

# Bystander suppression of allergic airway inflammation by lung resident memory CD8+ T cells

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CD8+ memory T cells have recently been recognized as playing a key role in natural immunity against unrelated viral infections, a phenomenon referred to as "heterologous antiviral immunity." We now provide data that the cellular immunological interactions that underlie such heterologous immunity can play an equally important role in regulating T helper 2 immune responses and protecting mucosal surfaces from allergen-induced inflammation. Our data show that CD8+ T cells, either retained in the lung after infection with influenza virus, or adoptively transferred via the intranasal route can suppress allergic airway inflammation. The suppression is mediated by IFN- $\gamma$ , which acts to reduce the activation level, T helper 2 cytokine production, airways hyperresponsiveness, and migration of allergen-specific CD4+ T cells into the lung, whereas the systemic and draining lymph node responses remain unchanged. Of note, adoptive transfer of previously activated transgenic CD8+ T cells conferred protection against allergic airway inflammation, even in the absence of specific-antigen. Airway resident CD8+ T cells produced IFN- $\gamma$  when directly exposed to conditioned media from activated dendritic cells or the proinflammatory cytokines IL-12 and IL-18. Taken together these data indicate that effector/memory CD8+ T cells present in the airways produce IFN- $\gamma$  after inflammatory stimuli, independent of specific-antigen, and as a consequence play a key role in modifying the degree and frequency of allergic responses in the lung.

Recent investigations have shown that memory CD8+ T cells are retained in peripheral tissues for long periods after infection (1, 2). It is normally hypothesized that these memory T cells allow the host to respond efficiently to subsequent infections against the same virus; however, Chen *et al.* (3) have identified a secondary role for these CD8+ T cells as demonstrated by their activation and contribution to protective immunity against unrelated viral infections. The term "heterologous antiviral immunity" is used to describe this phenomenon. Intriguingly, it has long been known that viral infections have the potential to influence unrelated immune responses for better or worse. Experimental and epidemiological data show that infection of asthmatic individuals with respiratory viruses (4) such as influenza A (5) and respiratory syncytial virus (6) can greatly exacerbate the development and pathology of asthma. However, in international epidemiological studies of exposure rates, the indication is that some types of infection may actually decrease the prevalence of asthma (7–9). The term "hygiene hypothesis" was coined (10) to describe the inverse relationship between decreased incidence of diseases such as tuberculosis (11) and asthma. It was suggested that the removal of such diseases in the western world removed a key immunoregulatory influence, which has led to the increased incidence of allergic airway disease. The precise nature of these immunoregulatory influence(s) has remained elusive, although experimental investigations have identified the inflammatory cytokine IFN- $\gamma$  as an important mediator of the phenomenon (12).

We hypothesized that viral infections, which stimulate the conditions for heterologous immunity, may have the potential to influence the cellular processes involved in the development of allergen-induced airway inflammation. In particular, we inves-

tigated the effect of previous influenza A virus infection on the development of allergic airway inflammation in mice.

We report here that local production of IFN- $\gamma$  by CD8+ T cells, either resident in the airways after influenza infection, or specifically adoptively transferred into the airways, can regulate allergic immune responses. Specifically, effector/memory CD8+ T cells reduce the activation status and suppress the migration of allergen-specific CD4+ T cells into the airways. The consequence of this immunomodulation is reduced eosinophil migration, airways hyperresponsiveness, and T helper 2 (Th2) cytokine production. This work identifies a key mechanism whereby CD8+ T cells, which underpin heterologous antiviral immunity, may have the equally important role of regulating the development of allergic immune responses in the lung and preventing the development of asthma. In addition, our intranasal (i.n.) adoptive transfer study demonstrates that localization of effector/memory cells in the lung and airways is a powerful regulator of local responses and should be considered in future vaccine design and cell therapy.

## Materials and Methods

**Mice.** C57BL/6J mice were originally obtained from The Jackson Laboratory. Strain 318 mice, transgenic for a T cell antigen receptor (TCR) specific for H-2 D<sup>b</sup> plus fragment 33–41 of the lymphocytic choriomeningitis virus glycoprotein (LCMV<sub>33–41</sub>) were kindly provided by H. Pircher (University of Freiburg, Freiburg, Germany). Breeding of all mice was carried out at the Biomedical Research Unit of the Wellington School of Medicine. All animal experimental procedures were approved by the Wellington School of Medicine Animal Ethics Committee and conducted in accordance with the guidelines of the University of Otago (Dunedin, New Zealand).

