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CD8 T Cells Inhibit Respiratory Syncytial Virus (RSV) Vaccine-Enhanced Disease[1]

Matthew R. Olson* and Steven M. Varga[2]*†

Vaccination of children with a formalin-inactivated (FI) respiratory syncytial virus (RSV) vaccine led to exacerbated disease including pulmonary eosinophilia following a natural RSV infection. Immunization of BALB/c mice with FI-RSV or a recombinant vaccinia virus (vv) expressing the RSV attachment (G) protein (vvG) results in a pulmonary Th2 response and eosinophilia after RSV challenge that closely mimics the RSV vaccine-enhanced disease observed in humans. The underlying causes of RSV vaccine-enhanced disease remain poorly understood. We demonstrate here that RSV M2-specific CD8 T cells reduce the Th2-mediated pathology induced by vvG-immunization and RSV challenge in an IFN-γ-independent manner. We also demonstrate that FI-RSV immunization does not induce a measurable RSV-specific CD8 T cell response and that priming FI-RSV-immunized mice for a potent memory RSV-specific CD8 T cell response abrogates pulmonary eosinophilia after subsequent RSV challenge. Our results suggest that the failure of the FI-RSV vaccine to induce a CD8 T cell response may have contributed to the development of pulmonary eosinophilia and augmented disease that occurred in vaccinated individuals. ***The Journal of Immunology,*** **2007, 179: 5415–5424.**

Respiratory syncytial virus (RSV)[3] is the leading cause of lower respiratory tract infection and hospitalization in children <5 years of age (1). During the 1960s a formalin-inactivated RSV (FI-RSV) vaccine was used in a series of clinical trails. A subsequent natural RSV exposure resulted in ~80% of the vaccine recipients requiring hospitalization as well as several deaths (2–4). Histological examination of the lung tissue from the deceased children revealed extensive mononuclear cell infiltration and pulmonary eosinophilia (2–4).

BALB/c mice immunized with a recombinant vaccinia virus (vv) that expresses the RSV attachment (G) protein (vvG) develop pulmonary eosinophilia following RSV challenge, a response similar to the vaccine-enhanced disease observed in the children from the FI-RSV vaccine trials (5, 6). Although BALB/c mice do not mount a RSV G-specific CD8 T cell response, vaccination with the RSV G protein does elicit a mixed Th1 and Th2 CD4 T cell response comprised predominately of CD4 T cells expressing Vβ14 TCR that are required for the development of pulmonary eosinophilia after RSV challenge (5–8). Although the manifestations of RSV vaccine-enhanced disease are similar in vvG- and FI-RSV-immunized mice, the underlying immune mechanisms that induce the disease differ (9–13).

Recent work has suggested that priming mice for both a CD4 and a CD8 T cell response alters the resulting secondary memory response following RSV challenge (14–16). Mice immunized with a recombinant vv expressing a RSV G protein engineered to contain the immunodominant RSV CD8 T cell epitope (vvG/M2) from the M2 protein ($M2_{82–90}$) do not exhibit pulmonary eosinophilia post-RSV challenge (14). These data suggest that CD8 T cells play a key role in inhibiting the Th2 response and pulmonary eosinophilia. However, the mechanism by which CD8 T cells mediate the inhibition of RSV vaccine-enhanced pulmonary eosinophilia is currently unknown.

We demonstrate in this study that RSV M2-specific CD8 T cells inhibit vvG-induced pulmonary eosinophilia by reducing the numbers of $Vβ14^+$ and Th2 CD4 T cells in the lung after RSV challenge. Because the introduction of a concurrent RSV-specific CD8 T cell response inhibits pulmonary eosinophilia in vvG-immunized mice (14), we questioned the ability of FI-RSV to prime a CD8 T cell response. We show that FI-RSV immunization does not elicit a detectable acute CD8 T cell response as measured by MHC class I tetramers, as well as the lack of an increased RSV-specific CD8 T cell response following RSV challenge. Importantly, we demonstrate that subsequent priming for an M2-specific CD8 T cell response in FI-RSV-immunized mice results in an almost complete abrogation of pulmonary eosinophilia after RSV challenge. Taken together, these data suggest that the vaccine-enhanced disease, including pulmonary eosinophilia, exhibited by FI-RSV vaccinees may have been due in part to the inability of this vaccine to prime a RSV-specific CD8 T cell response.

Materials and Methods

Virus and mice

The RSV A2 strain was a gift from B. S. Graham (National Institutes of Health, Bethesda, MD) and was propagated in HEp-2 cells (American Type Culture Collection (ATCC). Recombinant vv were a gift from T. J. Braciale (University of Virginia, Charlottesville, VA) and J. L. Beeler (U.S. Food and Drug Administration, Bethesda, MD) and were propagated in BSC-40 cells (ATCC). Female BALB/cAnNCr mice between 6 and 10 wk of age were purchased from the National Cancer Institute (Bethesda, MD). Each mouse was scarified with 3×10^6 PFU of recombinant vv using ~100 light strokes of a 22-gauge needle. Immunized mice were rested for

*Department of Microbiology and †Interdisciplinary Graduate Program in Immunology, University of Iowa, Iowa City, IA 52242

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[2] Address correspondence and reprint requests to Dr. Steven Varga, Department of Microbiology, 51 Newton Road, University of Iowa, Iowa City, IA 52242. E-mail address: steven-varga@uiowa.edu

[3] Abbreviations used in this paper: RSV, respiratory syncytial virus; BAL, bronchial alveolar lavage; β-gal, β-galactosidase; F, fusion; GKO, IFN-γ deficient; FI-RSV, formalin inactivated-RSV; ICS, intracellular cytokine staining; vv, vaccinia virus; WT, wild type.

3 wk before infection with 1–3 $\times$ 10^6 PFU of RSV under light anesthesia with halothane. All mouse experiments have been evaluated and approved by the University of Iowa Animal Care and Use Committee on a yearly basis and are in compliance with institutional, state, and federal policies on the care of laboratory animals.

Tissue isolation and preparation

The bronchoalveolar lavage (BAL) was collected from immunized and RSV-challenged mice by cannulation of the trachea and lavage with three successive 1ml washes of PBS. Cytospin 2 (Shandon) preparations of BAL cells were stained with Diff-Quik (Baxter Healthcare) before analysis. Differential cell counts were performed on at least 200 cells based on morphology and staining characteristics. Following collection of the BAL, the blood was perfused from the lungs by injecting 5 ml of PBS through the right ventricle. After perfusion, lungs were isolated and pressed through a wire mesh screen (Cellector; Bellco Glass) to create a single cell suspension for use in intracellular cytokine staining (ICS), tetramer staining, or in vitro restimulation experiments. Spleens and lymph nodes were isolated from mice and disassociated into single cell suspensions by pressing the tissue between the ends of two frosted glass slides (Surgipath).

