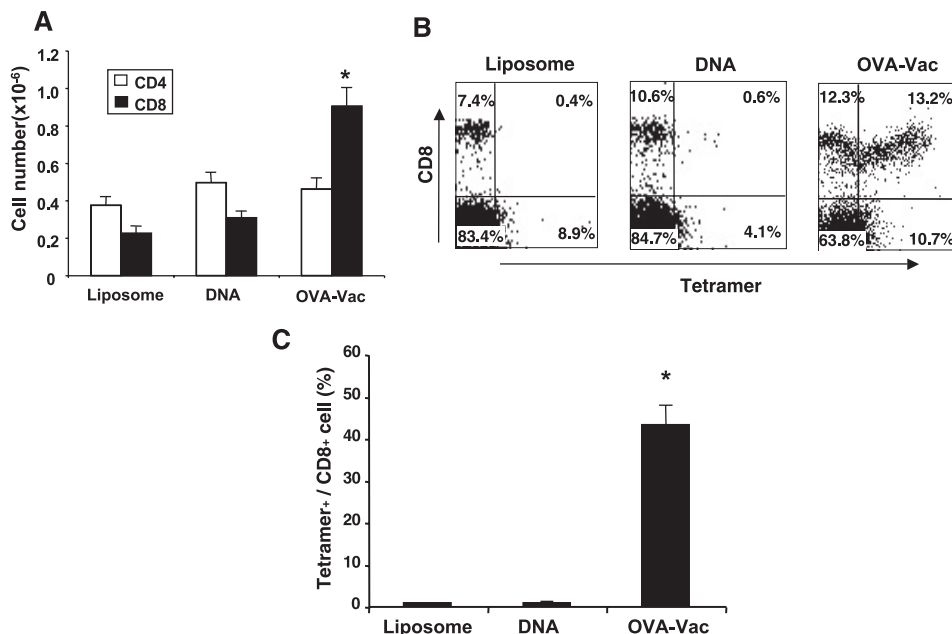
Adoptive transfer of CD8⁺ T cells from OVA-CLDC-treated mice

Lungs of C57BL/6 or IFN- γ ^{-/-} mice immunized with OVA-CLDC were removed, lung cells were isolated as described above, and CD8⁺ T cells were purified by negative selection using immunomagnetic cell-sorting (MACS; Miltenyi Biotec). The purity of the isolated population was routinely >95% CD8⁺ T cells. The cells that were retained on the column were also collected for the cell transfer and used as control (non-CD8⁺ T cells; <5% CD8⁺). Recipient mice were sensitized twice with OVA plus alum on days 1 and 14. Isolated CD8⁺ T cells (5×10^6) or non-CD8⁺ T cells as a control were injected via the tail vein into OVA-sensitized recipient mice followed by three allergen challenges via the airways on days 28–30. Assays of lung function and immunological responses were conducted on day 32.

Statistical analysis

All results were expressed as the mean $\pm$ SEM. The *t* test was used to compare differences between two groups, while ANOVA and the Tukey-Kramer multiple-means comparison test were used for comparisons between three or more groups. Statistical analyses using nonparametric analysis (Mann-Whitney *U* test or Kruskal-Wallis test) were also performed to

FIGURE 3. Effects of immunization on the numbers of CD8⁺ and CD4⁺ T cells in the lungs of OVA-sensitized and -challenged mice. Cells were isolated from the left lung lobes of OVA-sensitized and -challenged mice ($n = 8/\text{group}$) that were immunized with OVA-CLDC (OVA-Vac), CLDC alone (DNA), or liposomes alone (liposome) as described in *Materials and Methods*. **A**, The mean number of total CD4⁺ and CD8⁺ T cells per lung lobe is shown, as assessed by flow cytometry. **B**, A representative dot plot of OVA-specific CD8⁺ T cells in the lung of vaccinated and nonimmunized mice is shown. **C**, The mean percentage ($\pm$ SEM) of OVA-specific CD8⁺ T cells in immunized and nonimmunized mice. *, $p < 0.05$ compared with liposome and DNA.



confirm that the differences remained significant. The p values for significance were set to 0.05 for all tests with statistical software (JMP; SAS Institute).

Results

Modulation of airway responses following OVA-CLDC immunization

In the model used in these studies, OVA sensitization and inhalational challenge (OVA/OVA) resulted in significant increases in airway responsiveness, increased numbers of airway eosinophils, increases in IL-4, IL-5, and IL-13 levels in BAL fluid, and increased goblet cell metaplasia (Fig. 2). To assess the effects of immunization, mice were sensitized with OVA via the i.p. route, immunized, then subjected to inhalational challenge with OVA. The immunization groups included mice immunized with OVA/CLDC, with CLDC alone, or with cationic liposomes alone.

Following immunization with OVA-CLDC, development of AHR was significantly reduced (Fig. 2A). In addition, immunization with OVA-CLDC also significantly reduced airway eosinophilia and goblet cell metaplasia (Fig. 2, B, D, and E). Moreover, production of IL-13, IL-4, and IL-5 was also significantly suppressed in BAL fluid obtained from OVA-CLDC-immunized mice compared with untreated control animals (Fig. 2C). Concurrently, BAL concentrations of IFN- γ were significantly increased in OVA/CLDC-immunized mice compared with control mice.