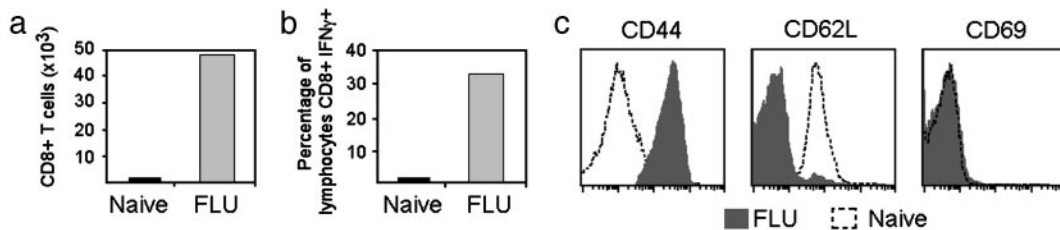
**Infection with Influenza Virus.** On day 0 mice were anesthetized by i.p. injection of a mixture of ketamine and xylazine (Phoenix, Auckland, New Zealand), and 12 hemagglutinating units of A/HKx31 (H3N2) influenza A virus, in a 30- $\mu$ l volume of PBS, was administered by i.n. inoculation.

**Purification of *Nippostrongylus brasiliensis*-Derived Allergen (NES).** L3 infective larvae were washed in sterile PBS and then washed several more times in an antibiotic mixture [RPMI medium 1640/1/20 penicillin–streptomycin/1/100 gentamycin (GIBCO/BRL)]. The worms were then cultured in medium (RPMI medium 1640/1/100 penicillin–streptomycin/1/100 gentamycin) supplemented with 1% glucose at 37°C for 48 h. The supernatant was concentrated and amount of protein NES determined by absorbance at 280 nm.

Abbreviations: NES, *Nippostrongylus brasiliensis*-derived allergen; TCR, T cell antigen receptor; BAL, bronchoalveolar lavage; LPS, lipopolysaccharide; i.n., intranasal; DC, dendritic cell; Th2, T helper 2; IMDM, Iscove's modified Dulbecco's medium.

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**Fig. 1.** A population of CD8<sup>+</sup> T cells with an effector/memory phenotype remain in the airways after influenza virus infection. After influenza infection (25 days), BAL was performed on naive and previously infected mice. (a) Total CD8<sup>+</sup> T cell numbers in pooled samples were determined by flow cytometry. (b) Pooled BAL cells were stimulated at 37°C by anti-CD3 in the presence of IL-2 and anti-CD28 for a total of 6 h. IFN- $\gamma$  production by CD8<sup>+</sup> T cells was determined by flow cytometry. (c) Surface expression of CD44, CD62L, and CD69 was determined for gated CD8<sup>+</sup> cells in the postinfection group by flow cytometry. CD8<sup>+</sup> T cells from pooled peripheral lymph nodes were used as naive controls. Insufficient cells were present in the airways of naive mice to measure IFN- $\gamma$  production or surface marker staining. The data shown are from independent experiments using three to five mice per group. Each experiment was repeated three times with similar results. Values are representative of individual mice.

**NES-Induced Airway Inflammation.** Mice were injected i.p. with 0.2  $\mu$ g of NES in 200  $\mu$ l of alum adjuvant (Serva) 14 days after influenza- or mock-infection. Then 7 days later mice were anesthetized, and 5  $\mu$ g of NES in a 50- $\mu$ l volume of PBS was administered i.n.

**Quantitative and Qualitative Measurement of Inflammatory Cells in the Bronchoalveolar Lavage (BAL).** At 4 days after i.n. challenge with NES, mice were killed with a lethal dose of anesthetic, and BAL was performed as described (13).

**Histological Analysis.** Lungs were fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin wax. Sections were cut and stained with hematoxylin and eosin by using standard histological protocols.

**Measurement of Airway Responsiveness.** On day 4 after i.n. challenge with NES, mice were placed in individual unrestrained whole body plethysmograph chambers (Buxco Electronics, Petersfield, U.K.). Airway responsiveness was assessed in mice by inducing airflow obstruction with aerosolized methylcholine-chloride and enhanced pause (PenH) measured by using BIOSYSTEM XA software (Buxco Electronics, Sharon, CT).

**Serum IgE ELISA.** A sandwich ELISA was used to measure total IgE levels in the serum as described (12).

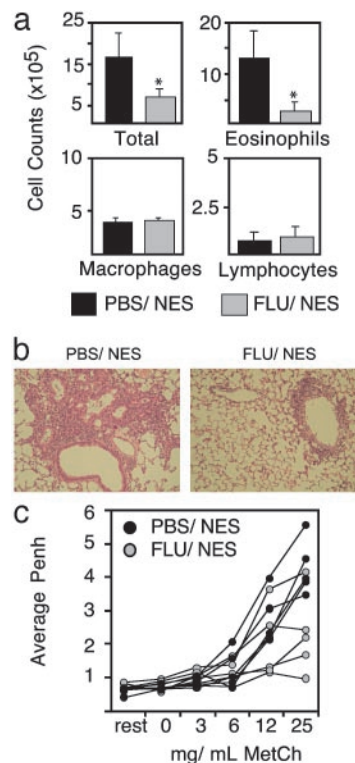
**Fluorescence-Activated Cell Sorter Analysis.** Anti-CD44-phycoerythrin (PE), anti-CD62L-PE, anti-CD69-PE, anti-IFN- $\gamma$ -PE, and anti-IL-4-FITC Abs were obtained from Pharmingen. Anti-CD8 (2.43) and anti-CD4 (GK1.5) (kindly provided by Fred Finkelman, Cincinnati Veterans Affairs Medical Center, Cincinnati) were affinity-purified from hybridoma culture supernatants. Flow cytometric analysis was performed on a FACSort (Becton Dickinson).

