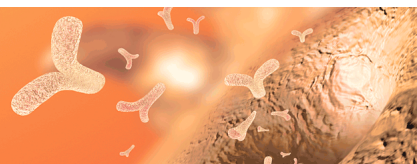




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Allergen-Specific CTL Require Perforin Expression To Suppress Allergic Airway Inflammation

Noriyuki Enomoto,*¹ Evelyn Hyde,* Joel Zhi-Iong Ma,*² Jianping Yang,* Elizabeth Forbes-Blom,* Brett Delahunt,[†] Graham Le Gros,* and Franca Ronchese*

Allergen-specific CTL have a protective effect on allergic airway inflammation, a function thought to be mediated by cytokines, especially IFN- γ . However, the contribution of cytotoxic function to this protective effect has not been investigated. We examined the contribution of cytotoxic function to the therapeutic effect of allergen-specific CTL in allergic airway inflammation. We used a murine model of allergic airway inflammation in which mice were sensitized to OVA and then challenged with the same Ag via the intranasal route. CTL were elicited in these mice by immunization with dendritic cells (DC) or by adoptive transfer of in vitro-activated CD8⁺ T cells. Hallmark features of allergic asthma, such as infiltration of eosinophils in the bronchoalveolar lavage fluid and mucus production, were assessed. Suppression of allergic airway inflammation by allergen-specific CTL was critically dependent on the expression of perforin, a key component of the cytotoxic machinery. Both perforin-sufficient and perforin-deficient allergen-specific CTL were recovered from the lungs of allergen-sensitized mice and upregulated CD69 expression and secreted the cytokines IFN- γ and TNF- α upon intranasal allergen challenge. However, only perforin-sufficient CTL inhibited eosinophil infiltration in the airway, mucus production, and cytokine accumulation in the bronchoalveolar lavage fluid. Treatment with allergen-specific CTL, but not their perforin-deficient counterparts, was also associated with a decrease in the number of DC in the mediastinal lymph node. Our data suggest that the cytotoxic function of allergen-specific CD8⁺ T cells is critical to their ability to moderate allergic airway inflammation. *The Journal of Immunology*, 2012, 188: 000–000.

Asthma remains one of the most prevalent airway diseases in the world. Hallmark features of asthma include chronic airway inflammation and subsequent airflow obstruction with cough, dyspnea, wheezing, and impaired respiratory function.

Clinical and experimental investigations demonstrated that CD4⁺ Th2 lymphocytes play an essential role in the pathogenesis of asthma (1, 2). Production of the cytokines IL-4, IL-5, and IL-13 by Th2 cells in the airway induces recruitment of eosinophils, as well as the mucus production and airway hyperresponsiveness that

are characteristic of asthmatic disease. Cytokine production by Th2 cells is triggered by exposure to inhaled allergens, which are taken up and presented in an immunogenic form by airway dendritic cells (DC). Through their Ag-presenting function, DC play a critical and nonredundant role in the effector phase of the allergic airway response (3), as shown by mouse experiments in which airway DC depletion was sufficient to ablate all signs of allergic airway inflammation (4).

In contrast to CD4⁺ T cells, the role of $\alpha\beta$ CD8⁺ T cells in asthma remains controversial. Experimental models of allergic airway inflammation revealed that type 2 CD8⁺ T cells, or Tc2 cells, can exacerbate inflammation by secreting type 2 cytokines such as IL-5 and IL-13 (5–7). In contrast, conventional type 1 Tc1 cells ameliorate (8–13) inflammation and airway hyperresponsiveness. The mechanisms of these CD8⁺ T cell-mediated effects are thought to involve CD8⁺ T cell-derived cytokines, especially IFN- γ . Similarly to CD8⁺ T cells, $\gamma\delta$ T cells can also inhibit airway inflammation (14) by an IFN- γ -dependent mechanism (15). However, although a protective effect of IFN- γ on eosinophilic inflammation was suggested in some studies, others revealed that IFN- γ may exacerbate disease (reviewed in Refs. 2, 16). No other mechanisms have been proposed to explain the regulatory effect of CD8⁺ T cells during allergic airway inflammation.

Given the evidence that DC play a pivotal role in asthma (4, 17–20), we hypothesized that the inhibitory effect of CD8⁺ T cells on airway inflammation might be mediated via effects on DC function. We and others have shown that activated CTL can kill Ag-presenting DC (21–23), thereby limiting their ability to induce further T cell activation (24). We propose that, in a similar fashion, killing of allergen-presenting DC in the airway might result in termination of Ag presentation to CD4⁺ Th2 cells and resolution of the inflammatory response. In this study, we examined the role of CD8⁺ T cell cytotoxic function in the inhibition of allergic inflammation. We report that allergen-specific CTL must express

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Abbreviations used in this article: AB-PAS, Alcian blue and periodic acid-Schiff; Alm, alum adjuvant; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CMTMR, (5-(and-6)-((4-chloromethyl)benzoyl)amino) tetramethylrhodamine; DC, dendritic cell; i.n., intranasal(ly); LN, lymph node; low-E, low endotoxin; MLN, mediastinal lymph node; OVA-AF488, Alexa Fluor 488-labeled OVA; OVA/Alm, OVA in alum adjuvant; PKO, perforin knockout; WT, wild type.

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perforin, a critical component of the CTL cytotoxic granule, to reduce eosinophilic inflammation and mucus production in the airway, whereas IFN- γ is not critical. Thus, our data highlight cytotoxic function as a critical mechanism for the control of allergic airway inflammation.

Materials and Methods

Mice

All mice were bred at the Malaghan Institute of Medical Research. C57BL/6J and perforin knockout (PKO) mice were originally from Jackson Laboratories (Bar Harbor, ME). CD45-congenic B6.SJL-Ptpr^a mice were from the Animal Resources Centre (Perth, Australia). OT-I mice carry a transgenic TCR specific for H-2K^b + fragment 257–264 of chicken OVA, whereas Line 318 (L318) mice carry a transgenic TCR specific for H-2D^b + gp33. OT-I mice that are PKO (PKO OT-I) were generated by conventional breeding. All experimental protocols were approved by the Victoria University Animal Ethics Committee and performed according to institutional guidelines.

In vitro culture media and reagents

All cultures were in complete medium composed of IMDM, 2 mM glutamax, 1% penicillin-streptomycin, 5×10^{-5} M 2-ME, and 5% FBS (all from Invitrogen). The OVA_{257–264} (SIINFEKL) and gp33 (KAVYNFATM) peptides were from Sigma-Genosys. LPS from *Escherichia coli* was from Sigma-Aldrich. OVA Grade V was from Sigma-Aldrich, low endotoxin (low-E) Endo-free OVA was from Profos, and Alexa Fluor 488-conjugated OVA (OVA-AF488) was from Molecular Probes, Invitrogen.

Sensitization and challenge

Six- to eight-week-old female mice were sensitized by i.p. injection of 2 μ g OVA in 1.36 mg alum adjuvant (SERVA) in a total volume of 200 μ l on days 0 and 14. Control mice received alum adjuvant (vehicle) only. On day 24, mice were anesthetized and instilled with 100 μ g OVA in 50 μ l PBS intranasally (i.n.). In some experiments, the same amounts of low-E OVA or OVA-AF488 were used, as indicated.