In contrast, immunization with CLDC alone or liposomes alone failed to suppress AHR or goblet cell metaplasia in sensitized mice (Fig. 2, A, D, and E). However, immunization with CLDC alone did significantly suppress airway eosinophilia as well as production of IL-4, IL-5, and IL-13 in BAL fluid (Fig. 1C). Concentrations of IFN- γ were not however increased in BAL of mice immunized with CLDC alone. Administration of liposomes alone had no effect on any of the parameters measured. Thus, suppression of AHR, goblet cell metaplasia, and production of IFN- γ following OVA-CLDC immunization was an Ag-specific response, whereas suppression of Th2 cytokines and eosinophil infiltration appeared to result from nonspecific effects elicited by the CLDC immunization.

Vaccination induces expansion in numbers of OVA-specific CD8⁺ T cells in the lungs

Experiments were first conducted to assess the effects of immunization on T cell responses in the lungs and airways. Lung mononuclear cells were isolated from immunized and control mice and the numbers of CD4⁺ and CD8⁺ T cells were determined by flow cytometry. We found that the numbers of CD8⁺ T cells in the lungs were significantly increased in OVA/CLDC-immunized mice compared with untreated but sensitized mice (Fig. 3A). However, the number of CD4⁺ cells was not increased in the lungs of immunized mice compared with controls. In addition, administration of CLDC alone or liposomes alone did not alter the numbers of CD8⁺ T cells or CD4⁺ T cells in the lungs.

Next, the Ag specificity of lung CD8⁺ T cells was assessed using tetramer staining. As shown in Fig. 3, B and C, the numbers of OVA-specific CD8⁺ T cells were markedly increased (up to 44% of total CD8⁺ T cells) in the lungs of immunized mice compared with untreated control animals or mice immunized with CLDC or liposomes only. (0.73 and 0.88% of total CD8⁺ T cells, respectively).

Ag specificity of vaccine-induced airway immune responses

Experiments were then conducted to determine whether specific induction of CD8⁺ T cell responses, as opposed to induction of CD4⁺ T cell responses, was required for reducing airway inflammation and hypersensitivity. Mice were therefore immunized with CLDC and either intact OVA protein, MHC class I SIINFEKL peptide, or the MHC class II peptide OVA₃₂₃₋₃₃₉. We found that all three immunizations significantly reduced the numbers of eosinophils and Th2 cytokine production (IL-13, IL-4, and IL-5) in BAL (Fig. 4, B and C), consistent with the nonspecific effects elicited by CLDC alone noted above (Figs. 1 and 2). However, only immunization with OVA-CLDC or SIINFEKL-CLDC significantly suppressed AHR and goblet cell metaplasia (Fig. 4, A, D, and E), consistent with CD8⁺ T cell-mediated effects. Moreover, flow cytometric analysis of lung cells from SIINFEKL-CLDC-immunized mice showed similar levels of OVA-specific CD8⁺ T