**Intracellular Cytokine Staining.** Intracellular staining was performed as described (13).

**In Vivo IFN- $\gamma$  Neutralization.** At 14 days after influenza- or mock-infection, mice were injected with 0.5 mg of anti-IFN- $\gamma$  (XMG-D6, kindly provided by Fred Finkelman) i.p. for 3 consecutive days and then again every third day until the mice were killed. Anti-IFN- $\gamma$  Ab was affinity-purified from hybridoma culture supernatants as described above.

**Preparation of CD8<sup>+</sup> T Cells for Adoptive Transfer.** Spleens from L318 transgenic mice were removed, homogenized into a single cell suspension, and lysed to remove RBCs. Cells were resuspended at  $1 \times 10^6$ /ml in 1 ml of Iscove's modified Dulbecco's

medium (IMDM) (Sigma-Aldrich) and dispensed into 24-well plates (Falcon) previously coated with 10  $\mu$ g/ml anti-CD3. IMDM (1 ml) containing 10 units/ml IL-2 and 10 ng/ml IL-6 (x63J-mIL6) was added to each well. Cells were incubated for 5 days and cytokines replenished on days 2 and 4 by replacing 1 ml of media per well. On day 5 cells were harvested and cultured in 24-well plates containing 10 units/ml IL-2, with replacement of



**Fig. 2.** Allergen-induced migration of eosinophils into the airways is reduced in mice previously infected with influenza virus. Mice were infected i.n. with influenza virus or mock-infected with PBS. Groups were sensitized i.p. (14 days later) with 0.2  $\mu$ g of NES in alum adjuvant followed by an i.n. challenge of 5  $\mu$ g of NES in PBS 7 days later. (a) Infiltration of inflammatory cells into the airways was determined by differential cell counts of total BAL cells. (b) Hematoxylin/eosin staining of formalin-fixed, paraffin-embedded tissue 4 days after i.n. NES challenge. (c) After NES challenge (4 days), mice were exposed to increasing doses of nebulized methylcholine, and airway hyper-responsiveness was measured in an unrestrained whole body plethysmograph. All data shown are from representative experiments using three to five mice per group. These experiments were repeated with similar results. \*,  $P < 0.03$ , as determined by the Student  $t$  test between the PBS/NES and FLU/NES groups.

IL-2 after 24 h. Cells were washed in IMDM before use and determined to be >80% CD8+ by flow cytometry.

**Preparation of Dendritic Cell (DC) Supernatant.** Bone marrow cells from C57BL/6J mice were cultured in six-well plates (Falcon) at  $4 \times 10^5$  cells per ml in IMDM (Sigma-Aldrich) containing 20 ng/ml IL-4 and 20 ng/ml granulocyte/macrophage colony-stimulating factor, as described (14). Cultures were provided with fresh IMDM and cytokines every 2 days and incubated at 37°C for a total of 7 days. Lipopolysaccharide (LPS, 100 ng/ml) from Sigma was added to the media 16 h before harvest. The supernatant was removed, filtered, and stored at -20°C until required.