Culture and adoptive transfer of bone marrow DC

To generate DC, bone marrow cells from C57BL/6 mice were cultured at 4×10^5 cells/ml in complete medium containing 20 ng/ml murine rIL-4 and 10 ng/ml murine rGM-CSF for 7 d, as described (25), and activated by adding 100 ng/ml LPS during the last 24 h of culture. DC were collected from culture by gentle pipetting and were $\geq 80\%$ CD11c⁺ by flow cytometry. They were loaded with 10 μ M peptide or PBS (vehicle) for 2 h at 37°C, and 1×10^6 cells were instilled i.n. into anesthetized mice in 50 μ l PBS.

Culture and adoptive transfer of CTL

To generate CTL, lymph nodes (LN) were removed from OT-I mice or PKO OT-I mice, cocultured with DC/SIINFEKL for 4 d, and expanded in 100 U/ml human rIL-2 for an additional 2 d (26). A similar protocol was used to generate L318 CTL (27), except that DC/gp33 were used. These protocols generated populations that were uniformly CD62L^{low}CD44^{hi}, highly cytotoxic (28, 27, 26), and produced large amounts of IFN- γ and TNF- α when activated in vitro with specific peptide (27). Cells were harvested on day 6, and 5×10^6 cells were injected into recipient mice through the lateral tail vein.

Bronchoalveolar lavage analysis

Mice were euthanized by nonrecovery anesthesia, the trachea was cannulated, and lungs were lavaged with 1 ml PBS at room temperature. After erythrocyte lysis, the recovered cells were washed, counted, and spun onto a glass slide. The cells were stained with Diff-Quik (Dade Behring), and differential counts were performed on 200 cells at 200 $\times$ magnification (29). Bronchoalveolar lavage fluid (BALF) samples were stored at -20°C for the measurement of cytokines.

Measurement of cytokines

The concentrations of cytokines (IFN- γ , IL-4, IL-5, IL-10, IL-12p70, IL-13, TNF- α , and IL-17) in BALF were evaluated using a bead multiplex immunoassay and Bio-plex suspension array system (Bio-Rad Life Sciences) or Milliplex Map Mouse Cytokine Panel (Millipore), according to the manufacturers' instructions. The concentration of TGF- β was determined using a sandwich ELISA (Quantikine, R&D Systems).

Lung histopathology

On day 3 after i.n. challenge, lungs were recovered after bronchoalveolar lavage (BAL) and fixed in 10% formalin before embedding in paraffin. Mucus-containing goblet cells were detected by staining of 4- μ m-thick slices with Alcian blue and periodic acid-Schiff (AB-PAS). All bronchioles with a diameter of 250–500 μ m were counted in both lungs. Bronchioles were scored as mucus producing when the number of mucus-producing cells was ≥ 11 cells/mm, as determined by measuring the circumference of the airway at basement membrane level using ImageJ software (National Institutes of Health).

DC elimination assay in vivo

This assay was carried out as described (30), except that DC were injected i.v. and were recovered from the mediastinal LN (MLN). Briefly, bone marrow DC were cultured as described above, harvested from culture, washed, labeled with CFSE (Molecular Probes) or (5-(and-6)-((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR) (Molecular Probes), loaded with SIINFEKL or no peptide (PBS), and washed extensively. Recipient mice were injected i.v. with a mixture of 4×10^6 CFSE-labeled DC/SIINFEKL and 4×10^6 CMTMR-labeled DC/PBS. Forty-eight hours after the injection, MLN were removed, digested in Liberase TH and DNase I (both from Roche Applied Science), and analyzed by flow cytometry for the presence of live CFSE⁺ and CMTMR⁺ cells. The survival of CFSE⁺ DC/SIINFEKL was calculated as a percentage of the CMTMR⁺ DC/PBS population in each sample and normalized to the relevant controls, as described (30). Specific DC killing (%) was calculated as $(100 - \text{surviving DC } [\%])$.

Tracking of airway APC

To track OVA in airway APC, mice were anesthetized and instilled with 100 μ g OVA-AF488 in 50 μ l PBS i.n. Twenty-four hours after the instillation, MLN were removed, digested in Liberase CI and DNase I, and analyzed by flow cytometry for the presence of OVA-AF488⁺ DC (identified as DAPI⁺CD11c^{high}MHCII⁺ cells).

In vivo IFN- γ neutralization

In some experiments, mice were injected with 500 μ g the IFN- γ -neutralizing mAb XMG-D6, produced in-house from hybridoma culture supernatants and tested in vivo for the ability to neutralize the IFN- γ -induced upregulation of MHC class II expression on lung macrophages. The mAb was injected i.p. 4 h before i.n. OVA challenge on day 24.

Preparation of lung cell suspensions

Mice were sensitized with OVA in alum adjuvant (OVA/Alm) and challenged i.n. with 100 μ g OVA-AF488. Twenty-four hours after challenge, mice were euthanized and perfused with ~ 15 ml PBS to remove blood from the lung vascular bed. Minced lung tissue was incubated in IMDM containing 0.5 mg/ml Liberase TL and 0.5 mg/ml DNase I for 45 min at 37°C; 10 mM EDTA was added to stop digestion. Remaining tissue fragments were broken down by passage through an 18G needle and a 70- μ m cell strainer (BD Falcon). The resulting cell suspension contained $>70\%$ CD45⁺ cells by flow cytometry (31).

Flow cytometry

Anti-Fc γ RII (2.4G2) was affinity purified from hybridoma culture supernatant and used to block FcRs before incubation with fluorescent Abs. Anti-CD45.2-FITC, anti-CD8-PerCP, and anti-CD69-PE were used to examine CTL activation in the lungs of mice injected with in vitro-activated CTL. Anti-CD11c-PE-Cy7, anti-CD11c-AF700, anti-MHCII-AF647, and anti-CD11b-PerCP-Cy5.5 were used to identify DC and macrophage populations in lungs and MLN. All Abs were from BD Pharmingen or eBioscience. All samples were analyzed on FACSCalibur or LSRII multicolor flow cytometers (BD Biosciences) using CellQuest (BD Biosciences) and FlowJo (Tree Star) software. Live cells were identified by propidium iodide (BD Pharmingen) or DAPI (Molecular Probes) dye exclusion.

Statistical analysis

Data from multiple experiments were expressed as mean $\pm$ SEM. Data were analyzed using one-way ANOVA with Bonferroni correction or using a two-sided unpaired *t* test. Statistical analyses were performed using Prism 5, GraphPad Software. All tests were performed at the 0.05 significance level.

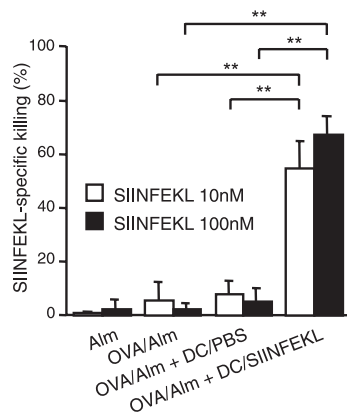


FIGURE 1. Sensitization with OVA/Alm does not elicit OVA-specific CTL responses. Mice were sensitized i.p. with OVA/Alm on days 1 and 14 and treated i.n. with 1×10^6 DC/PBS or DC/SIINFEKL on day 17. OVA-specific killing was evaluated in vivo using spleen cell targets injected i.v. on day 24. Target cells were recovered from the MLN 24 h after injection and were quantified by flow cytometry; nonimmunized mice were used as controls to determine background killing. The bar graph shows mean $\pm$ SEM for four mice/group. The data shown are representative of two independent experiments that gave similar results. $**p < 0.01$.