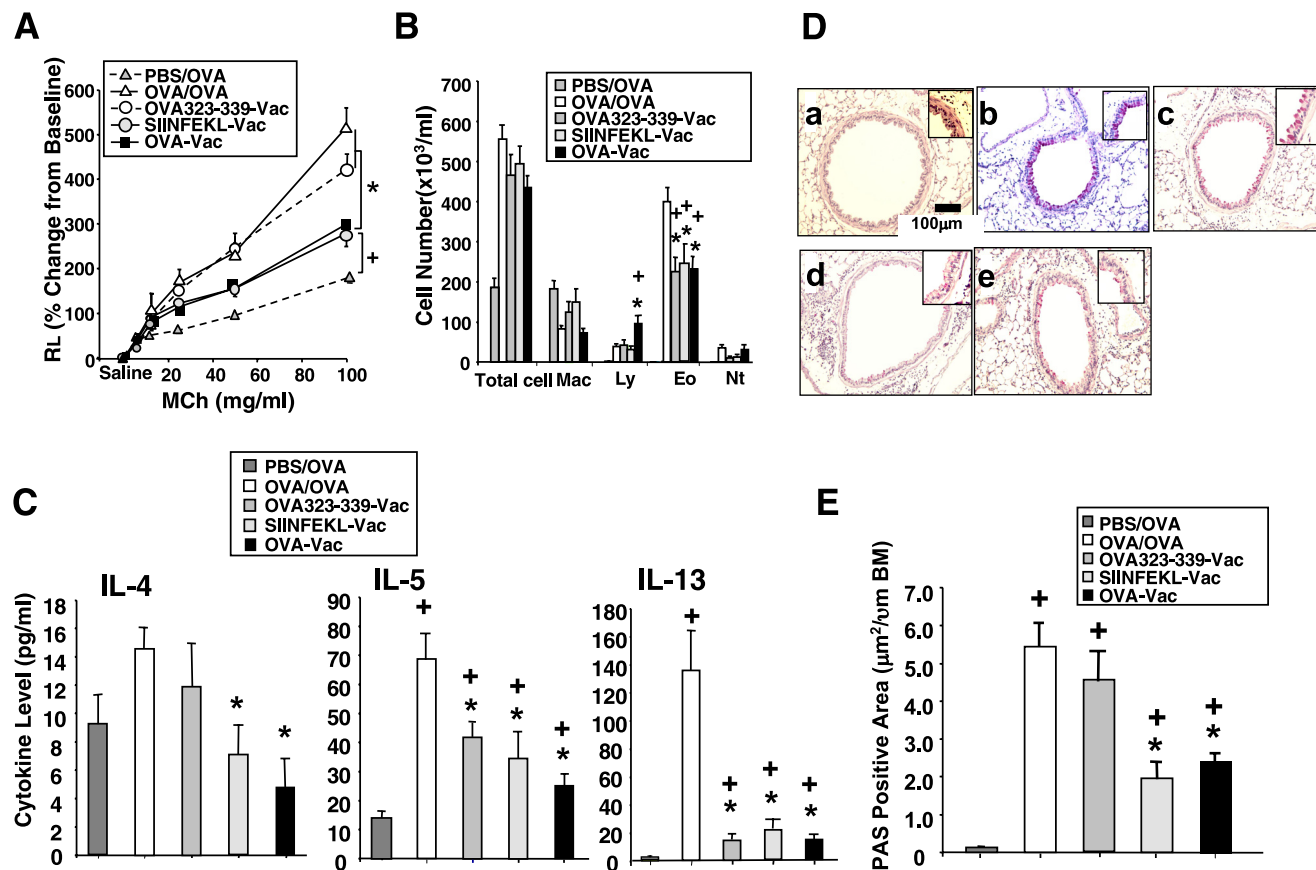


FIGURE 4. Effects of immunization on airway resistance (AHR) in OVA-sensitized and -challenged mice. Mice were sensitized and challenged with OVA (OVA/OVA) or Sham-sensitized followed by airway OVA challenge (PBS/OVA). Mice were immunized with OVA protein plus CLDC (OVA-Vac), OVA₂₅₇₋₂₆₄ peptide (MHC class I epitope, SIINFEKL) plus CLDC (SIINFEKL-Vac), or OVA₃₂₃₋₃₃₉ peptide (MHC class II epitope) plus CLDC (OVA₃₂₃₋₃₃₉-Vac), and functional and immunological responses were assessed. Airway resistance (AHR) was assessed in A, while cell composition in BAL was assessed in B and BAL cytokine concentrations were assessed in C. D, Representative histological sections with PAS staining are shown for the following mice: PBS/OVA, no immunization (a), OVA/OVA immunized with liposome alone (b), OVA/OVA immunized with OVA₃₂₃₋₃₃₉ plus CLDC (c), OVA/OVA immunized with OVA₂₅₇₋₂₆₄ plus CLDC (d), and OVA/OVA immunized with OVA protein plus CLDC (e). E, Image analysis of PAS⁺ areas in lung histological sections is shown. Mac, Macrophages; Ly, lymphocytes; Eo, eosinophils; Nt, neutrophils. The results for each group ($n = 8$) are expressed as mean $\pm$ SEM. Significant differences are shown as *, $p < 0.05$ compared with OVA/OVA or liposome alone. +, $p < 0.05$ compared with PBS/OVA.

cells as in mice immunized with whole OVA ($52 \pm 4\%$ and $49 \pm 5\%$ of tetramer-positive cells CD8⁺ T cells, respectively).

IFN- γ production by OVA-specific CD8⁺ T cells

The functionality of the OVA-specific CD8⁺ T cells induced by OVA-CLDC immunization was assessed by evaluating intracellular IFN- γ production. As shown in Fig. 5, IFN- γ production was significantly increased in OVA-specific (tetramer-positive) CD8⁺ T cells isolated from the lungs of OVA-CLDC-immunized mice compared with tetramer-negative CD8⁺ T cells following in vitro stimulation with PMA/ionomycin or SIINFEKL. As noted previously (Fig. 2, B and C), immunization with CLDC alone induced too few tetramer-positive CD8⁺ T cells for analysis. These data demonstrate that Ag-specific CD8⁺ T cells were a major source of IFN- γ production following stimulation with OVA Ag.

Effects of adoptively transferred CD8⁺ T cells from immunized mice

To directly assess the effects of CD8⁺ T cells from immunized mice on airway responses, we adoptively transferred purified lung CD8⁺ T cells from naive and immunized mice into sensitized mice before inhalational challenge with OVA. Adoptive transfer of CD8⁺ T cells but not non-CD8⁺ cells from OVA-CLDC-immunized mice significantly reduced both AHR and the number of

eosinophils in BAL fluid (Fig. 6). In contrast, adoptive transfer of CD8⁺ T cells from nonimmunized mice failed to affect AHR or eosinophil numbers (data not shown). Thus, these results indicated that the suppressive effects of immunization were mediated primarily by Ag-specific CD8⁺ T cells.

Role of IFN- γ in suppressive effects of CD8⁺ T cells

Experiments were conducted to investigate the role of CD8⁺ T cell-generated cytokines in mediating the effects of immunization in suppressing AHR and pulmonary inflammation. Given the known key role of IFN- γ in reversing or deviating Th2 immune responses, we determined whether IFN- γ was necessary for vaccine efficacy. First, we investigated the effects of immunization in IFN- γ -deficient mice. We observed that immunization was ineffective in IFN- γ ^{-/-} mice in suppressing AHR or the number of eosinophils in the airways compared with immunization of WT mice (Fig. 7).

Next, we used adoptive transfer experiments to directly assess the requirement for IFN- γ production by OVA-specific CD8⁺ T cells. In this study, lung CD8⁺ T cells were isolated from immunized IFN- γ ^{-/-} mice and adoptively transferred into OVA-sensitized WT mice before OVA challenge (Fig. 8). In addition, reciprocal experiments were conducted in which lung CD8⁺ T cells from immunized WT mice were transferred into OVA-sensitized and -challenged IFN- γ ^{-/-} mice (Fig. 9). We observed that the