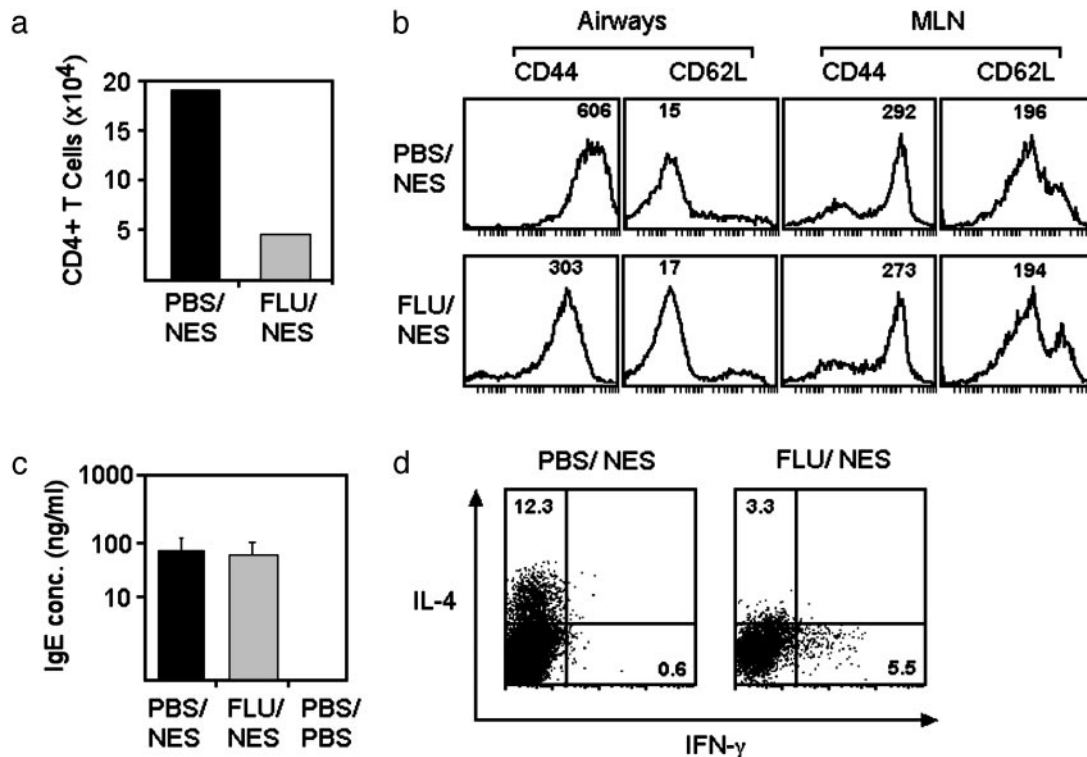
**Activation of BAL Lymphocytes with Conditioned Media.** Approximately  $5 \times 10^5$  BAL cells were immediately fixed *ex vivo* in 4% paraformaldehyde, and the remaining BAL cells were incubated at 37°C for 2 h to remove macrophages. Floating lymphocytes were resuspended at  $5 \times 10^5$  cells per ml in either DC supernatant or media supplemented with 100 ng/ml IL-18 (Pepro-Tech, Rocky Hill, NJ) and/or 10 ng/ml IL-12 (PeproTech). These cells were then plated in 24-well plates (Falcon) at  $5 \times 10^5$  cells per well and incubated overnight at 37°C. Monensin (Calbiochem-Novabiochem) was added in the last 2 h of the culture.

**Statistics.** Statistical significance was analyzed by the Student *t* test. Unless otherwise indicated, data represent mean  $\pm$  SD, with statistical significance defined at  $P < 0.05$ .

## Results and Discussion

### Memory CD8+ T Cells Remain in the Airways After Influenza Infection.

We extensively analyzed the cell types present in the airways of naive mice and compared them to cell types found in the airways of mice that had recovered from an infection with influenza virus. In our model of experimental influenza infection, infectious virions were cleared within 7–9 days, and the primary immune response subsided within 14 days (data not shown). Negligible numbers of haemopoietic cells ( $< 2 \times 10^4$  total cells per BAL) were found in the airways of naive mice and were comprised of predominantly alveolar macrophages. In striking contrast, a significant population of CD8+ T cells could still be detected in the airways of mice 25 days after infection with influenza virus (Fig. 1*a*) and remained for >100 days (data not shown). A large proportion of the CD8+ T cells isolated from the airways at day 25 after infection had the potential to produce IFN- $\gamma$  after 6-h restimulation on anti-CD3, as shown by intracellular staining (Fig. 1*b*). Furthermore, these cells expressed high levels of CD44 and low levels CD62L and CD69 (Fig. 1*c*), had a small resting size as determined by flow cytometry, and specifically lysed influenza-peptide-pulsed target cells after *ex vivo* activation with plate-bound anti-CD3 (data not shown). Together these data indicated that after the resolution of influenza virus infection, a population of influenza-specific CD8+ memory T cells resided in the airways. These data are supported by recent studies showing that virus-specific CD8+ T cells reside for long periods in both lymphoid and nonlymphoid tissues (1). Moreover, studies by Hogan *et al.* (15) identified a



**Fig. 3.** Previous infection with influenza virus reduces CD4+ T cell migration into and activation within the airways. Mice were infected i.n. with influenza virus or mock-infected with PBS. Both groups were sensitized i.p. (14 days later) with 0.2  $\mu$ g of NES in alum adjuvant followed by an i.n. challenge of 5  $\mu$ g of NES in PBS 7 days later. (a) The numbers of CD4+ T cells infiltrating the airways was determined by flow cytometric analysis of pooled BAL cells taken from mice 4 days after i.n. NES challenge. (b) The expression of CD44 and CD62L on gated CD4+ T cells from pooled BAL and mediastinal lymph node samples was determined by flow cytometry. The numerical indices represent the geometric mean of the fluorescence intensity for each sample. (c) Total IgE levels in the serum of mice that had been sensitized and challenged to NES, with or without previous influenza infection, was measured by ELISA. Serum IgE in mice that had not been sensitized and challenged to NES was below the limit of detection. (d) Airway lymphocytes were stimulated for 6 h with DCs pulsed with NES, and IL-4/IFN- $\gamma$  production was determined by flow cytometry. All data shown are from representative experiments using four to six mice per group. Each experiment was repeated with similar results.



substantial population of antigen-specific T cells in the lung that persisted for several months after recovery from an influenza virus infection.