Intracellular cytokine staining

Lung mononuclear cells (between 2 $\times$ 10^6 and 3 $\times$ 10^6 cells) were incubated in the presence or absence of 1 μM of either $M2_{82–90}$ or $G_{183–195}$ peptide for 5 h in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich). After incubation, cells were washed with staining buffer (PBS, 5% FCS, and 0.025% sodium azide) and blocked with purified anti-FcγRII/III mAb (clone 2.4G2; BD Pharmingen). After blocking for 15 min, cells were washed with staining buffer and incubated with optimal concentrations of anti-CD4 PerCP-Cy5.5 (clone RM4-5; BD Pharmingen), CD8 Pe-Cy7 (clone 53-6.7; eBioscience), and CD90.2-FITC (clone 53-2.1; eBioscience) for 30 min at 4°C. Cells were then washed twice with staining buffer and fixed and erythrocytes were lysed with FACS lysing solution (BD Biosciences). Cells were then washed in permeabilization buffer (staining buffer containing 0.5% saponin; Sigma-Aldrich) and stained with an optimal concentration of anti-IFN-γ-PE (clone XMG1.2; eBioscience). Cells were washed an additional time with permeabilization buffer and with staining buffer before analysis on a BD FACSCanto flow cytometer. Data were analyzed using FlowJo software (version 8.1.1; Tree Star).

Plaque assay

Lungs were harvested from recombinant vv-expressing β-galactosidase (vvβ-gal)-, vvG-, or vvG/M2-immunized mice on day 4 or day 7 post-RSV challenge in 1 ml of serum-free RPMI 1640. Lung tissue was homogenized using a tissue homogenizer (Ultra Turrax T25; IKA) and lung homogenates were then centrifuged at 2000 rpm for 10 min. Cell-free supernatants from these samples were snap frozen in liquid nitrogen and thawed samples were then subjected to plaque assay on Vero cells. Briefly, dilutions of lung homogenates were incubated on Vero cells (ATCC) in 6-well plates (BD Falcon) for 1.5 h at 37°C with gentle rocking. Cells were then overlaid with 4 ml of 1% agarose (SeaKem ME agarose; Cambrex) in Eagle's MEM (Cambrex) and allowed to incubate for 5 days at 37°C. After 5 days of incubation, cells were overlaid again with 2 ml of 1% agarose in Eagle's MEM containing a final concentration of 0.01% neutral red (Sigma-Aldrich) and allowed to incubate an additional 24 h, after which the number of plaques were counted.

Tetramer staining

Single cell suspensions isolated from various tissues were blocked with purified anti-FcγRII/III mAb for 15 min. Cells were then washed with staining buffer and incubated with optimal concentrations of $M2_{82–90}$- or $F_{85–93}$-specific H-$2k^d$ allophycocyanin-conjugated tetramers (obtained from the National Institutes of Health Tetramer Core Facility, Bethesda, MD) for 45 min at 4°C. After tetramer staining, cells were washed twice with staining buffer and incubated with optimal concentrations of anti-CD4 PerCP-Cy5.5, CD8 PE-Cy7, CD90.2 PE, and anti-Vβ14 FITC (clone 14-2 BD Pharmingen) mAbs. Cells were washed two additional times with staining buffer before analysis on a BD FACSCanto flow cytometer. Data were analyzed using FlowJo software.

In vitro restimulation and cytokine ELISA

Lungs were harvested from vvβ-gal-, vvG-, or vvG/M2-immunized mice 7 days after RSV challenge and prepared into single cell suspensions as described above. Lung cells (2 $\times$ 10^6) were aliquoted into uncoated 24-well plates (BD Falcon) or 24-well plates coated overnight with 10 μg/ml purified anti-CD3 in PBS (clone 145-2C11; eBioscience) or anti-Vβ14. After 48 h of incubation at 37°C and 5% CO_2, supernatants were harvested and stored at −80°C before analysis by ELISA for concentrations of IL-4, IL-5, IL-13, and IFN-γ. Briefly, 96-well plates (Nunc) were coated overnight at 4°C with 2 μg/ml purified anti-IL-4 (clone 11B11; eBioscience), IL-5 (clone TRFK5; eBioscience), IL-13 (clone 38213.11; R&D Systems), and anti-IFN-γ (clone XMG1.2; eBioscience). Plates were washed four times with PBS plus 0.1% Tween 20 (v/v) (Sigma-Aldrich) and subsequently blocked with RPMI 1640 containing 10% FCS for 2 h at room temperature. Plates were washed four additional times with PBS-Tween 20 before the addition of 50 μl of each individual sample, and standards were diluted to appropriate concentrations in PBS containing 5% FCS. Samples were incubated in plates for 2 h before being washed four times with PBS-Tween 20 and the application of secondary biotinylated mAbs (IL-4 clone BVD6-24G2, eBioscience; IL-5 clone TRFK4, eBioscience; IL-13, R&D Systems; and IFN-γ clone R4-6A2, eBioscience). After 2 h of incubation of biotinylated secondary mAbs, plates were washed and incubated an additional 30 min with 1 μg/ml avidin-peroxidase (Sigma-Aldrich). Plates were then washed four times with PBS-Tween 20 and developed with 3,3′,5,5′-tetramethylbenzidine (Sigma-Aldrich) in 0.05 M phosphocitrate buffer (pH 5.0) containing H_2O_2 for 5–10 min and treated with 2 N H_2SO_4 to stop the reaction. ELISA samples were analyzed by an ELx800 reader (BioTek Instruments) and data were tabulated using KC Junior (BioTek Instruments) software.

ELISPOT assays

ELISPOT assays for IL-5-secreting cells were performed based on established protocols (17, 18). Briefly, 96-well nitrocellulose-based microtiter plates (MultiScreen HA; Millipore) were coated overnight at 4°C with 50 μl/well of anti-IL-5 (clone TRFK5, eBioscience; to a final concentration of 2 μg/ml) diluted in PBS. After the plates were washed with PBS, all wells were blocked with 200 μl of RPMI 1640 containing 10% FCS for 2 h at 37°C. After washing once with PBS, 5 $\times$ 10^6 lung cells were serially diluted in RPMI 1640 with 10% FCS (200 μl/well total volume) and incubated in the presence or absence of 1 μM $G_{183–195}$ peptide for 40 h at 37°C with 5% CO_2. After incubation, the wells were washed in PBS-Tween 20, biotinylated anti-IL-5 (clone TRFK4, eBioscience; 2 μg/ml) was added, diluted in PBS containing 10% FCS (100 μl/well), and incubated overnight at 4°C. Plates were washed in PBS-Tween 20 and 100 μl of an anti-biotin mAb conjugated with peroxidase (1/250 dilution in PBS with 10% FCS; Jackson ImmunoResearch Laboratories) per well were added, followed by another overnight incubation at 4°C. Spots representing individual cytokine-secreting cells were visualized by developing with the substrate 3-amino-9-ethylcarbazole and counted using a ImmunoSpot reader and ImmunoSpot software (version 3, Cellular Technology. All assays were performed in triplicate. Mean numbers of cytokine-secreting cells were calculated from the triplicate assays.

Data analysis

Graphical and statistical analyses were performed using Prism software (version 4.0b, GraphPad Software). Data were analyzed using the Student's *t* test assuming equal variance. Differences were considered significant at $p < 0.05$.