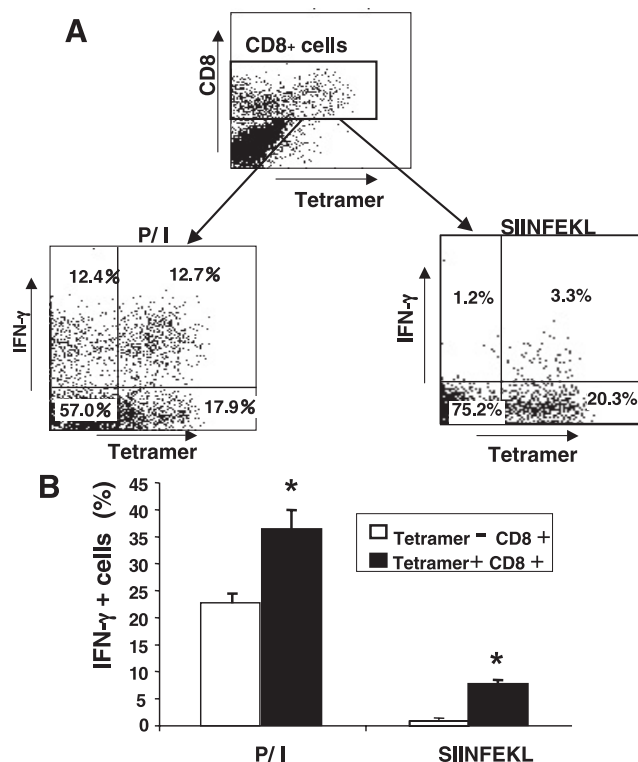


FIGURE 5. Immunization increases OVA-specific, IFN- γ -producing CD8⁺ T cells in the lungs of OVA-sensitized mice. Intracellular IFN- γ production by OVA-specific CD8⁺ lung T cells from mice immunized with OVA-CLDC was assessed by flow cytometry as described in *Materials and Methods*. **A**, A typical dot plot of CD8⁺ T cells analyzed for intracellular expression of IFN- γ is shown. **B**, The mean percentage ($\pm$ SEM) of IFN- γ -positive cells from either OVA-specific (tetramer-positive) CD8⁺ T cells or tetramer-negative CD8⁺ T cells isolated from the lungs of OVA-sensitized mice ($n = 8$ /group) is plotted. *, $p < 0.05$ compared with OVA-specific vs nonspecific CD8⁺ T cells.

transfer of CD8⁺ T cells isolated from the lungs of immunized IFN- γ ^{-/-} mice did not alter the development of AHR or the numbers of eosinophil in the BAL fluid of WT mice (Fig. 8). However, when CD8⁺ T cells isolated from the lungs of immunized WT mice were transferred to OVA-sensitized IFN- γ ^{-/-} mice, the development of AHR and numbers of eosinophils in BAL fluid were significantly suppressed (Fig. 9). Together, these data indicate that suppression of allergic airway disease by Ag-specific CD8⁺ T cells was mediated primarily by their production of IFN- γ .

Efficacy of immunization in response to secondary allergen challenge following established allergic airway inflammation and AHR

Previously, we showed that the pathways and responses to intervention leading to the development of AHR and airway eosinophilia after sensitization and primary challenge may differ from those resulting in the same responses when mice are subjected to secondary challenge, after initial lung allergic responses returned to baseline. To examine whether Ag-CLDC immunization remained effective even when allergic airway inflammation and AHR were previously established, vaccine was given to previously sensitized and challenged mice before secondary allergen challenge. As shown in Fig. 10, OVA-CLDC immunization but not CLDC alone reduced the development of AHR and eosinophilic inflammation in response to secondary allergen challenge, indicating that Ag-CLDC immunization remained effective even when