Results

Allergen-specific CTL decrease allergic airway inflammation

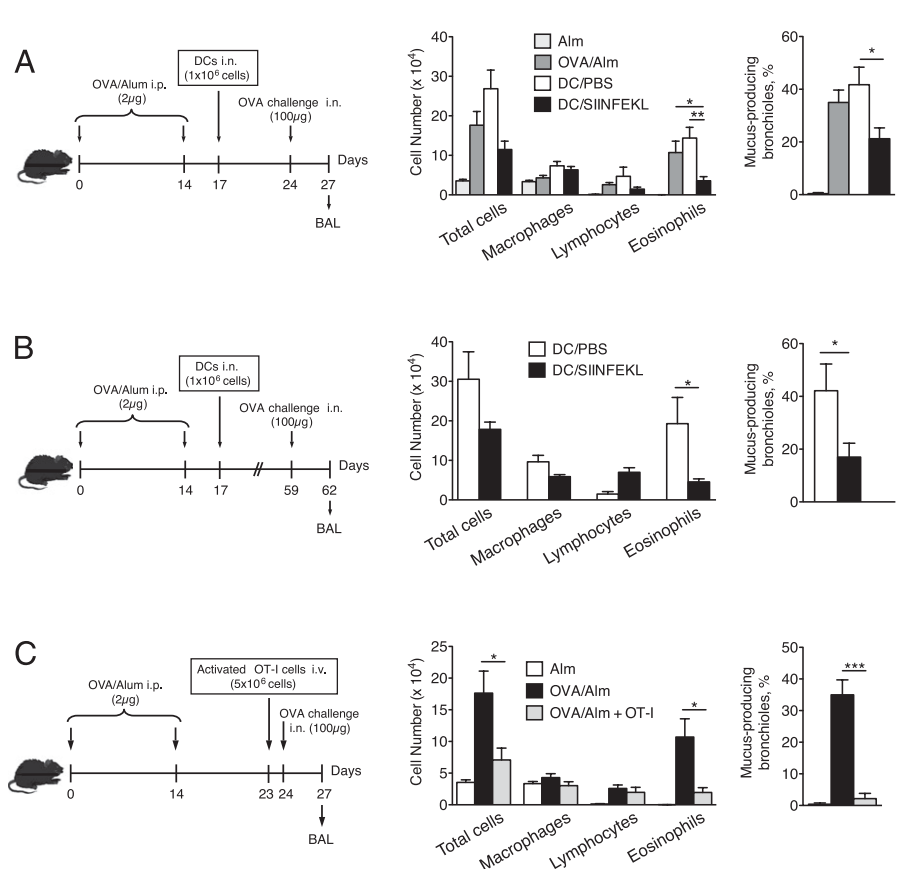
To evaluate the effects of allergen-specific cytotoxic function on allergic airway inflammation, we used a model in which mice were sensitized with OVA/Alm or vehicle alum adjuvant (Alm) on days 0 and 14 and then challenged i.n. with OVA on day 24. As shown in Fig. 1, this protocol of immunization did not induce activation

of OVA-specific CTL, because sensitized mice failed to kill spleen targets loaded with the MHC class I-binding peptide OVA_{257–264} and injected i.v. In contrast, mice sensitized with OVA/Alm and immunized i.n. on day 17 with DC/SIINFEKL, but not DC/PBS, generated strong OVA-specific cytotoxic responses (Fig. 1).

We then tested the effect of OVA-specific CTL induced by DC/SIINFEKL immunization on allergic airway inflammation. Mice were sensitized with OVA/Alm and immunized on day 17 with DC/SIINFEKL, DC/PBS, or no DC, according to the protocol in Fig. 2A. All mice were challenged i.n. with OVA on day 24. As shown in Fig. 2A, compared with mice sensitized with Alm, mice sensitized with OVA/Alm and not immunized with DC, and mice sensitized with OVA/Alm and immunized with DC/PBS, showed increased cellularity in BALF, as well as infiltration of eosinophils. In contrast, the number of BALF eosinophils was significantly lower in mice immunized with DC/SIINFEKL (Fig. 2A). Lung histopathology was also used to determine the effects of DC/SIINFEKL immunization on airway mucus production. The percentage of mucus-producing bronchioles was reduced in mice treated with DC/SIINFEKL compared with DC/PBS (Fig. 2A, Supplemental Fig. 1A). The levels of the Th2 cytokines IL-4, IL-5, and IL-13 were also significantly lower in the BALF of DC/SIINFEKL-treated mice compared with untreated or DC/PBS-treated mice (Supplemental Fig. 1A); some IFN- γ was detected in DC/SIINFEKL-treated mice but at low levels. The levels of IL-12p70, IL-10, TGF- β , and IL-17 were low and did not significantly change across any of the groups (Supplemental Fig. 1A). Therefore, immunotherapy to elicit allergen-specific CTL can specifically decrease allergic airway inflammation.

To validate the long-term effects of DC treatment, mice were sensitized with OVA/Alm on days 0 and 14, treated with DC i.n. on

FIGURE 2. Intranasal treatment with DC loaded with an MHC class I allergen epitope or adoptive transfer of allergen-specific CTL ameliorates allergic airway inflammation. Mice were sensitized with OVA/Alm or Alm and treated with DC/PBS or DC/SIINFEKL (A, B), or injected with OVA-specific OT-I CTL (C), according to the protocols shown (left panels). Mice were challenged with OVA i.n., and the numbers of cells in individual BALF were evaluated 3 d later by cytopsin and differential counting. Mucus-producing goblet cells in bronchioles were evaluated on histological specimens using AB-PAS staining. Bar graphs (middle panels) show mean $\pm$ SEM of the number of cells/BALF, whereas bar graphs (right panels) show mean $\pm$ SEM of the percentage of mucus-producing bronchioles in both lungs. BALF data in A refer to 9–14 mice/group, pooled from three of several independent experiments that gave similar results. Mucus production data refer to four to six mice/group and are representative of three independent experiments that gave similar results. BALF and mucus production data in B are for seven mice/group. C, BALF data are for seven to nine mice/group, pooled from three of several independent experiments that gave similar results. Mucus production data are for four to six mice/group, pooled from two independent experiments that gave similar results. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.