Results

Kinetics of RSV-specific T cell responses

RSV infection of vvG- or vvM2-immunized mice elicits a potent RSV-specific memory CD4 or CD8 T cell response, respectively. However, the kinetics of these memory T cell responses are largely undefined. In this study we demonstrate that there are similar numbers of total cellular infiltrate in the lungs of mice undergoing a primary RSV infection (vvβ-gal-immunized) or mice undergoing a memory CD4 (vvG-immunized), a memory CD8 (vvM2-immunized), or both a memory CD4 and memory CD8 T cell response (vvG/M2-immunized; Fig. 1*A*). Although the total number of cells in the lung is similar between each of these groups, there are large differences in the total number of CD4 and CD8 T cells present. Mice immunized with vvG elicit a robust CD4 T cell response after RSV challenge (Fig. 1*B*). In contrast, mice immunized with vvG/M2 exhibit a slight decrease in the total number of CD4 T cells in the lung (Fig. 1*B*). Immunization of mice with vvM2 or vvG/M2 induces the influx of more CD8 T cells as compared with vvβ-gal- or vvG-immunized mice (Fig. 1*C*). As predicted, only

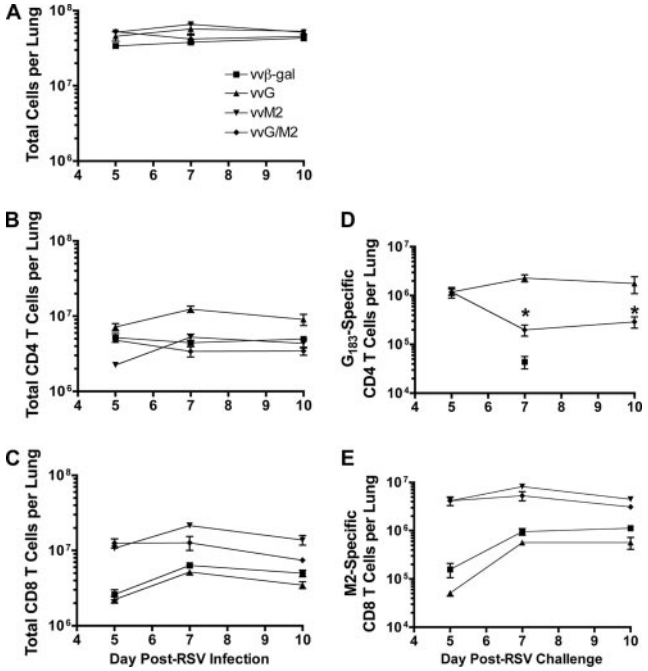

FIGURE 1. T cell kinetics after recombinant vv immunization and RSV challenge. BALB/c mice were scarified with 3×10^6 PFU vvβ-gal, vvG, vvM2, or vvG/M2. Three weeks after challenge, all groups were challenged intranasally with 2.6×10^6 PFU of RSV and their lungs were analyzed for the total number of cells (*A*) and the total number of CD4 (*B*), CD8 (*C*), G_{183}-specific CD4 (*D*), and $M2_{82}$-specific CD8 T cells (*E*) by flow cytometry on days 5, 7, and 10 post-RSV challenge. Error bars represent SEM of 3–4 mice per group. Data are representative of two individual experiments.

mice immunized with vvG or vvG/M2 induce a reproducibly detectable G_{183}-specific CD4 T cell response (Fig. 1*D*), whereas all mice mount a detectable $M2_{82}$-specific CD8 T cell response with increased numbers in vvM2- or vvG/M2-immunized mice (Fig. 1*E*). Interestingly, there are significantly ($p < 0.05$) fewer G_{183}-specific CD4 T cells in vvG/M2-immunized mice compared with those immunized with vvG (Fig. 1*D*). These data suggest that M2-specific CD8 T cells may inhibit the G_{183}-specific CD4 T cell response.

RSV-specific CD8 T cells inhibit vvG-induced pulmonary eosinophilia

Previous work has demonstrated that RSV M2-specific CD8 T cells can inhibit eosinophilia in the lung parenchyma (14). To determine whether M2-specific CD8 T cells also inhibit pulmonary eosinophilia in the airways, mice were immunized with vvβ-gal, vvG, vvM2, or vvG/M2 and subsequently challenged with RSV 3 wk later. Mice undergoing a RSV G-specific memory CD4 T cell response (vvG-immunized) exhibited pulmonary eosinophilia by day 5 after RSV challenge, whereas mice undergoing only a M2-specific memory response (vvM2 immunized) or a concurrent RSV G-specific CD4 and M2-specific memory CD8 T cell response (vvG/M2-immunized) had significantly reduced levels of pulmonary eosinophils both in percentages ($p < 0.05$; Fig. 2*A*) and total numbers ($p < 0.05$; Fig. 2*B*) at all time points examined. Mice immunized with a 1:1 mixture of vvG and vvM2 also showed a significant reduction ($p < 0.05$) in eosinophilia at the peak of the eosinophilic response (Fig. 3, *A* and *B*) compared with vvG-immunized mice, thus demonstrating that this inhibitory effect is not dependent on having the $M2_{82-90}$ CD8 T cell epitope expressed within the G protein.

To ensure that vvG/M2-immunized mice and mice immunized with a 1:1 mixture of vvG plus vvM2 mount similar memory $M2_{82}$- and G_{183}-specific T cell responses following RSV challenge, we enumerated the frequency of these cells by ICS for IFN-γ. There was no significant difference ($p > 0.05$) in the numbers of G_{183}-specific Th1 CD4 T cells in the lungs of vvG/M2-immunized and vvG plus vvM2-immunized mice at day 7 post-RSV challenge, indicating that the introduction of the $M2_{82}$ epitope did not negatively influence the RSV G-specific CD4 or M2-specific CD8 T cell memory response (Fig. 3, C and *D*). Similarly, there was no significant difference ($p > 0.05$) in the total numbers of $M2_{82}$-specific CD8 T cells in vvM2-, vvG plus vvM2-, or vvG/M2-immunized mice at day 7 post-RSV challenge (Fig. 1*D*). However, there were ~7- to 10-fold fewer G_{183}-specific Th1 CD4 T cells in the lungs of vvG/M2 or vvG plus vvM2-immunized mice compared with vvG-immunized mice (Fig. 1*C*), suggesting that $M2_{82}$-specific CD8 T cells inhibited the trafficking and/or expansion of these cells.

Viral clearance in immunized mice

CD8 T cells play a critical role in viral clearance by killing virus-infected cells in the host. Mice immunized with vvG/M2 mount both a memory CD4 and CD8 T cell response after RSV challenge and, therefore, might be expected to clear RSV faster than vvG-immunized mice that mount only a memory CD4 T cell response after RSV challenge. To determine whether vvG/M2-immunized mice eliminate virus faster than vvG-immunized mice, lung tissue from vvβ-gal-, vvG-, and vvG/M2-immunized mice was collected either 4 or 7 days post-RSV challenge and analyzed for RSV viral titers by plaque assay. Both vvG- and vvG/M2-immunized mice had significantly reduced viral titers at day 4 postchallenge ($p < 0.05$) compared with vvβ-gal-immunized mice (Fig. 4). Surprisingly, vvG-immunized mice had slightly less virus in the lung than vvG/M2-immunized mice at day 4 postchallenge, although this is not significantly different ($p > 0.05$). Despite this small difference, both vvG- and vvG/M2-immunized mice had no detectable virus

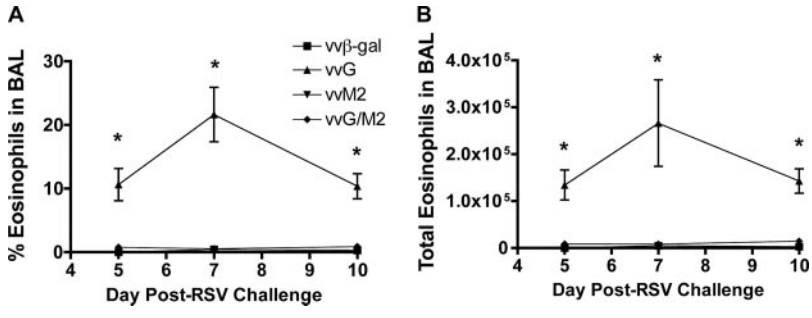

FIGURE 2. Eosinophil kinetics in recombinant vv-immunized mice after RSV challenge. Mice were immunized and challenged with RSV as described in Fig. 1. BAL was harvested 5, 7, and 10 days post-RSV challenge and analyzed for the frequency (*A*) and total numbers (*B*) of eosinophils by quantitative morphometry. Error bars represent SEM of 3–4 mice per group. Data are representative of two individual experiments.