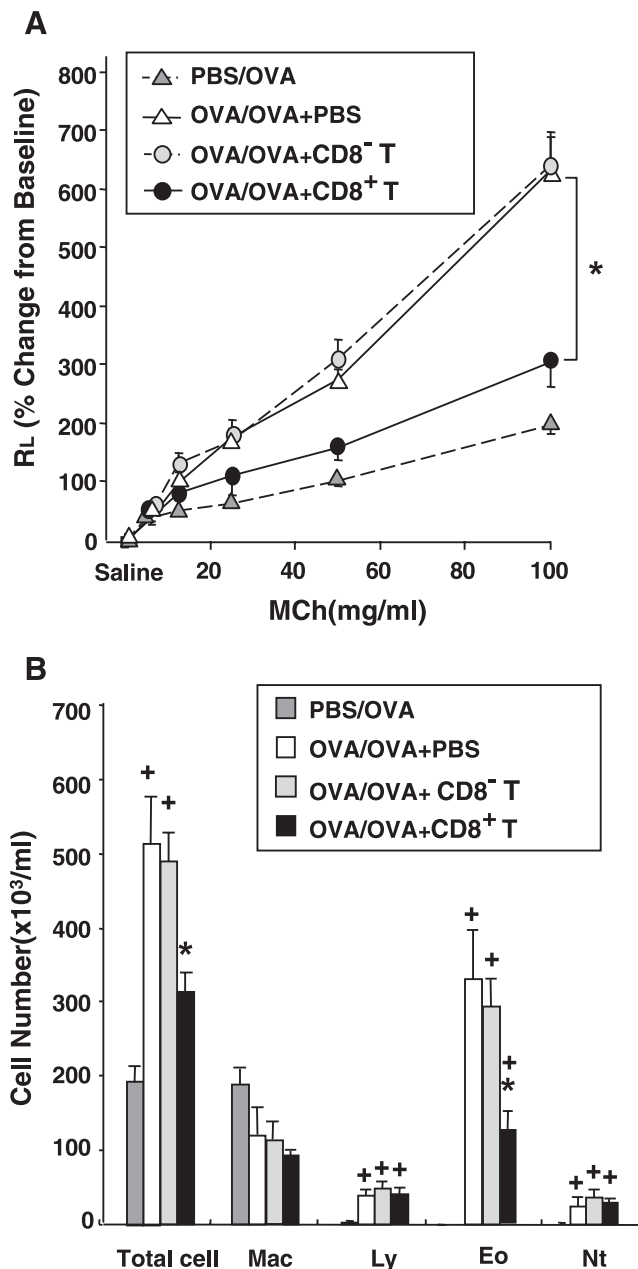


FIGURE 6. Effects of adoptive transfer of lung CD8⁺ T cells recovered from OVA-immunized mice on airway responses to OVA. Lung CD8⁺ T cells from OVA-CLDC-immunized mice were transferred into recipient mice before OVA challenge but after sensitization (OVA/OVA + CD8⁺ T). Control mice received the same number of lung CD8⁻ lung T cells (OVA/OVA + CD8⁻ T) but were not immunized but challenged with OVA (PBS/OVA) or not given adoptively transferred cells (OVA/OVA + PBS). RL (**A**) and cellular composition of BAL fluid (**B**) were assessed as described in *Materials and Methods*. Mac, Macrophages; Ly, lymphocytes; Eo, eosinophils; Nt, neutrophils. The results for each group ($n = 8$) are expressed as mean $\pm$ SEM. Significant differences are shown as *, $p < 0.05$ compared with OVA/OVA and OVA/OVA + non-CD8⁺ T cells and +, $p < 0.05$ compared with PBS/OVA.

administered to mice that had already undergone sensitization and primary challenge.

Discussion

Among the key findings from the studies reported here were that immunization or vaccination with a cationic liposome-DNA and

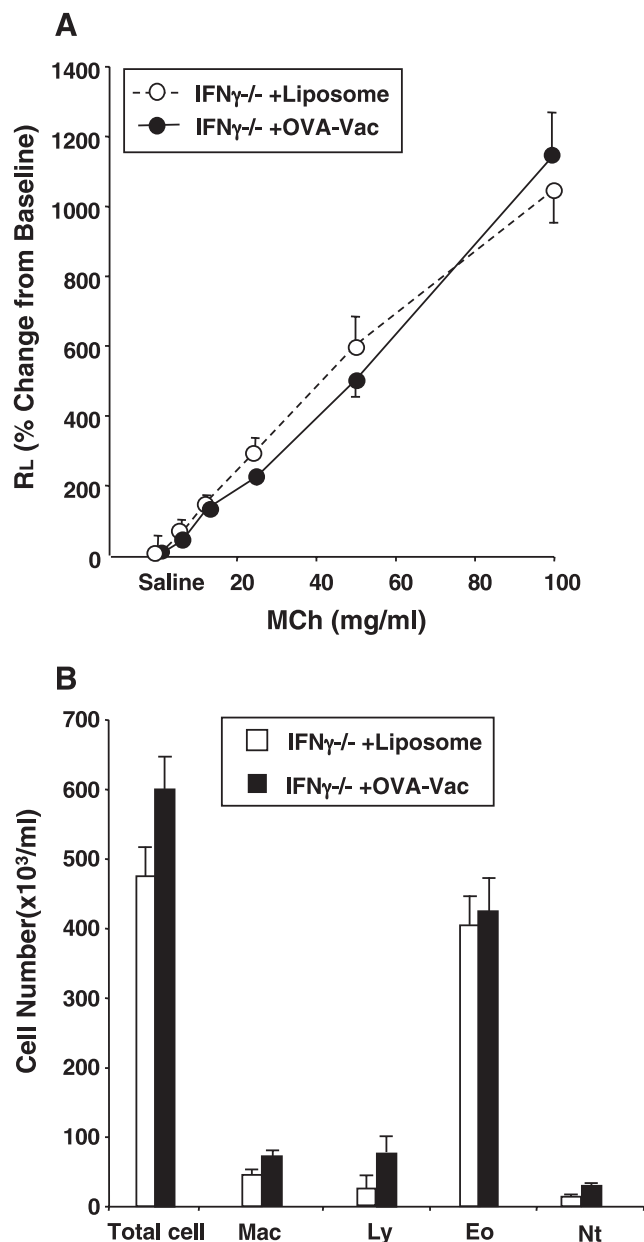


FIGURE 7. The effects of immunization on airway responses in IFN- γ -deficient mice. IFN- γ ^{-/-} mice were sensitized and challenged with OVA as described for WT mice, then immunized with OVA-CLDC (or liposome alone control) (IFN- γ ^{-/-} + OVA-Vac and IFN- γ ^{-/-} + liposome, respectively). Airway resistance (A) and BAL cellular composition (B) were then determined as described in *Materials and Methods*. Mac, Macrophages; Ly, lymphocytes; Eo, eosinophils; Nt, neutrophils. Results for each group ($n = 8$) are expressed as the mean $\pm$ SEM.

allergen complex effectively suppressed airway allergic responses, even in animals with established sensitivity to the allergen in the lungs, by generating Ag-specific CD8⁺ T cells. This finding was unexpected inasmuch as previous studies have suggested that CD4⁺ T cells play the dominant role in regulating airway inflammation in asthma. Moreover, vaccine-elicited CD8⁺ T cells were able to suppress airway inflammation through an IFN- γ -dependent process. These results suggest alternative mechanisms for generation of therapeutic immunity in patients with asthma and indicate that potent immunization protocols that generate strong CD8⁺ T cell responses may in fact be capable of suppressing or reversing established disease.