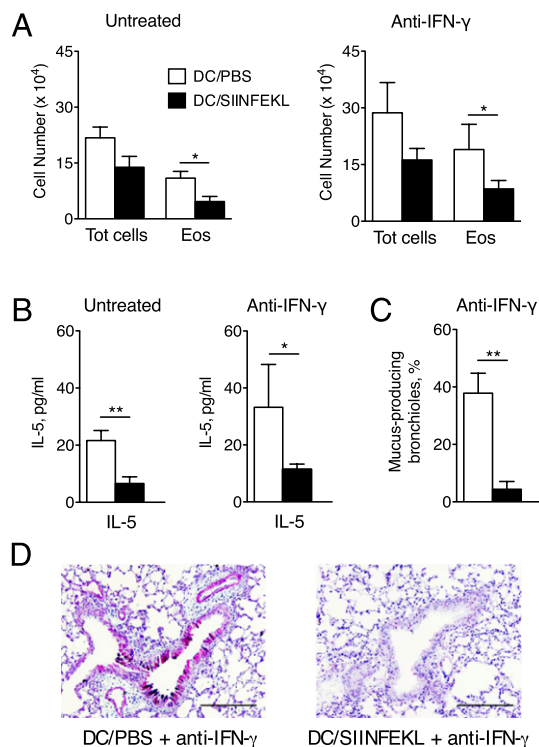


FIGURE 3. Allergen-specific CTL ameliorate allergic airway inflammation independently of IFN- γ . Mice were sensitized and treated with DC as described in Fig. 2A. The IFN- γ -neutralizing mAb XMG-D6 was injected i.p. 4 h before i.n. OVA challenge on day 24. The number of cells (A) and the concentration of IL-5 (B) in individual BALF were evaluated 3 d after OVA challenge. C, Percentages of mucus-producing bronchioles in both lungs. D, Representative bronchioles in lung sections stained with AB-PAS. Scale bars, 200 μ m. Bar graphs show mean $\pm$ SEM for 4–10 mice/group. Data are from one representative experiment of two that gave similar results. * p < 0.05, ** p < 0.01.

day 17, and challenged i.n. with OVA 6 wk later (Fig. 2B). BAL was performed 3 d after OVA challenge. Again, eosinophil numbers were decreased in the BALF of mice treated with DC/SIINFEKL compared with DC/PBS, and the percentage of mucus-producing

bronchioles was decreased (Fig. 2B). Therefore, allergen-specific CTL induced by appropriate DC immunization mediate long-term protection against allergic airway inflammation.

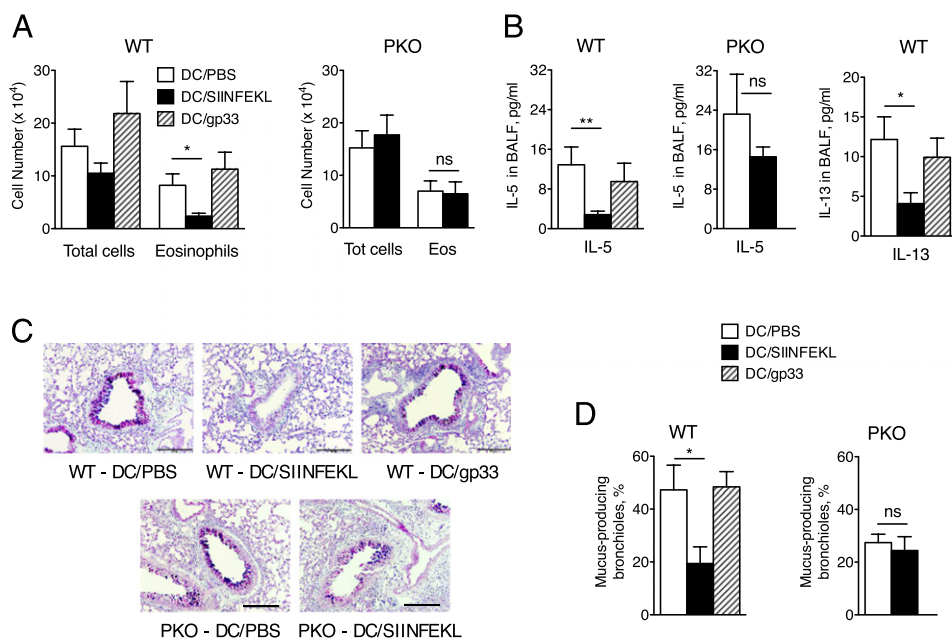
To directly assess the role of allergen-specific CTL in the reduction of allergic airway inflammation, we used in vitro-activated OVA-specific CD8⁺ T cells from OT-I mice. Activated OT-I CTL were transferred i.v. into OVA/Alm-sensitized mice 1 d prior to i.n. OVA challenge (Fig. 2C). Total cell and eosinophil numbers in BALF and the percentage of mucus-producing bronchioles were significantly decreased in mice treated with OT-I CTL compared with OVA-sensitized controls (Fig. 2C, Supplemental Fig. 1B). The levels of IL-4, IL-5, and IL-13 were also significantly lower in OT-I CTL-treated mice compared with untreated mice. In contrast, IFN- γ was significantly increased, suggesting that OT-I CTL were the likely source of this cytokine (Supplemental Fig. 1B).

Therefore, allergen-specific CTL appear to decrease the activation of Th2 cells in the lung and airway, as indicated by decreased eosinophil infiltration, mucus production, and Th2 cytokines.

IFN- γ is not required for the inhibition of allergic airway inflammation by allergen-specific CTL

It was reported that CD8⁺ T cells can suppress allergic airway inflammation in an IFN- γ -dependent fashion (10, 32). In our experiments, IFN- γ production by CD8⁺ T cells did not appear to correlate with their ability to inhibit allergic airway inflammation (Supplemental Fig. 1A, 1B). Nonetheless, to further evaluate the role of IFN- γ in our model, we used the IFN- γ -neutralizing mAb XMG-D6 to block IFN- γ in vivo. One i.p. dose of 500 μ g XMG-D6 was sufficient to completely prevent the IFN- γ -dependent upregulation of MHC class II on lung macrophages in mice treated with OT-I CTL and challenged with OVA i.n. (data not shown). To establish the effects of IFN- γ on allergic airway inflammation, the XMG-D6 mAb was injected i.p. 4 h before i.n. OVA challenge on day 24. Although IFN- γ neutralization slightly attenuated the protective effects of DC/SIINFEKL, this treatment was still able to significantly reduce total cell and eosinophil numbers in BALF (Fig. 3A), whereas macrophage, neutrophil, and lymphocyte numbers were not affected (data not shown). Even after IFN- γ neutralization, DC/SIINFEKL treatment decreased IL-5 in BALF (Fig. 3B) and mucus-producing bronchioles in the lungs (Fig. 3C, 3D). These results indicated that IFN- γ is neither necessary nor

FIGURE 4. Treatment with DC loaded with an MHC class I allergen epitope fails to ameliorate allergic airway inflammation in PKO hosts. PKO or WT mice were sensitized and challenged with low-E OVA and treated with DC, as described in Fig. 2A. The number of cells (A) and the concentration of IL-5 and IL-13 (B) were evaluated in individual BALF 3 d after low-E OVA challenge. Combined data from two separate experiments including 5–14 mice/group are shown. C, Mucus-producing goblet cells in bronchioles were identified using AB-PAS staining, as described in Fig. 2. Panels show representative bronchioles. Scale bars, 200 μ m. D, Bar graphs show the percentages of mucus-producing bronchioles in both lungs for groups of five to seven mice. * p < 0.05, ** p < 0.01. ns, Not significant.



sufficient for the inhibition of allergic airway inflammation by allergen-specific CTL.