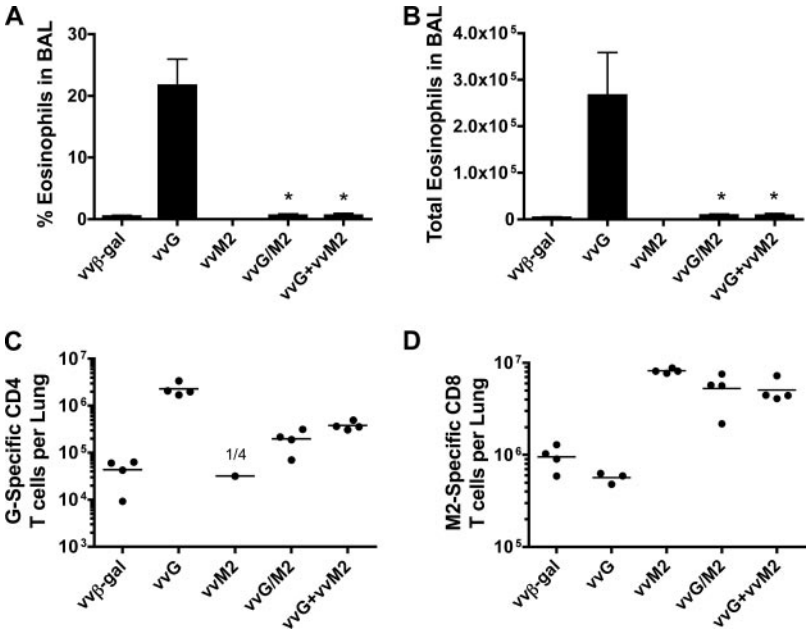

FIGURE 3. M2-specific CD8 T cells inhibit RSV vaccine-enhanced pulmonary eosinophilia. BALB/c mice were scarified with 3×10^6 PFU of vvβ-gal, vvG, vvM2, and vvG/M2 or a 1:1 mixture of vvG and vvM2. Three weeks after scarification these mice were challenged intranasally with 1.6×10^6 PFU of RSV. The percentage (*A*) and total eosinophils (*B*) from the BAL were enumerated by quantitative morphometry 7 days post-RSV challenge. Total numbers of RSV G_{183}-specific CD4 T cells (*C*) and $M2_{82}$-specific CD8 T cells 7 days post-RSV challenge (*D*) were quantified by intracellular cytokine staining for IFN-γ after 5 h of stimulation with G_{183} or $M2_{82}$ peptides, respectively. Error bars represent the SEM from four mice per group. Data are representative of four individual experiments. *, $p < 0.05$, significantly different from vvG-immunized mice.

in the lungs at day 7 postchallenge, whereas there was still detectable levels of virus in the lungs of vvβ-gal-immunized mice undergoing an acute RSV infection (Fig. 4). Taken together, these data demonstrate that the reduction of pulmonary eosinophilia in vvG/M2-immunized mice (Fig. 2 and 3) is not due to accelerated viral clearance.

RSV-specific CD8 T cells reduce the number of Th2 cells in the lung

We have previously demonstrated that the oligoclonal RSV G_{183}-specific Vβ14$^+$ CD4 T cell response generated in vvG-immunized mice is required for development of RSV vaccine-enhanced pulmonary eosinophilia (8). To determine whether this subset of CD4 T cells is decreased in mice with a concurrent RSV $M2_{82}$-specific CD8 T cell response, we examined both the frequency and total number of Vβ14$^+$ CD4 T cells in the lungs of vvβ-gal-, vvG-, or vvG/M2-immunized mice on day 7 post-RSV challenge. Mice immunized with vvG/M2 demonstrated a modest decrease in the percentage of pulmonary Vβ14$^+$ CD4 T cells compared with vvG-immunized mice (Fig. 5, *A* and *B*), however; there was a significant decrease in the total numbers of these cells present in vvG/M2-immunized mice compared with vvG-immunized mice (Fig. 5C; $p < 0.05$), consistent with the decrease in the total number CD4 T cells we observe at this time (Fig. 1*B*).

To further elucidate the mechanism by which CD8 T cells inhibit pulmonary eosinophilia, we asked whether a concurrent RSV $M2_{82}$-specific CD8 T cell response would reduce the number of RSV G_{183}-specific Th2 cells in the lungs of vvG/M2-immunized mice after RSV challenge. We measured the number of IL-5-secreting cells in the lungs of vvβ-gal-, vvG-, or vvG/M2-immunized mice 5 days post-RSV challenge. We demonstrate here that the total number of G_{183}-specific IL-5-secreting cells in mice with a concurrent RSV $M2_{82}$-specific CD8 T cell response is significantly reduced ($p < 0.05$) to a level similar to

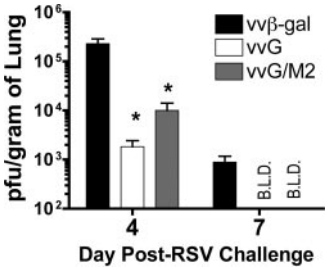

FIGURE 4. RSV M2-specific CD8 T cells do not enhance viral clearance. BALB/c mice were scarified with 3×10^6 PFU of vvβ-gal, vvG, or vvG/M2 and subsequently intranasally challenged with 3×10^6 PFU RSV 3 wk later. Lungs were harvested 4 and 7 days postchallenge and were analyzed for viral titers by plaque assay on Vero cells. B.L.D., Below limit of detection (100 PFU). Error bars represent the SEM of four individual mice per group. Data represent three pooled experiments.