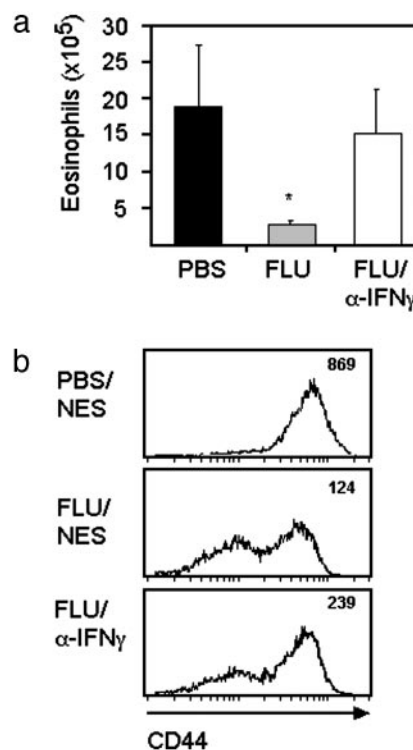
**Previous Infection by Influenza Virus Suppresses the Induction of Allergic Airway Inflammation.** It has previously been shown that memory CD8<sup>+</sup> T cells can influence the outcome of unrelated viral infections (3); however it is unclear whether such memory cells can influence the development of allergy. We tested whether the presence of influenza-specific effector/memory CD8<sup>+</sup> T cells could modify a subsequent allergen-induced, Th2-dependent allergic immune response in the airways. After influenza infection (14 days), mice were sensitized for a subsequent Th2 immune response by i.p. immunization with allergens extracted from NES absorbed on alum adjuvant. Later (7 days) these sensitized mice were challenged i.n. with NES in PBS, and subsequent airway inflammation was analyzed. The i.n. challenge with NES induced substantial infiltration of lymphocytes and eosinophils into the airways with the peak response occurring at day 4 (Fig. 2*a* and data not shown). Mice that had previously been infected with influenza showed a dramatic reduction in the degree of NES-induced infiltration of eosinophils in both the airways (Fig. 2*a*) and the lung parenchyma (Fig. 2*b*) in comparison to uninfected, but similarly NES-challenged mice. We have found a comparable suppression >100 days after infection and in addition have tested other allergen models such as ovalbumin and found a similar suppression after influenza infection (data not shown). The hallmark feature of allergic asthma is airways hyperresponsiveness, and accordingly, we sought to investigate whether this IL-13-dependent response was also reduced after influenza infection. In line with the reduced infiltration of eosinophils, the response to increasing doses of methyleholine was similarly reduced in mice which had previously been infected with influenza (Fig. 2*c*). We have also investigated whether the timing of allergen sensitization relative to the infection was important and found that the outcome was comparable irrespective of whether mice were sensitized before or after infection (data not shown). Taken together, these data show that the presence of CD8<sup>+</sup> memory T cells in the lung is associated with suppressed allergen-induced airway inflammation.

**Previous Infection with Influenza Virus Reduces Allergen-Induced CD4<sup>+</sup> T Cell Migration into, and Activation Within, the Airways.** Allergen-specific CD4<sup>+</sup> T cells producing IL-4 and IL-5 have been demonstrated to be major inducers of airway eosinophilia (16). In further support of this, consistently elevated numbers of CD4<sup>+</sup> T cells have been found in BAL fluids and bronchial biopsies from asthmatics (17). We therefore investigated the effect of previous influenza infection on CD4<sup>+</sup> T cell migration, activation state, and cytokine production in the airways. After sensitization and challenge with NES, we found that mice previously infected with influenza had reduced numbers of CD4<sup>+</sup> T cells in the airways (Fig. 3*a*). Analysis of extended time points confirmed that this result was not due to delayed entry of CD4<sup>+</sup> T cells into the airways (data not shown). Furthermore, the expression of CD44 on the CD4<sup>+</sup> T cells that migrated into the airways was lower in comparison with uninfected but similarly challenged control mice (Fig. 3*b*). Expression of CD62L was comparable between groups (Fig. 3*b*). No such reduction in CD44 expression was detected on CD4<sup>+</sup> T cells from the draining lymph node (Fig. 3*b*), nor was there any change in serum IgE levels (Fig. 3*c*). These data indicate that suppression of allergen-specific CD4<sup>+</sup> T cell activation is limited to the lung environment after influenza infection. These data were confirmed with two TCR-transgenic T cell adoptive transfer models where specific T cells can be tracked *in vivo* by using mAbs