The suppression of airway inflammation requires perforin expression by allergen-specific CTL

Because IFN- γ was not required for the inhibition of allergic airway inflammation by allergen-specific CTL, we examined the role of cytotoxic activity. We used PKO mice, because CTL from PKO mice exhibit defective cytotoxic ability, and our previous studies showed that PKO mice are unable to generate cytotoxic responses that kill DC in vivo (24). Defective killing was confirmed in PKO mice sensitized with OVA/Alm and immunized with DC/SIINFEKL (Supplemental Fig. 2A, 2B). In contrast, similarly immunized wild-type (WT) mice and mice treated with neutralizing anti-IFN- γ mAb (Supplemental Fig. 2C) generated good cytotoxic responses.

PKO and WT C57BL/6 mice were sensitized with low-E OVA/Alm to exclude allergen-nonspecific effects, treated with DC/PBS or DC/SIINFEKL i.n., and their responses to i.n. challenge with low-E OVA were compared. To control for the Ag specificity of the CTL response, some WT mice were also immunized with DC and the irrelevant peptide gp33 from lymphocytic choriomeningitis virus. As shown in Fig. 4A, compared with DC/PBS, treatment

with DC/SIINFEKL significantly decreased BALF eosinophil numbers in WT mice, whereas treatment with DC/gp33 had no effect. In contrast, in PKO mice, treatment with DC/SIINFEKL did not affect airway eosinophilia (Fig. 4A) or the numbers of other BALF cell populations (data not shown). Treatment with DC/SIINFEKL, but not DC/gp33, led to a decrease in IL-5 and IL-13 levels in the BALF of WT mice (Fig. 4B), whereas DC/SIINFEKL treatment did not affect IL-5 levels in the BALF of PKO mice. Similarly, in WT mice, the percentage of mucus-producing bronchioles was decreased after treatment with DC/SIINFEKL compared with treatment with DC/PBS or DC/gp33 (Fig. 4C, 4D). In contrast, treatment with DC/SIINFEKL failed to decrease the percentage of mucus-producing bronchioles in the airway of PKO mice. Therefore, perforin expression is required for the suppression of airway inflammation by allergen-specific CTL.

Further experiments were carried out using an adoptive transfer model of in vitro-activated CTL to confirm the importance of allergen specificity and to establish whether perforin must be expressed by the CTL themselves or by other immune cell populations. Treatment of sensitized WT B6.SJL-Ptprca mice with perforin-sufficient OT-I CTL decreased the number of eosinophils in BALF and the percentage of mucus-producing bronchioles in

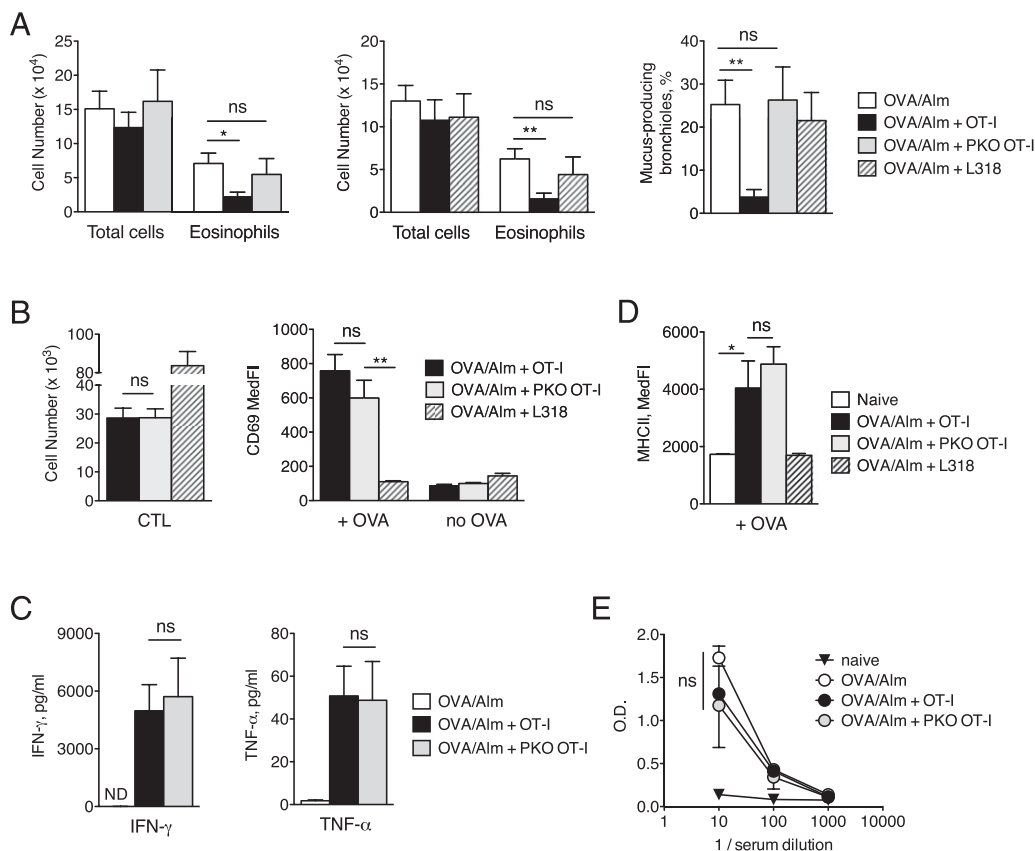


FIGURE 5. The inhibition of allergic airway inflammation requires perforin expression by allergen-specific CTL. WT mice were sensitized and challenged with low-E OVA and treated with OT-I CTL, PKO OT-I CTL, or L318 CTL, according to the protocol in Fig. 2C. **A**, The number of cells in individual BALF and the percentages of mucus-producing bronchioles in both lungs were evaluated 3 d after low-E OVA challenge. Bar graphs refer to 7–11 mice/group and show mean $\pm$ SEM for two combined experiments of several that gave similar results. **B**, CTL numbers and expression of CD69 on CTL in lung were evaluated 1 d after low-E OVA challenge. Bar graphs show numbers (left panel) and median fluorescence intensity (right panel) $\pm$ SEM for groups of three to five mice. Data are from one of two independent experiments that gave similar results. **C**, IFN- γ and TNF- α concentrations were measured 3 d after low-E OVA challenge. **D**, The expression of MHC class II on CD11b⁺CD11c⁻ lung macrophages was examined 1 d after low-E OVA challenge. Data are presented as average median fluorescence intensity $\pm$ SEM for groups of three to five mice. Data are from one of two independent experiments that gave similar results. **E**, Blood samples for OVA-specific IgE measurements were collected 1 d after low-E OVA challenge. Symbols show average OD $\pm$ SEM for groups of five mice; data are from one of two independent experiments that gave similar results. * p < 0.05, ** p < 0.01. ND, not detected; ns, not significant.

the lungs, whereas treatment with PKO OT-I CTL or with L318 CTL of irrelevant specificity was ineffective (Fig. 5A). OT-I CTL, PKO OT-I CTL, and L318 CTL could all be recovered from the lung tissue of mice undergoing allergic inflammation 1 d after i.n. challenge (Fig. 5B); these OT-I CTL and PKO OT-I CTL also had upregulated CD69 expression, indicating recognition of cognate Ag within the lung (Fig. 5B, Supplemental Fig. 3A). Analysis of BALF 72 h after OVA challenge showed high levels of IFN- γ and measurable levels of TNF- α in mice treated with OT-I and PKO OT-I CTL (Fig. 5C), further confirming that both CTL populations were activated by allergen administration. Similar data were obtained at 24 h after OVA challenge (data not shown). In contrast, IFN- γ was undetectable in the BALF of mice treated with L318 CTL (data not shown) or not treated with CTL (Fig. 5C). In line with the observed IFN- γ production, MHC class II expression on lung CD11b⁺CD11c⁻ cells was increased in mice treated with OT-I or PKO OT-I CTL, but not in mice treated with L318 CTL or not treated with CTL (Fig. 5D, Supplemental Fig. 3B). Lastly, the concentration of OVA-specific serum IgE was elevated in OVA/Alm mice compared with controls, but it was not further affected by treatment with OT-I CTL or PKO OT-I CTL (Fig. 5E).