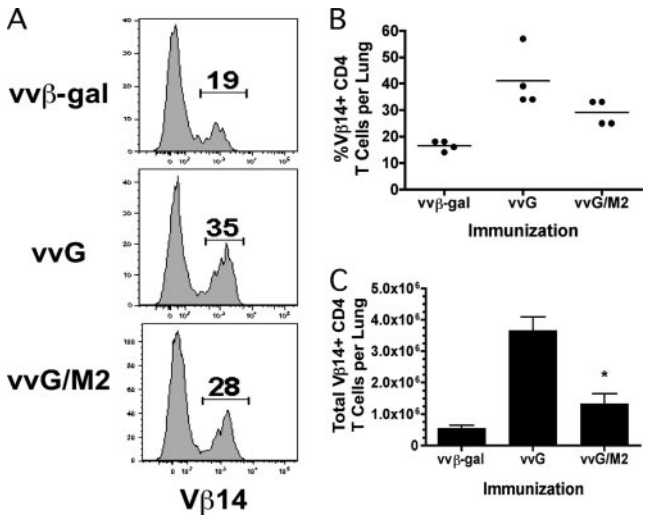

FIGURE 5. Reduced number of Vβ14$^+$ CD4 T cells in vvG/M2-immunized mice. BALB/c mice were scarified with 3×10^6 PFU of vvβ-gal, vvG, or vvG/M2. Three weeks later, these mice were challenged intranasally with 1.6×10^6 PFU of RSV. Mice were harvested 5 days post-RSV challenge and lung CD4 T cells were analyzed for the frequency of Vβ14$^+$ (*A* and *B*) or the total number of Vβ14$^+$ CD4 T cells (*C*) by flow cytometry. *, $p < 0.05$; significantly different from vvG-immunized mice. Error bars represent the SEM from four mice per group. Data are representative of three individual experiments.

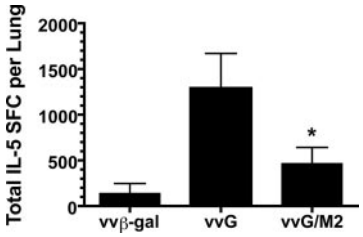

FIGURE 6. Reduced number of RSV G_{183}-specific IL-5 secreting cells in vvG/M2-immunized mice. BALB/c mice were scarified with 3×10^6 PFU of vvβ-gal, vvG, or vvG/M2. Three weeks later, these mice were challenged intranasally with 1.6×10^6 PFU of RSV. Mice were harvested 5 days post-RSV challenge and the frequency of IL-5-producing cells in the lung was quantitated by ELISPOT assay. Error bars represent the SEM from four mice per group. *, $p < 0.05$; significantly different from vvG-immunized mice. Data are representative of three individual experiments. SFC, Spot-forming cells.

that of vvβ-gal-immunized negative controls (Fig. 6). We also asked whether the total Th2 response was reduced in vvG/M2-immunized mice compared with vvG-immunized controls. Mice were immunized with vvβ-gal, vvG, or vvG/M2 and subsequently challenged intranasally with RSV. Seven days after infection, cells were harvested from the lung and restimulated in vitro with either anti-Vβ14 or anti-CD3 for 48 h. After stimulation, supernatants were harvested and analyzed for the presence of Th2- (IL-4, IL-5, and IL-13) or Th1-associated (IFN-γ) cytokines by ELISA. Fig. 7, *A–C* demonstrate a profound reduction in the capacity of cells isolated from the lungs of vvG/M2-immunized mice to produce all of the prototypical Th2 cytokines following anti-CD3 stimulation compared with cells recovered from vvG- or vvβ-gal-immunized mice (Fig. 7, *A–C*). In contrast, there was an increase in the capacity of cells from vvG/M2-immunized mice to produce IFN-γ when stimulated with anti-CD3 (Fig. 7*D*), likely due to the increased number of M2-specific CD8 T cells in the lungs of vvG/M2-immunized mice (Fig. 1*D*). Consistent with our previous observations (8), anti-Vβ14 stimulation of lung mononuclear cells isolated from vvG-immunized mice 7 days post-RSV challenge results in IL-5 and IL-13 production but little IL-4 production. The production of IL-4, IL-5, and IL-13 is reduced in mice with a concurrent RSV $M2_{82}$-specific CD8 T cell response after anti-Vβ14 stimulation. There is a slight reduction in the capacity of cells from vvG/M2-immunized mice to produce IFN-γ when stimulated with anti-Vβ14 mAb (Fig. 7*D*), consistent with the reduction in the number of Vβ14$^+$ CD4 T cells in the lungs of these mice (Fig. 5*C*). Taken together, these data suggest that RSV M2-specific CD8 T cells inhibit vvG-induced pulmonary eosinophilia by decreasing the total number of Vβ14$^+$ Th2 cytokine-secreting cells in the lungs after RSV challenge.

Inhibition of RSV vaccine-enhanced pulmonary eosinophilia requires RSV $M2_{82}$-specific memory CD8 T cells

Having established that CD8 T cells inhibit the RSV G_{183}-specific Th2 CD4 T cell response, we next wanted to determine whether this inhibition occurs at priming or during the memory recall response. To determine whether the inhibition of the RSV G_{183}-specific Th2 response occurs after vv priming, mice were immunized with vvβ-gal, vvG, or vvG/M2 and, 8 days after priming, we examined the draining inguinal lymph nodes and spleen for the presence of Th2 cells by IL-5 ELISPOT assay. Fig. 8 demonstrates that there was no significant decrease ($p > 0.05$) in the number of RSV G_{183}-specific IL-5 producing cells in the draining inguinal lymph nodes (Fig. 8*A*) or spleen (Fig. 8*B*) in vvG/M2-immunized mice as compared with vvG-immunized mice. These data provide further evidence that CD8 T cell inhibition of RSV vaccine-enhanced pulmonary eosinophilia occurs at the secondary effector stage and requires memory RSV-specific CD8 T cells.

Several additional observations suggest that CD8 inhibition of the Th2 response occurs during the memory recall response. One of these observations is that vv-specific CD8 T cells generated immediately after priming in vvG-immunized mice do not inhibit the generation of the RSV G_{183}-specific Th2 response that ultimately leads to development of pulmonary eosinophilia after RSV challenge. A second observation is that