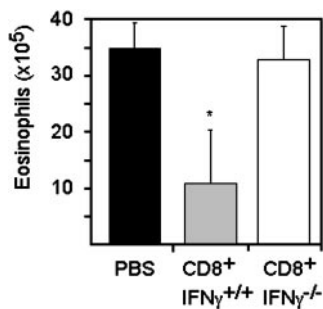
directed against the specific V $\alpha$ V $\beta$  TCR chains (data not shown).

To establish whether the CD4<sup>+</sup> T cells in the airways exhibited full Th2 effector function, we isolated lymphocytes from the airways of mice previously infected with influenza or mock-infected with PBS, and restimulated them for 6 h by using DCs pulsed with NES. We found that NES-specific CD4<sup>+</sup> T cells showed strong Th2 polarization as evidenced by a large proportion of cells producing IL-4 (Fig. 3*d*). In striking contrast, a reduced proportion of CD4<sup>+</sup> T cells isolated from the airways of mice previously infected with influenza produced IL-4, and in addition, a proportion of NES-specific cells producing IFN- $\gamma$  were also detected (Fig. 3*d*). Taken together with the reduced total number of CD4<sup>+</sup> T cells detected in the airways after influenza infection, overall Th2 cytokine production is reduced even further. This reduction in Th2 cytokine production is likely to be responsible for the previously observed reduction in eosinophil accumulation and development of airway hyperresponsiveness.

**IFN- $\gamma$  Mediates the Influenza-Induced Inhibition of Allergic Airway Inflammation.** IFN- $\gamma$  is produced in large quantities by CD8<sup>+</sup> T cells during influenza virus infection (18). IFN- $\gamma$  is also capable of inhibiting the differentiation of *in vitro*-derived Th2 cells (19)



**Fig. 4.** Anti-IFN- $\gamma$  treatment abrogates influenza-induced suppression of eosinophil migration and CD44 expression on allergen-induced CD4<sup>+</sup> T cells in the airways. Mice were infected i.n. with influenza virus or mock-infected with PBS. All groups were sensitized i.p. (14 days later) with 0.2  $\mu$ g of NES in alum adjuvant followed by an i.n. challenge of 5  $\mu$ g of NES in PBS 7 days later. The anti-IFN- $\gamma$  group received 0.5 mg of XMG-D6 Ab at days 14, 15, 16, 19, and 22 after infection. (a) Infiltration of eosinophils into the airways was measured 4 days after i.n. NES challenge. (b) Expression of CD44 on gated CD4<sup>+</sup> T cells was determined by fluorescence-activated cell sorter analysis of pooled BAL samples. The numerical indices represent the geometric mean of the fluorescence intensity for each sample. The data shown are from representative experiments using three to five mice per group. Each experiment was repeated with similar results. Statistical significance ( $P < 0.02$ ) as determined by the Student  $t$  test between the PBS/NES and FLU/NES groups is designated by \*.



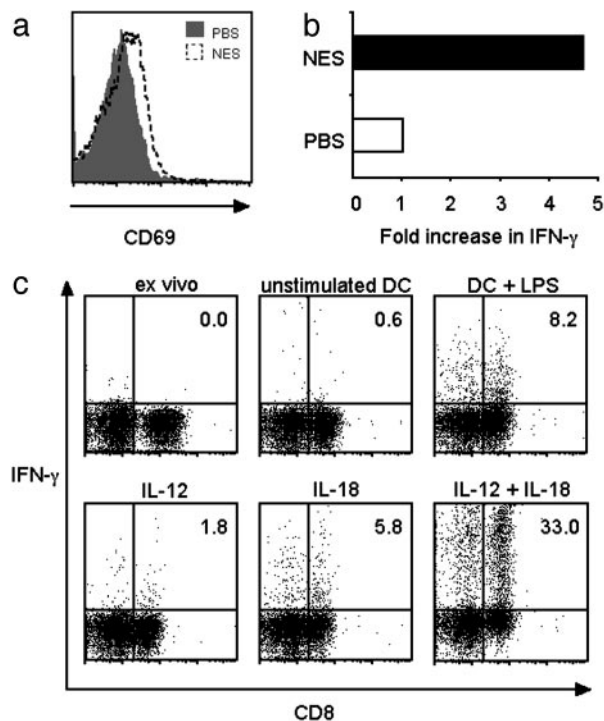
**Fig. 5.** Adoptive transfer of activated CD8+IFN-γ+ T cells via the i.n. route inhibits NES allergen-induced airway eosinophilia in an IFN-γ-dependent manner. Splenocytes from L318 × IFN-γ<sup>-/-</sup> or WT L318 mice were stimulated *in vitro* with anti-CD3 in the presence of IL-2 and IL-6 for 5 days. The proportion of CD8+ T cells present in each culture was determined by flow cytometry. PBS, 5 × 10<sup>5</sup> CD8+ IFN-γ<sup>+/+</sup> cells or 5 × 10<sup>5</sup> CD8+ IFN-γ<sup>-/-</sup> cells were administered i.n. to C57BL/6J mice previously sensitized i.p. with 0.2 μg of NES in alum. At 1 days after adoptive transfer, mice were challenged i.n. with NES in PBS. Infiltration of eosinophils into the airways was measured 4 days after NES challenge by BAL. The data shown are from a representative experiment using three to five mice per group. The experiment was repeated three times with similar results. Statistical significance ( $P < 0.02$ ) as determined by the Student *t* test between the PBS and CD8 IFN-γ<sup>+/+</sup> groups is designated by \*.