Together, these results suggest that OT-I CTL and PKO OT-I CTL can both migrate to the lung and respond to allergen challenge. However, only perforin-sufficient OT-I CTL can suppress allergic airway inflammation. In addition, production of IFN- γ and TNF- α do not appear to correlate with the suppressive effect.

Allergen-specific treatments decrease the numbers of DC in the MLN

DC are critical for Th2 activation in the lung and consequent inflammation (4). In addition, DC are sensitive to perforin-dependent CTL-mediated killing (21, 24). Therefore, we considered the possibility that allergen-specific CTL may reduce inflammation by killing allergen-presenting DC in vivo. We examined DC number in the lung-draining MLN, because lung and airway-derived DC loaded with inhaled Ag can be demonstrated in MLN (33) (Supplemental Fig. 4). In addition, differences in the number of lung DC due to CTL-mediated killing should also be reflected in the MLN (24).

OVA/Alm-sensitized mice were instilled i.n. with OVA-AF488 on day 24. Twenty-four hours later, MLN were excised and digested, and the total number of DC was evaluated by flow cytometry (Fig. 6A). DC were examined as a whole population, rather than separately as MLN resident and tissue-derived DC as shown in Supplemental Fig. 4, because the distinction between these two populations becomes less clear in LN undergoing immune responses. As shown in Fig. 6C, DC numbers were decreased in the MLN of OT-I CTL-treated mice compared with controls; this was the consequence of a combined decrease in MLN cellularity and percentage of DC in MLN (Fig. 6B). Some decrease in DC numbers was also observed in mice treated with PKO OT-I CTL, but this was not statistically significant. In addition, we noted a decrease in the OVA-AF488 median fluorescence intensity in OT-I-treated mice (Fig. 6C), suggesting a selection against DC that had taken up high levels of OVA; however, this was not statistically significant. Together, these data suggest that successful treatment with allergen-specific CTL correlates with a decrease in the number of DC in MLN.

Discussion

In this article, we report that allergen-specific CTL can therapeutically ameliorate allergic airway inflammation by a mechanism that requires intact cytotoxic function. The therapeutic effect of CTL is mediated at the effector phase of the response, involves

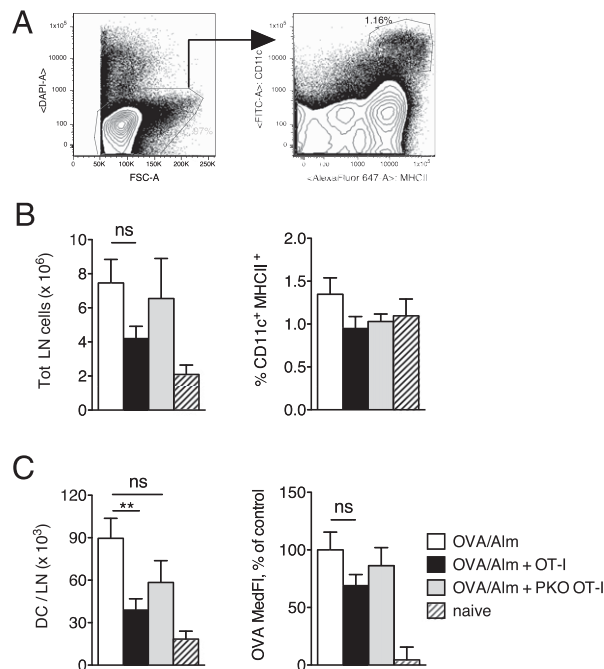


FIGURE 6. Allergen-specific CTL decrease the numbers of DC in MLN. Mice were sensitized with OVA/Alm and treated with OT-I CTL or PKO OT-I CTL, according to the protocol in Fig. 2C. Mice were challenged i.n. with 100 μ g low-E OVA-AF488, and MLN were harvested 24 h after challenge. **A**, DC were identified on the basis of CD11c and MHC-II expression. **B**, Total LN cellularity and percentage of DC in each treatment group. **C**, Number of DC/LN and uptake of fluorescent OVA by DC, as the percentage of the OVA/Alm group. Bar graphs show mean $\pm$ SEM of 8–10 mice/group from two combined experiments. $^{**}p < 0.01$. ns, not significant.

a decrease in the Th2 response in BALF, and is associated with a decline in the number of DC in the MLN. Together, these observations support the notion that specific CTL may suppress allergic airway inflammation by killing allergen-presenting DC and presumably removing the APC that elicit CD4⁺ T cell activation and effector function in the lung.

Numerous studies reported the protective function of both allergen-specific and nonspecific CTL on allergic airway inflammation. In most of those studies, the effect of CTL was found to be mediated by IFN- γ (34, 10, 32), either directly or indirectly via the induction of IL-12 production by lung DC (12). Our experiments revealed the presence of variable levels of IFN- γ in the BALF of mice treated with specific CTL; however, these did not correlate with suppression of allergic airway inflammation. IL-12 was not detected. In addition, although treatment with IFN- γ -neutralizing mAb was effective and successfully blocked the biological effects of IFN- γ on lung macrophages, it could not reverse the protective effect of allergen-specific CTL, suggesting that IFN- γ was not required for this immunotherapeutic effect. IFN- γ had an inhibitory effect in some cases, but only when using CTL that were not allergen specific together with allergen preparations that contained endotoxin (N. Enomoto and F. Ronchese, unpublished observations). This is unlike the current study, which used low-E allergen preparations. Together, these results suggest that IFN- γ cannot, by itself, suppress airway inflammation, but presumably cooperates with additional mediators or signaling pathways that are elicited by endotoxin treatment. Therefore, our studies suggest that CTL can inhibit airway inflammation by at least two separate mechanisms, one that is allergen nonspecific and endotoxin and IFN- γ dependent, and a second, previously unappreciated mechanism

that is allergen specific and endotoxin and IFN- γ independent, and requires perforin expression by CTL.

Perforin differs from other cytotoxic granule proteins, such as granzyme A, for which a proinflammatory function has also been reported (35), in that its only known function is in cell-mediated cytotoxicity. Thus, the protective effects of allergen-specific CTL on allergic airway inflammation are likely to be due to the direct killing of a cell population that is critical to the allergic response. Importantly, this protective effect is mediated at the effector stage of the allergic response; therefore, it is clearly distinct from other mechanisms invoking immune deviation of allergic Th2 cells into type 1 effector cells (36). The requirement for cytotoxic function may also reconcile the protective effects of the type 1 CTL used in our study with the exacerbation observed when type 2 CD8⁺ T cells were transferred into sensitized mice (5, 37), because Tc2 cells may lack full cytotoxic function (38, 39).