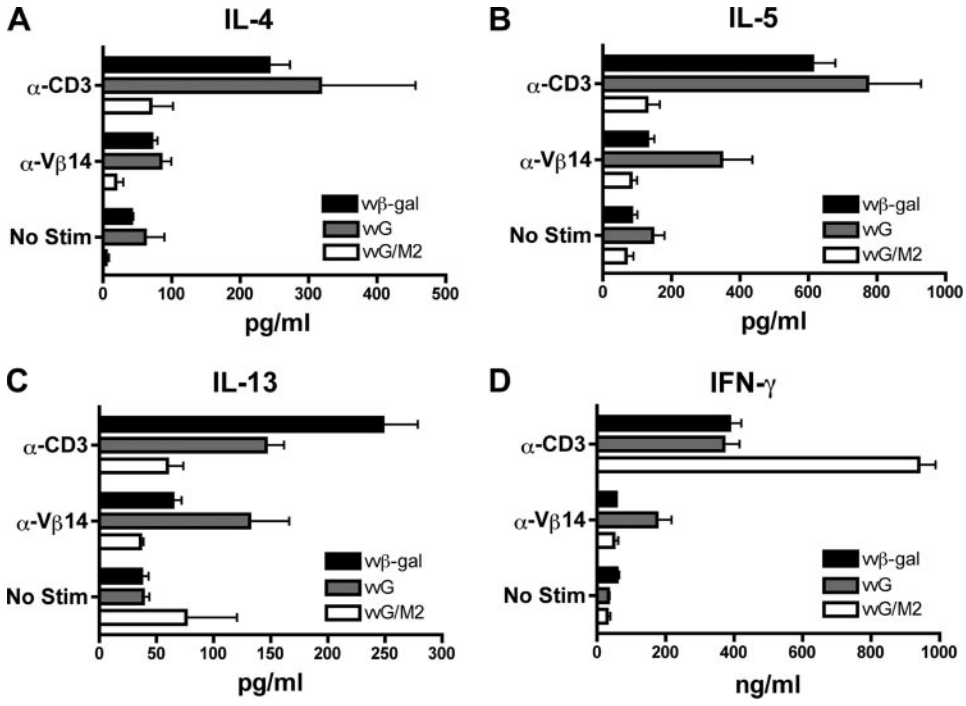

FIGURE 7. Reduced Th2 response in the lungs of vvG/M2-immunized mice after RSV challenge. BALB/c mice were immunized with 3×10^6 PFU of vvβ-gal, vvG, or vvG/M2 and 3 wk later were intranasally challenged with 2.6×10^6 PFU RSV. Seven days postchallenge, lungs were harvested and single cell suspensions were restimulated in vitro with anti-CD3 (α-CD3) or anti-Vβ14 (a-Vβ14) or left unstimulated (No Stim) for 48 h. Supernatants were then harvested and analyzed for IL-4 (*A*), IL-5 (*B*), IL-13 (*C*), or IFN-γ (*D*) by ELISA. Error bars represent the SEM of four mice per group. Data represent three individual experiments.

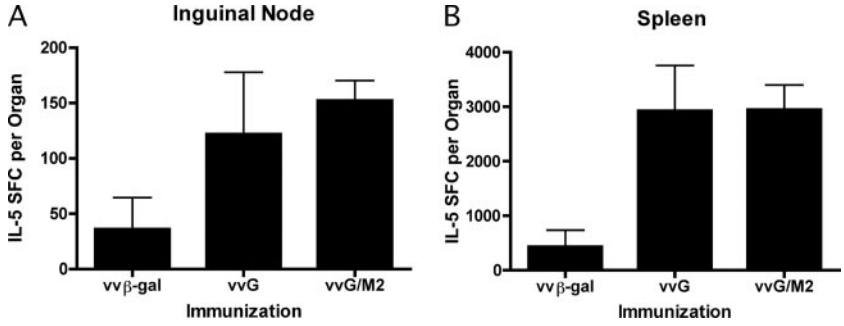

FIGURE 8. RSV $M2_{82}$-specific CD8 T cells do not inhibit the priming of RSV G_{183}-specific Th2 cells. BALB/c mice were scarified with 3×10^6 PFU of vvβ-gal, vvG, or vvG/M2. Inguinal lymph nodes (*A*) and spleens (*B*) were harvested 8 days postscarification and analyzed for IL-5-producing cells by ELISPOT assay. Error bars represent the SEM from four mice per group. Data are representative of three individual experiments. SFC, Spot-forming cells.

primary $M2_{82}$-specific CD8 T cells generated after RSV challenge of vvG-immunized mice do not inhibit pulmonary eosinophilia (Fig. 1, *A* and *B*). Taken together, these data suggest that CD8 T cell inhibition of pulmonary eosinophilia occurs during the secondary response of $M2_{82}$-specific memory cells and not during the acute vv priming in vvG/M2-immunized mice.

IFN-γ is not required for M2-specific CD8 T cell inhibition of RSV vaccine-enhanced pulmonary eosinophilia

IFN-γ is a key effector molecule produced by CD8 T cells. Additionally, IFN-γ production by CD8 T cells has been demonstrated to play a role in the suppression of Th2-mediated immunopathology in several asthma and allergy models (19, 20). Previous work has demonstrated that mice immunized with a recombinant vv expressing the RSV fusion (F) protein undergo both a memory CD4 and memory CD8 T cell response after RSV challenge (21, 22). Depletion of CD8 T cells or IFN-γ from vvF-immunized mice after RSV challenge resulted in a substantial increase in pulmonary eosinophilia (15). These data suggest that RSV F-specific CD8 T cells may inhibit pulmonary eosinophilia by an IFN-γ-dependent mechanism. To determine whether M2-specific CD8 T cells also inhibit pulmonary eosinophilia in an IFN-γ-dependent manner, both WT and IFN-γ-deficient (GKO) mice were immunized with vvβ-gal, vvG, and vvG/M2 and subsequently challenged intranasally with RSV 3 wk later. Interestingly, all recombinant vv-immunized GKO mice exhibited an ~5-fold increase the numbers of pulmonary eosinophils after RSV challenge as compared with their WT counterparts (Fig. 9, *A* and *B*). However, vvG/M2-immunized GKO mice had significantly ($p < 0.05$) less pulmonary eosinophilia compared with vvG-immunized GKO mice (Fig. 9, *A* and *B*).

The reduction of pulmonary eosinophilia in vvG/M2-immunized GKO mice compared with vvG-immunized GKO mice is not due to an increase in the number of RSV M2-specific CD8 T cells as demonstrated by similar numbers of these cells in wild-type (WT) and GKO vvG/M2-immunized mice by tetramer staining (Fig. 9*C*). In contrast, there is a slight reduction in the number of TNF-α-producing Th1 CD4 T cells in GKO vvG/M2-immunized mice compared with their WT counterparts (Fig. 9*D*). However, there is not a 1:1 correlation in the production of TNF-α and IFN-γ by CD4 T cells at this time point (data not shown). Taken together, these data suggest that IFN-γ production plays some role in suppressing RSV vaccine-enhanced pulmonary eosinophilia (as seen by comparing all WT groups to their GKO counterparts); however, IFN-γ is not required for M2-specific CD8 T cells to inhibit this eosinophilic disease.

FI-RSV does not induce a M2- or F-specific CD8 T cell response

FI-RSV immunization followed by subsequent RSV infection leads to the development of pulmonary eosinophilia in multiple strains of mice and humans with varying MHC haplotypes (3, 4, 23). Based on our results using the vvG immunization system, we hypothesized that this widespread sensitization for enhanced disease is due to the inability of the FI-RSV vaccine to generate a concurrent CD8 T cell response that would inhibit pulmonary eosinophilia upon subsequent RSV infection. BALB/c mice undergoing an acute RSV infection mount a CD8 T cell response directed against two CD8 T cell epitopes located within the M2