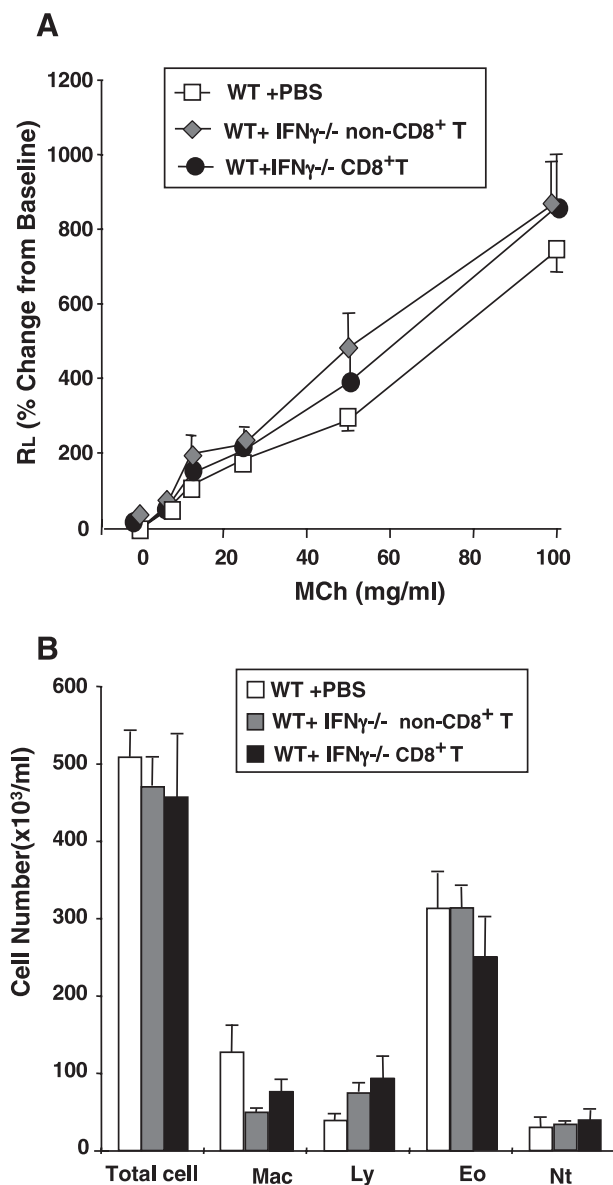


FIGURE 8. Effects of purified lung CD8⁺ IFN- γ ^{-/-} T cells from immunized mice on the airway responses to OVA in WT mice. Purified lung CD8⁺ T cells from immunized and naive IFN- γ ^{-/-} mice were adoptively transferred into sensitized WT recipient mice before OVA challenge (WT + IFN- γ ^{-/-} CD8⁺ T) as described in *Materials and Methods*. Control mice received an equivalent number of CD8⁺ T cells from the same immunized donor mice (WT + IFN- γ ^{-/-} CD8⁺ T) or PBS (WT + PBS). A, RL (AHR) in response to inhaled MCh was assessed, while in B the cell composition of BAL fluid was assessed. Mac, Macrophages; Ly, lymphocytes; Eo, eosinophils; Nt, neutrophils. Results for each group ($n = 8$) are expressed as mean $\pm$ SEM.

Conceptually, a prevailing theory is that allergic asthma is the result of an imbalance in Th1/Th2 function, with Th2 responses dominating, even from early infancy (25). Although the necessity for this imbalance as a functional prerequisite for the development of allergic asthma has been questioned (26), clinical and animal data support the essential (but perhaps not exclusive) role for Th2 immunity, at least as it relates to BAL and lung cytokine levels, airway eosinophilia, goblet cell metaplasia, and allergen-specific IgE. Thus, there remains a strong rationale for enhancing Th1 responses in controlling the allergic lung disease. Moreover, epidemiological studies lumped under the “hygiene hypothesis” suggest