Treatment with perforin-sufficient CTL resulted in a significant decrease in the number of DC in the MLN. The number of DC in MLN is thought to reflect the migration of lung-derived DC, as indicated by the presence of inhaled OVA-AF488 in this cell population (33). In addition, our previous experiments showed that decreased numbers of DC in LN is the consequence of decreased numbers of DC in nonlymphoid tissues (24). Together, these data support the possibility that lung DC are the likely target of CTL activity, and that decreased allergic inflammation is the consequence of decreased Ag presentation to disease-mediating Th2 cells in the airway. Importantly, this observation also implies that the same DC populations that present OVA to CD4⁺ Th2 cells can also cross-present Ag to CD8⁺ T cells and become targets of CTL activity. We cannot rule out that additional cell populations that are involved in allergic airway inflammation might also become targets of allergen-specific CTL. Mast cells and basophils are reported to have cross-presenting function (40, 41); however, their sensitivity to CTL-mediated killing in vivo has not been reported, and their loss would not be expected to result in the marked phenotype described in this article. In contrast, airway DC are reportedly critical in allergic inflammation (4). In CTL-treated mice, changes in numbers of DC were already observed within 24 h of allergen challenge, a time that appears compatible with the profound effect on airway inflammation observed at day 3 following i.n. challenge.

In addition to reducing eosinophil accumulation and goblet cell hyperplasia, the removal of allergen-presenting DC might be expected to have additional effects on airway inflammation. Airway hyperresponsiveness was not examined in our study, but it is known to correlate with increased eosinophils and mucus production (42, 43), and it is also dependent on an intact lung DC compartment (4), suggesting that it may be decreased in our CTL-treated mice. Prolonged allergen presentation by DC may be required to support the retention of disease-mediating memory Th2 cells in tissues (44–46). The removal of these DC may lead to a decrease in the memory cell population in lymphoid and nonlymphoid tissues. Thus, the effects of reducing the numbers of allergen-presenting DC may provide multiple therapeutic benefits and lead to a sustained reduction of allergic airway inflammation.

Lastly, our findings highlight the importance of the context in which the first allergen exposure takes place and whether this is conducive to the activation of allergen-specific CTL (47). For example, exposure to allergen in a context that supports CTL activation, such as i.p. immunization with high Ag doses, leads to weaker allergic responses than when CTL are not induced (48). The resolution of inflammation may also be accelerated. We propose that establishing whether environmental exposure to allergens results in the activation of allergen-specific effector CTL

may, in some cases, help to explain the appearance and severity of allergic inflammation.

In summary, we report that treatment with allergen-specific CTL suppresses allergic airway inflammation and mucus production via a perforin-dependent mechanism. We suggest that this previously unappreciated mechanism of regulation of the immune response may be relevant to the pathogenesis of allergic asthma. Unlike steroid treatment, immune therapy using allergen-specific CTL is not expected to be broadly immunosuppressive, and it may lead to improved treatment for asthma and other allergic diseases.

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Disclosures

The authors have no financial conflicts of interest.

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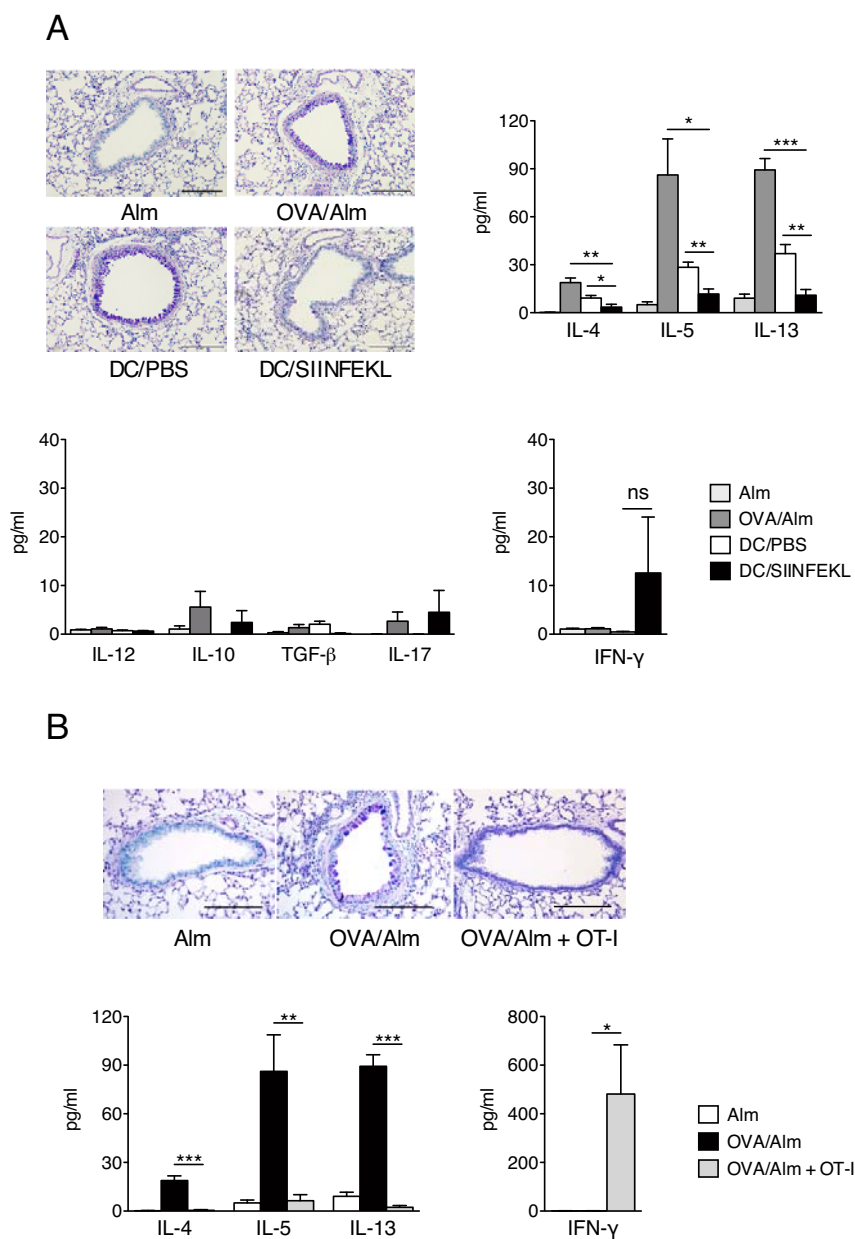


Figure S1. Intranasal treatment with DC loaded with an MHC-I allergen epitope, or adoptive transfer of allergen-specific CTL, reduce goblet cell hyperplasia and Th2 cytokines in the airway