and regulating Ab isotype switching (20). We therefore investigated the role of IFN-γ during the influenza-induced suppression of CD4+ T cell activation and consequent reduction of allergic airway eosinophilia. For this purpose, IFN-γ activity was neutralized by using *in vivo* mAb treatment 14 days after infection and throughout NES-allergen sensitization and i.n. challenge. Neutralization of IFN-γ restored NES-induced allergic airway eosinophilia in mice previously infected with influenza (Fig. 4a). We have previously found that IFN-γ neutralization has no effect on the development of airway eosinophilia in uninfected animals (data not shown). IFN-γ also plays a role in the influenza-induced reduction of CD44 expression on allergen-specific CD4+ T cells, as this was partially restored in anti-IFN-γ mAb-treated mice (Fig. 4b). These data strongly indicate that IFN-γ plays a role in the reduction of Th2 immune responses and airway eosinophilia in mice that had recovered from previous influenza infection. A similar role for IFN-γ has been shown by using a model in which bacillus Calmette–Guérin infection resulted in a reduced allergic airway eosinophilia (12).

**Adoptively Transferred *In Vitro* Cultured CD8+ T Cells Are Capable of Suppressing Allergic Airway Eosinophilia in an Antigen-Independent but IFN-γ-Dependent Manner.** We sought to determine whether the population of CD8+ T cells resident in the airways of mice previously infected with influenza virus was directly responsible for the reduction in allergic airway eosinophilia. To this end, we attempted to deplete CD8+ T cells *in vivo* by using an anti-CD8 mAb depletion protocol. Anti-CD8 mAb treatment completely removed CD8+ T cells from lymphoid organs; however, depletion of the memory CD8+ T cell population in the airways was not effective, even after using large doses of affinity purified mAb (data not shown). We thus developed a model involving the adoptive transfer of *in vitro*-activated CD8+ T cells directly into the airways as a means of investigating the potential of these cells for inhibiting allergic airway eosinophilia. Firstly, C57BL/6J mice were sensitized with an i.p. injection of NES absorbed on alum adjuvant. Six days later 5 × 10<sup>5</sup> *in vitro*-activated CD8+ T cells from LCMV<sub>33–41</sub> TCR transgenic or LCMV<sub>33–41</sub> TCR transgenic × IFN-γ<sup>-/-</sup> mice were administered via the i.n. route. The following day, mice were challenged i.n. with NES, and 4 days later the infiltration of eosinophils was determined (Fig. 5). Adoptive transfer of *in vitro*-activated CD8+ T cells before i.n.

administration of NES suppressed the development of an airway eosinophilia. This suppression depended on the action of IFN-γ as the adoptive transfer of *in vitro*-activated CD8+ IFN-γ<sup>-/-</sup> T cells failed to suppress the eosinophilia (Fig. 5). The adoptively transferred CD8+ T cells did not constitutively produce IFN-γ (data not shown) and remarkably did not require *in vivo* exposure to their specific antigen to mediate this suppression. Thus, it was likely that these cells were induced to secrete IFN-γ after nonspecific stimuli received after i.n. challenge with allergen.