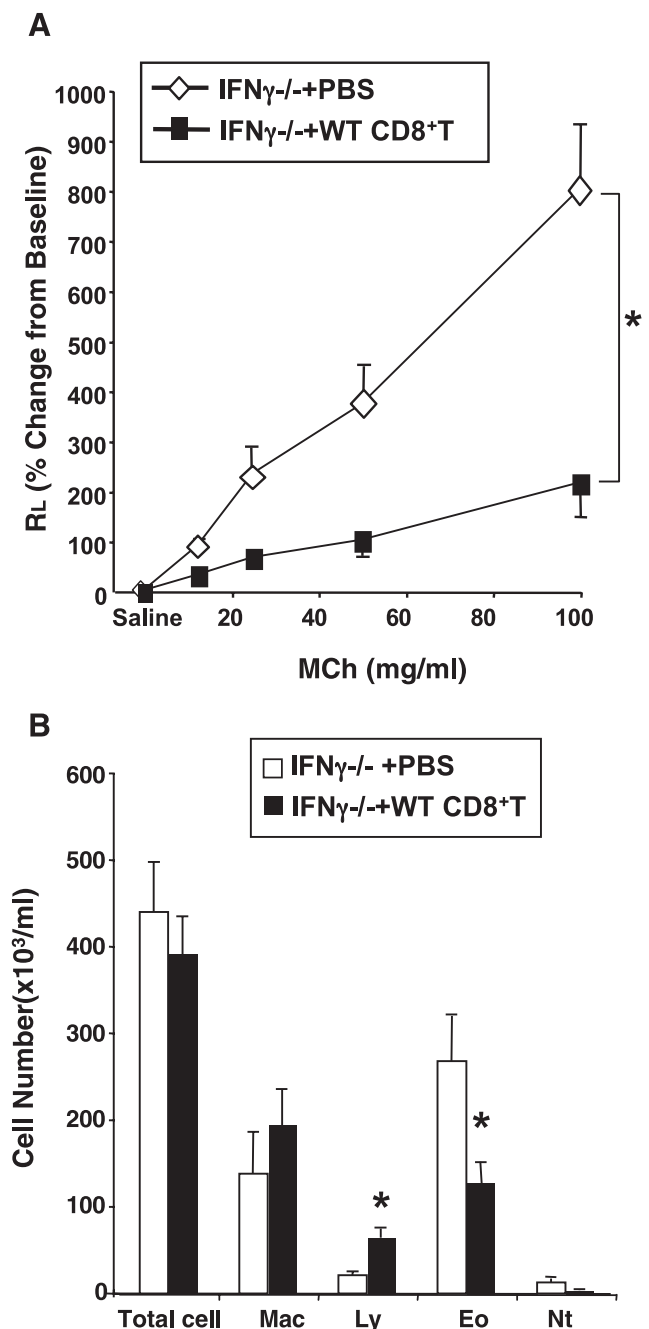


FIGURE 9. Effects of WT CD8⁺ T cells from immunized mice on the airway responses in IFN- γ ^{-/-} mice. Lung CD8⁺ T cells from OVA-immunized WT mice were purified and adoptively transferred into OVA-sensitized IFN- γ ^{-/-} recipient mice before OVA challenge (IFN- γ ^{-/-} + WT CD8⁺ T) as described in *Materials and Methods*. Control OVA-sensitized IFN- γ ^{-/-} mice received only PBS (IFN- γ ^{-/-} + PBS). **A**, RL in response to inhaled MCh challenge was assessed, while in **B**, the cellular composition of BAL fluid from immunized and control mice was assessed. Mac, macrophages; Ly, lymphocytes; Eo, eosinophils; Nt, neutrophils. The results for each group ($n = 8$) are expressed as mean $\pm$ SEM. Significant differences are shown as *, $p < 0.05$ compared with IFN- γ ^{-/-} + PBS.

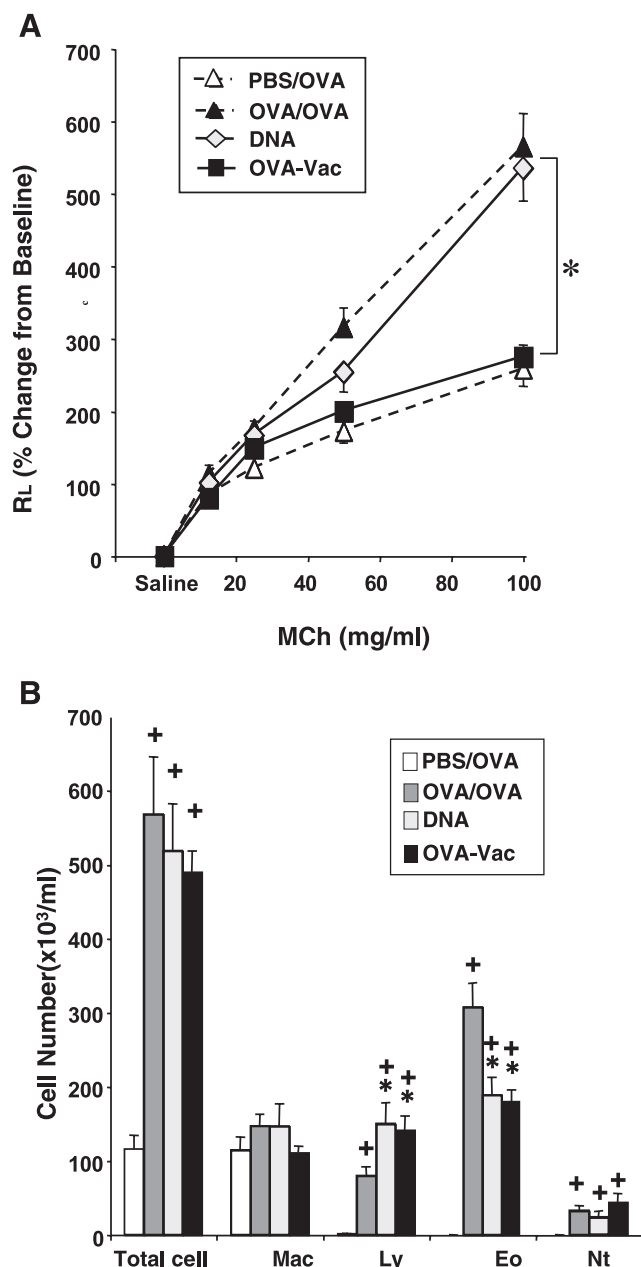


FIGURE 10. Effects of immunization on airway responses in previously established allergic airway disease. To establish primary allergic airway inflammation, mice were sensitized and challenged with OVA in the same way as in other experiments. Four weeks following the last OVA challenge after all responses returned to baseline, mice were rechallenged with OVA (secondary allergen challenge). Before secondary allergen challenge, mice received OVA-CLDC (OVA-Vac), CLDC, or PBS (OVA/OVA). Control mice received sham sensitization followed by primary and secondary allergen challenge. **A**, RL (AHR) in response to inhaled MCh was assessed, while in **B** the cell composition of BAL fluid was assessed. Mac, Macrophages; Ly, lymphocytes; Eo, eosinophils; Nt, neutrophils. Results for each group ($n = 8$) are expressed as mean $\pm$ SEM. Significant differences are shown as *, $p < 0.05$ compared with PBS.

a protective role for exposure to bacterial cell products and induction of Th1 immunity for reducing the risk of developing atopy or asthma (27).

Preventing or down-modulating allergic asthma by immunization is an attractive approach and has been investigated in several previous studies (9, 28, 29). In addition, immunotherapy using

hypomethylated CpG sequences of DNA have shown some initial successes in inducing tolerance in mouse models of allergic asthma (28, 29), but these beneficial effects were not realized in clinical trials in asthmatics (30). However, the use of vaccines and immunotherapy to manage asthma are still considered realistic treatment goals.