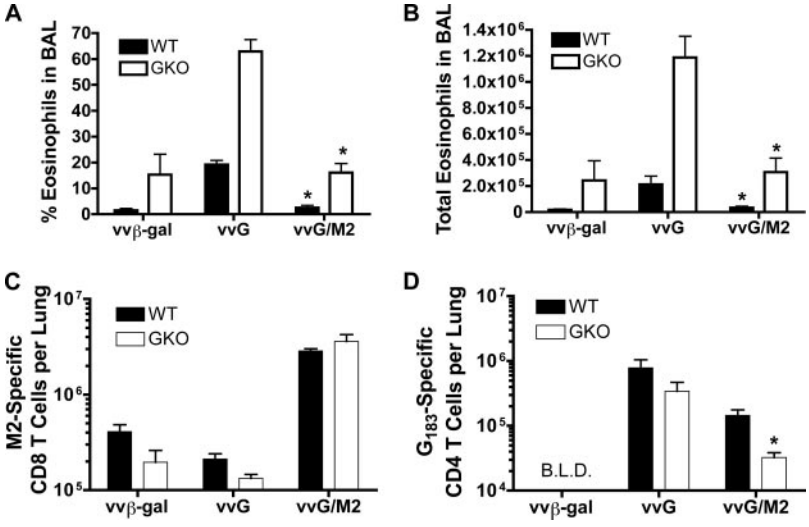

FIGURE 9. IFN-γ is not required for M2-specific CD8 T cells to inhibit RSV vaccine-enhanced pulmonary eosinophilia. BALB/c WT and IFN-γ deficient mice (GKO) were scarified with 3×10^6 PFU of vvβ-gal, vvG, or vvG/M2. Three weeks after immunization these mice were intranasally challenged with 2.6×10^6 PFU RSV. One week after challenge lungs and BAL were harvested. *A* and *B*, Frequency (*A*) and total number (*B*) of eosinophils in the BAL. *C*, Total number of $M2_{82}$-specific CD8 T cells as determined by tetramer staining. *D*, The total number of G_{183}-specific CD4 T cells as determined by TNF-α ICS. Error bars represent SEM based on 3–4 mice per group. *, $p < 0.05$; significantly different from vvG-immunized mice. These data represent two independent experiments. B.L.D., Below limit of detection.

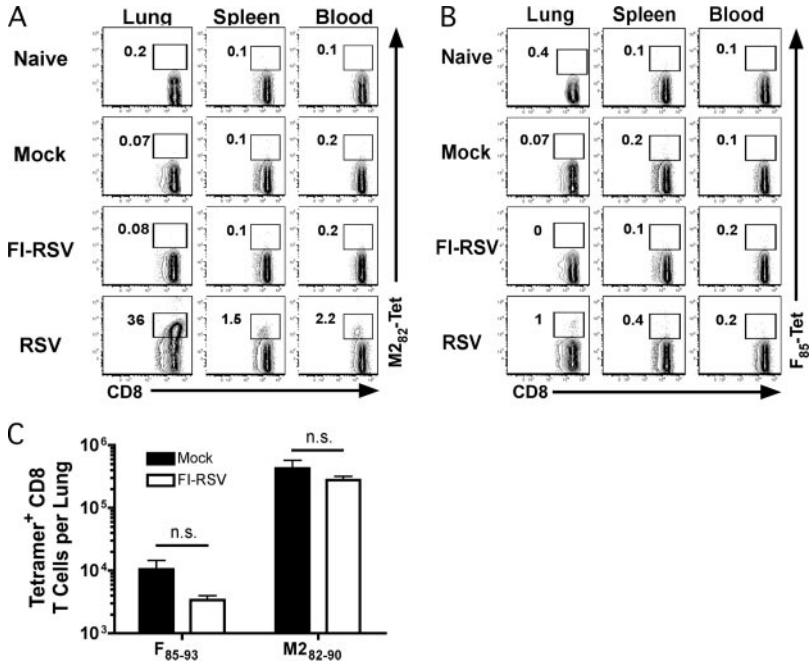

FIGURE 10. FI-RSV immunization does not induce a RSV-specific CD8 T cell response. *A* and *B*, BALB/c mice were i.m. immunized with a 1/200 dilution of FI-RSV or a mock supernatant preparation. Eight days postimmunization, lungs, spleens, and blood were harvested and subjected to $M2_{82}$- (*A*) or F_{85}-specific (*B*) tetramer (Tet) staining. Cells from each tissue were gated on lymphocytes based on forward scatter vs side scatter characteristics. Plots represent $CD8^+$-gated cells that fell within the lymphocyte gate. Numbers on plots represent the frequency of tetramer positive CD8 T cells. *C*, BALB/c mice were i.m. immunized with a 1/200 dilution of FI-RSV or a mock supernatant preparation. Four weeks postimmunization mice were intranasally challenged with 2.6×10^6 PFU of RSV and lungs were harvested 7 days later and stained with F_{85} or $M2_{82}$ MHC class I tetramers. Error bars represent the SEM from four mice per group. Data are representative of three individual experiments.

and F proteins, respectively (24, 25). To determine whether FI-RSV immunization generates a H-2^d-restricted CD8 T cell response, BALB/c mice were i.m. injected with FI-RSV or a mock treatment. Mice acutely infected with RSV were used as a positive control. Eight days after immunization or RSV infection, cells isolated from the lungs, spleens, blood, and lymph nodes were subjected to $M2_{82}$ and F_{85} H-2^d tetramer staining as well as ICS for IFN-γ using $M2_{82}$ and F_{85} peptides. We demonstrate here that there is a lack of $M2_{82}$- or F_{85}-specific CD8 T cells from the lungs, spleens, and blood of FI-RSV- and mock-immunized animals (Fig. 10, *A* and *B*). There were also no detectable $M2_{82}$- or F_{85}-specific CD8 T cells obtained from the inguinal or popiteal lymph nodes (data not shown). This is in contrast to RSV-infected animals showing readily detectable $M2_{82}$- and F_{85}-specific CD8 T cells in the lung, blood, and spleen (Fig. 10, *A* and *B*). These data were also confirmed by a lack of $M2_{82}$- or F_{85}-specific CD8 T cells in the lungs, spleens, blood, and lymph nodes of FI-RSV-immunized mice after peptide stimulation of cells and staining for intracellular IFN-γ (data not shown). Furthermore, we did not observe a significant up-regulation of CD8 T cell activation markers (CD11a, CD43, CD69) in any of the organs following mock or FI-RSV immunization (data not shown). Taken together, these data suggest that FI-RSV immunization does not elicit a RSV-specific CD8 T cell response in BALB/c mice.

To determine whether a small CD8 T cell response was generated below our limit of detection by ICS or tetramer staining, we attempted to recall potential $M2_{82}$- or F_{85}-specific CD8 T cell responses by challenging mock- or FI-RSV-immunized mice intranasally with RSV. Seven days postchallenge, the total number of M2- or F-specific CD8 T cells present in the lung was determined via MHC class I tetramer staining. We demonstrate here that there was no significant increase ($p > 0.05$) in the number of $M2_{82}$- or F_{85}-specific pulmonary CD8 T cells after RSV challenge (Fig. 10*C*). These data indicate that FI-RSV immunization does not induce a robust CD8 T cell response, which may have been a significant factor in why this vaccine induced enhanced immunopathology upon natural RSV infection.

M2-specific CD8 T cells inhibit FI-RSV-induced pulmonary eosinophilia

Although both vvG- and FI-RSV-immunized mice develop RSV vaccine-enhanced disease including pulmonary eosinophilia following RSV challenge, it is clear they do so by different mechanisms (9–13). Therefore, it is unclear whether or not inducing an M2-specific CD8 T cell response can inhibit FI-RSV-induced pulmonary eosinophilia. To determine whether a concurrent RSV $M2_{82}$-specific CD8 T cell response can inhibit FI-RSV-induced pulmonary eosinophilia, mock- and FI-RSV-immunized mice were immunized with vvβ-gal or vvM2 4 wk after the initial immunization. After an additional 4 wk, mice were intranasally infected with RSV. We demonstrate here that FI-RSV-immunized mice immunized with vvM2 and not vvβ-gal have significantly lower percentages (Fig. 11*A*; $p < 0.05$) and total numbers (Fig. 11*B*; $p < 0.05$) of pulmonary eosinophils compared with controls. Additionally, after in vitro restimulation of lung mononuclear cells from FI-RSV-immunized mice with anti-CD3, there was a significant decrease ($p < 0.05$) in the levels of Th2 cytokines produced by cells from FI-RSV plus vvM2-immunized mice compared with FI-RSV plus vvβ-gal-immunized mice (Fig. 11*C*). These data suggest that RSV M2-specific CD8 T cells reduce the FI-RSV-induced pulmonary Th2 response associated with enhanced eosinophilia. Furthermore, this suggests that the mechanisms that CD8 T cells use to regulate this eosinophilic disease in vvG- and FI-RSV-immunized mice are similar (see Fig. 7). These data provide evidence that one important failure of the FI-RSV vaccine may have been its inability to induce an RSV-specific CD8 T cell response.