**Airway-Resident Effector Memory CD8+ T Cells Can Be Activated to Produce IFN-γ in the Absence of Specific Antigen.** We observed that airway resident CD8+ T cells up-regulated surface expression of CD69 during NES-induced airway inflammation, indicating recent activation (Fig. 6a). Furthermore, CD8+ T cells isolated from the airways 6 h after i.n. NES challenge were found to produce IFN-γ as shown by direct *ex vivo* intracellular cytokine staining (Fig. 6b). To establish what factors were responsible for this activation, we isolated airway resident CD8+ T cells from previously infected mice and analyzed them *in vitro*. It was possible that the influenza-induced CD8+ T cells were cross-reactive with NES epitopes and were thus activated through TCR signaling; however, because suppression of allergic airway



**Fig. 6.** Airway resident effector memory CD8+ T cells can be stimulated to produce IFN-γ in the absence of specific antigen. After influenza infection (14 days) mice were sensitized i.p. with 0.2 μg of NES in alum adjuvant or alum adjuvant alone, followed by i.n. challenge with 5 μg of NES in PBS or PBS alone 7 days later. (a) The surface expression of CD69 on CD8+ T cells was determined by flow cytometry. (b) BAL was performed and lymphocytes were isolated 6 h after NES i.n. challenge. IFN-γ cytokine production by CD8+ T cells was determined directly *ex vivo* by intracellular cytokine staining and flow cytometry. (c) After influenza infection (25 days), airway resident lymphocytes were isolated by BAL and samples pooled. These lymphocytes were then cultured overnight in supernatant from unstimulated DCs, LPS-activated DCs, or in media supplemented with IL-18 and/or IL-12 overnight. IFN-γ production by CD8+ T cells was determined by flow cytometry. Numerical indices represent percentage of CD8+ T cells producing IFN-γ. The data shown are from representative experiments using three to five mice per group (a and b) or cells from pooled BAL samples of 25 mice (c). Each experiment was repeated with similar results.

inflammation was also found when moth cytochrome C<sub>88-103</sub> or OVA<sub>323-339</sub> peptides were used as allergens (data not shown), we reasoned that these effector memory CD8<sup>+</sup> T cells may be activated in the absence of specific antigen by inflammatory cytokines. We thus isolated airway-resident lymphocytes 25 days after influenza infection and analyzed the ability of various stimuli and supernatant fractions to activate memory CD8<sup>+</sup> T cells directly. For this purpose bone marrow-derived DCs were cultured *in vitro* for 6 days and then activated by addition of LPS. Supernatants were collected after 16 h of culture and filtered. Isolated airway resident lymphocytes were then cultured overnight in this conditioned media or in fresh media supplemented with recombinant IL-18 and/or IL-12. Supernatants from DCs activated with LPS induced significant production of IFN- $\gamma$  by CD8<sup>+</sup> T cells, as did the cytokines IL-18 and IL-12 (Fig. 6c). However, supernatant from resting DCs did not induce IFN- $\gamma$  production by the effector memory CD8<sup>+</sup> T cells (Fig. 6c). We also found that the *in vitro*-cultured CD8<sup>+</sup> T cells described in Fig. 5 responded to these stimuli in a comparable manner to the influenza-induced effector memory CD8<sup>+</sup> T cells (data not shown). IL-18 is produced by a variety of cell types in response to LPS (21), Fas ligand stimulation (22), or histamine (23). Furthermore, human macrophages have been shown to secrete IL-18 when infected *in vitro* with influenza A (24, 25). Thus, the response of the innate immune system to pathogens or allergens in the airways could be a potent source of IL-18, which may stimulate tissue-resident memory CD8<sup>+</sup> T cells to produce IFN- $\gamma$  and result in a localized IFN- $\gamma$  dominant cytokine milieu. Of importance, such a milieu is likely to make the airways refractory to Th2 responses, and indeed we have shown that IFN- $\gamma$  is directly responsible for inhibiting the development of Th2-mediated airway inflammation. Of note, a minor population of CD8<sup>-</sup> lymphocytes was also activated to produce IFN- $\gamma$  by

these stimuli. Further analysis showed that this population of cells was CD4<sup>+</sup> (Fig. 6c and data not shown). We consider it likely that these cells may also play a role in the heterologous immunity that protects against allergic airway inflammation; however because of the small size of this cell population, their role is likely to be secondary, but complementary, to the CD8<sup>+</sup> memory T cell response.

It was recently reported that the timing of respiratory syncytial virus infection during development has distinct effects on subsequent immune responses in mice (26). Indeed, experimental and epidemiological observations lead to the hypothesis that sites of infection, genetic predisposition, and the timing, type, and severity of infections are key factors in determining the development of asthma. The lung and airways may be imprinted by their infection history and consequently strongly bias the nature of subsequent immune responses. We provide evidence that strengthens this hypothesis and indicates a key role for peripheral tissue resident memory T cells in this process. In summary, our data suggest that virus-specific memory CD8<sup>+</sup> T cells resident in the airways can be activated in the absence of specific antigen to produce IFN- $\gamma$ , which suppresses subsequent allergic immune responses. The key mechanism appears to be local production of IFN- $\gamma$ , which acts to reduce allergen-induced infiltration and activation of CD4<sup>+</sup> T cells present within the lung and airways and as a consequence markedly reduces infiltration of eosinophils and development of airways hyperresponsiveness.

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