In the present study, immunization with the OVA-CLDC vaccine significantly suppressed allergic airway inflammation and AHR in mice. The suppression of AHR and goblet cell metaplasia was found to be primarily associated with expansion of Ag-specific, IFN- γ -producing CD8⁺ T cells in the airways. On the other hand, reduction of airway eosinophilia and Th2 cytokine production were elicited by nonspecific effects with the CLDC adjuvant alone. In previous studies, CLDC has been shown to be a potent activator of innate immunity, including activation of dendritic cells and NK cells (31). The nonspecific effects of immunization with CLDC alone most likely reflected the effects of NK cell-derived IFN- γ and possibly other cytokines produced by dendritic cells (32, 33). Thus, it appeared that AHR and goblet cell metaplasia were more resistant to suppression relative to eosinophilia and airway Th2 cytokine production and required the additive effects of both innate and adaptive (CD8⁺ T cell) immune responses.

These findings are important inasmuch as they suggest that effective immunotherapy of established allergic asthma may require both Ag-specific and Ag-nonspecific responses. For example, other investigators have found that plasmid DNA vaccines could decrease the number of eosinophils by redirecting the immune response to an IFN- γ -dominated Th1-type response (7, 34). In the present study, relatively small amounts of plasmid DNA were delivered (10 μ g per immunization) and this was sufficient to suppress eosinophilia and levels of airway Th2 cytokines.

It was surprising in this study to find that suppression of AHR and goblet cell metaplasia was mediated almost exclusively by IFN- γ -producing CD8⁺ T cells. Previous vaccine studies have identified a role for CD4⁺ T cells in protection from allergic airway disease (35). There may be several explanations for the differences in T cell mediators noted in these different studies. For example, most of the previous studies used immunization/vaccination strategies that were not capable of eliciting strong CD8⁺ T cell responses compared with what was elicited by the CLDC adjuvant (14). It is also likely that the OVA-CLDC vaccine did not elicit strong CD4⁺ T cell responses in the C57BL/6 strain of mice used in this study, since adoptive transfer experiments with non-CD8⁺ T cell populations which contain CD4⁺ T cells from OVA-CLDC-immunized lungs did not alter AHR or airway inflammation. Support for this notion comes from the fact that C57BL/6 mice mount poor Ab responses to OVA (36) and that mice immunized with the MHC class II peptide were not protected in this model as shown here. Thus, it is possible that CD8⁺ T cells were uniquely effective in this model following immunization because of the absence of CD4⁺ T cell responses. It is possible that in an outbred species such as humans, both CD4⁺ and CD8⁺ T cells may play a more balanced role in vaccine-induced protection.

IFN- γ is a potent suppressor of allergen-induced immunopathology in the lungs, an effect that is mediated in large measure by the ability of IFN- γ to suppress production of Th2 cytokines (37–39). In the present study, both the Ag-containing immunizations were largely dependent on production of IFN- γ . For example, OVA-CLDC did not show any effect in IFN- γ ^{−/−} mice and adoptive transfer experiments with CD8⁺ T cells from OVA CLDC-treated IFN- γ ^{−/−} donor mice into WT recipients also failed to suppress the responses. Thus, IFN- γ -producing CD8⁺ T cells likely play a pivotal role in the suppression of allergen-induced AHR and inflammation. However, because IFN- γ has been shown to be a critical element in memory CD8⁺ T cell development (40), we performed complementary experiments with adoptive transfer of OVA-CLDC-treated WT CD8⁺ T cells into IFN- γ ^{−/−} recipients. These results demonstrated that the effects of OVA-specific CD8⁺ T cells on suppressing AHR and airway eosinophilia were mediated by IFN- γ . Although the source of IFN- γ production by Ag-

nonspecific cells was not determined, it is likely that NK cells were the major producers, based on the results of previous studies with CLDC (31). Nonspecific activation of T cells by CLDC-elicited cytokines may also have accounted for the increased production of IFN- γ following PMA/ionomycin activation of T cells that were not OVA specific.

The findings from the present study also raise an important issue regarding the apparent dual functionality of CD8⁺ T cells in the development of allergic lung disease. We previously reported the critical role for IL-13-producing CD8⁺ T cells in promoting the development of allergen-induced AHR and airway inflammation (17, 20, 38). In contrast, in the present study, we found that CD8⁺ T cells in the lungs actually mediated suppression of these same allergic responses in the lungs. How then to resolve these apparently conflicting results regarding the role of CD8⁺ T cells in allergic lung disease? We suggest the explanation lies in the plasticity of CD8⁺ T cell functional development. As described previously for effector CD8⁺ T cells, signals from the environment can direct the peripheral functional development of CD8⁺ T cells into either IL-13- and IL-4-producing cells rather than IFN- γ -producing T cells (41).

Under the signals provided by immunization, Ag-specific CD8⁺ T cells are capable of developing into IFN- γ -producing cells that suppress airway inflammation and AHR. The default pathway in the absence of a signal from immunization may be for CD8⁺ T cells to produce IL-4 and IL-13 and promote the development of Th2-mediated airway inflammation. At present, it is unclear whether this functional plasticity is a property of all CD8⁺ T cells, only Ag-naïve CD8⁺ T cells, or different subsets of memory CD8⁺ T cells. However, it is clear that an improved understanding of the mechanisms involved in vaccine-induced modulation of CD8⁺ effector T cell functional differentiation in the lungs will be important for developing more effective immunotherapeutic approaches in the treatment of asthma.

Disclosures

The authors have no financial conflict of interest.

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