Mice were sensitized with OVA/Alm or Alm alone, and treated with DC/PBS or

DC/SIINFEKL (A), or injected with OVA-specific OT-I CTL (B), according to the protocols shown in Figure 2A and 2C. Three d after i.n. challenge, mucus-producing goblet cells in bronchioles were evaluated on histological specimens stained with AB-PAS. Representative bronchioles from each experimental group are shown; bars correspond to 200 μ m. The corresponding average data are shown in Figure 2A and 2C. The concentrations of IL-4, IL-5, IL-13, IL-10, TGF- β and IL-17 in BALF were evaluated 3 d after OVA challenge, while IL-12p70 and IFN- γ were assessed 6h and 24h after OVA challenge, respectively. (A) Bar graphs show mean + SEM for 5 mice/group. (B) Bar graphs show mean + SEM for groups of 7-9 mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

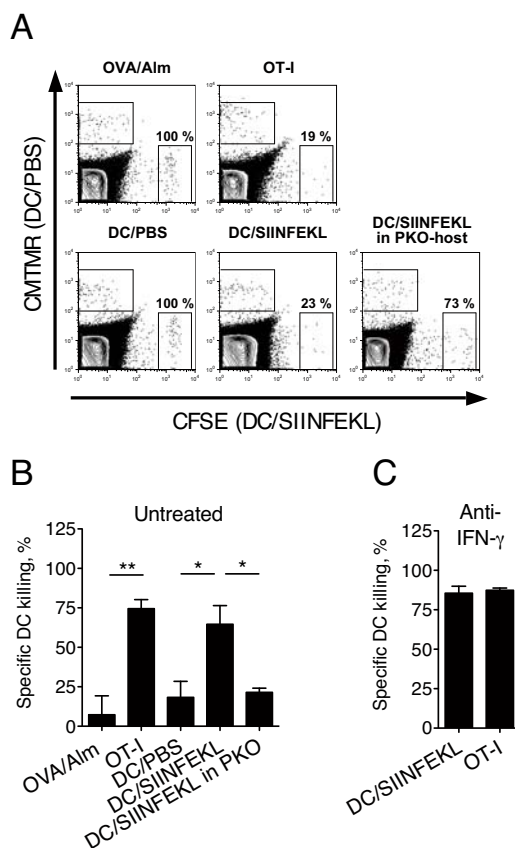


Figure S2. OVA-specific DC killing activity is not affected by treatment with IFN- γ -neutralizing mAb but is abrogated in PKO mice

WT or PKO mice were sensitized with OVA/Alm, and treated with OT-I CTL as described in Figure 1A, or with DC/PBS or DC/SIINFEKL as described in Figure 2A. On d 24, mice were injected i.v. with a mixture of target DC that were CFSE-labeled and loaded with SIINFEKL, or CMTMR-labeled and left untreated. Forty-eight h after target DC injection, MLN were collected and analyzed for the presence of fluorescent cells by flow cytometry. (A) Representative contour plots of live-gated total MLN cells from individual mice. The percentages of remaining CFSE⁺ DC/SIINFEKL are shown in each plot. (B) Normalized OVA-specific DC killing.

Bars show the mean $\pm$ SEM for 4 mice/group; **, $p < 0.01$. (C) Normalized OVA-specific DC killing in mice that had been injected i.p. with 500 μ g IFN- γ -neutralizing mAb 4 h before the i.v. transfer of target DC. Bars show mean $\pm$ SEM for 4-5 mice/group.

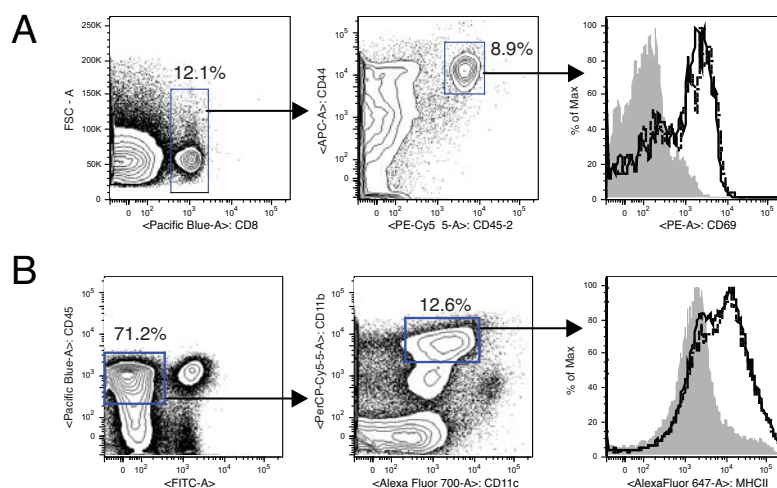


Figure S3: Expression of CD69 on CTL in lung tissue, and MHCII on lung macrophages in CTL-treated mice.

Mice were treated as in Figure 5. Lungs were collected one day after i.n. challenge with low-E OVA, and processed into single cell suspensions. (A) Live cells were identified by DAPI exclusion and donor CTL were identified as CD8⁺ and CD45.2⁺CD44^{high} cells. Expression of CD69 on the gated populations is shown in the histogram: L318 CTL, solid gray histogram; OT-I, black line; PKO OT-I, dashed black line. (B) Live cells were identified by DAPI exclusion, and macrophages were identified as low-autofluorescent CD45⁺ cells which were CD11b^{high} CD11c^{int}. Expression of MHCII on the gated populations is shown in the histogram: L318 CTL, solid gray histogram; OT-I, black line; PKO OT-I, dashed black line.

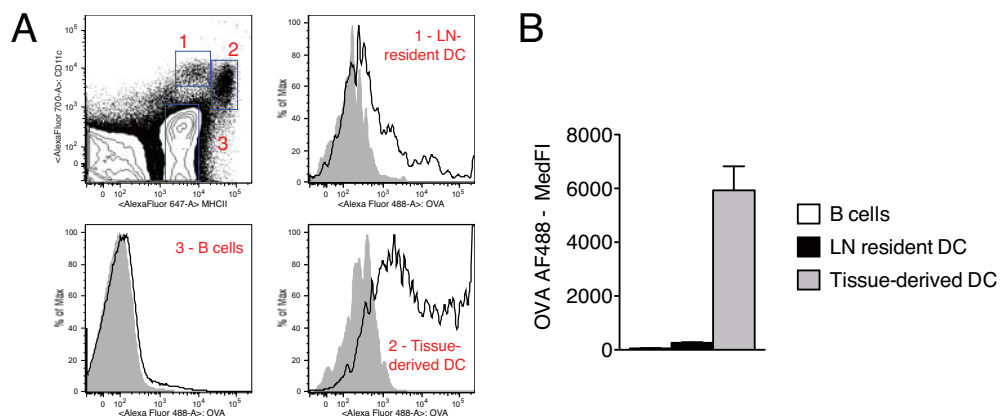


Figure S4. OVA administered i.n. is preferentially located in a subpopulation of tissue-derived DC in the MLN.

Panels on the left show gating of the various APC populations in LN cell suspensions from mice injected i.n. with 100 ug OVA-AF488 or PBS vehicle 24h previously. 1) LN resident DC; 2) tissue-derived DC; 3) B cells. Histogram overlays show the OVA-AF488 fluorescence of each APC population isolated from mice that received i.n. PBS (solid gray histograms) or OVA-AF488 (empty histograms). The bar graph on the right shows the average + SE of the OVA-AF488 Median Fluorescence Intensity for each APC population; data refer to groups of 4 mice.