We have demonstrated in this study that RSV M2-specific CD8 T cells inhibit both vvG- and FI-RSV-induced pulmonary eosinophilia after RSV challenge. However, it was unknown whether M2-specific CD8 T cells also inhibit the systemic disease (as measured by excessive weight loss) that is observed in vvG- and FI-RSV-immunized mice after RSV challenge. To address this question, mock- and FI-RSV-immunized mice from Fig. 11, *A* and *B* were weighed each day after RSV challenge to assess weight loss.

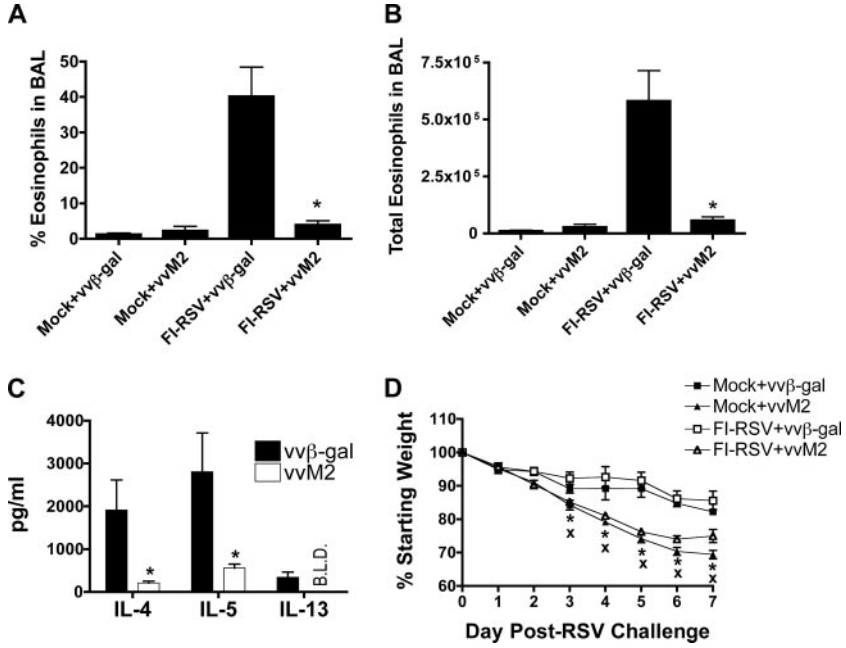

FIGURE 11. Induction of a RSV-specific CD8 T cell response in FI-RSV immunized mice abrogates pulmonary eosinophilia and pulmonary Th2 responses. *A* and *B*, BALB/c mice were i.m. immunized with a 1/200 dilution of FI-RSV or a mock supernatant preparation. Four weeks postimmunization mice were scarified with vvβ-gal or vvM2. An additional 4 wk postscarification mice were challenged with 2.6×10^6 PFU of RSV and 7 days later BAL was harvested and analyzed for the percentage (*A*) or total numbers (*B*) of eosinophils by quantitative morphometry. *C*, Lung mononuclear cells (2×10^6) from each group of mice were stimulated in vitro with α-CD3 for 48 h. Supernatants from these cultures were analyzed for IL-4, IL-5, and IL-13 by ELISA. B.L.D., Below limit of detection (<50 pg/ml). *D*, Weight loss of individual mice was tracked each day after RSV challenge. *, $p < 0.05$; significantly different from FI-RSV plus vvβ-gal-immunized mice. ×, $p < 0.05$; significantly different from Mock + vvβ-gal-immunized mice. Error bars represent the SEM from four mice per group. Data are representative of three individual experiments.

Mock plus vvβ-gal- and FI-RSV plus vvβ-gal-immunized mice lost little weight after RSV challenge (Fig. 11*D*). In contrast, mock plus vvM2- and FI-RSV plus vvM2- immunized mice lost ~30% of their body weight by day 7 post-RSV challenge (Fig. 11*D*). Results similar to these were also noted in vvG- and vvG/M2-immunized mice, whereas vvG/M2-immunized mice lost substantially more weight than vvG-immunized mice (data not shown). These data indicate that although M2-specific CD8 T cells inhibit RSV vaccine-enhanced pulmonary eosinophilia, they exacerbate systemic disease.

Discussion

A series of FI-RSV vaccine trials conducted during the 1960s resulted in enhanced disease, including pulmonary eosinophilia after natural RSV exposure (2–4). Despite the fact that >40 years have passed since the failed vaccine trial, there is still no licensed RSV vaccine. During this time, the establishment of the BALB/c mouse model has substantially increased our understanding of the underlying mechanisms that contribute to RSV vaccine-enhanced disease.

We have previously demonstrated that RSV challenge of vvG-immunized mice induces the expansion in numbers of an oligoclonal G-specific CD4 T cell response dominated by CD4 T cells expressing the Vβ14 TCR (7, 8). In addition, we showed that in vivo depletion of these Vβ14$^+$ CD4 T cells ameliorated disease (8). In this report we show that mice undergoing a concurrent RSV-specific memory CD8 T cell response exhibit a dramatically reduced total number of pulmonary Vβ14$^+$ CD4 T cells (Fig. 5*C*). Based on our previous results in vvG-immunized mice, this decrease in the total number of Vβ14$^+$ CD4 T cells is consistent with the reduced pulmonary eosinophilia we observe. However, FI-RSV-immunized mice do not develop this Vβ14$^+$-skewed CD4 T cell response after RSV challenge (13), yet RSV-specific CD8 T cells are still capable of inhibiting the resulting pulmonary eosinophilia (Fig. 11, *A* and *B*). These data suggest that RSV $M2_{82}$-specific CD8 T cells do not act specifically on the Vβ14$^+$ CD4 T cell population but rather on the entire CD4 T cell population as a whole (note decreases in total CD4 T cells and G_{183}-specific Th1 and Th2 CD4 T cell responses (Figs. 1, *B* and *D*, and 6 and 7)). A similar global inhibition of CD4 T cells also occurs in a model of lymphocytic choriomeningitis virus (LCMV)-induced wasting disease. In this model, intracranial infection with LCMV results in rapid mortality in immune-sufficient mice (26, 27). In contrast, mice lacking β_2-microglobulin (B_2m) that exhibit a profound decrease in their numbers of CD8 T cells have partial and delayed mortality after intracranial LCMV infection and suffer from a wasting disease (28). This disease is similar to that seen in vvG-immunized mice after RSV challenge and results in weight loss, malaise, and ruffled fur (28). Adoptive transfer of purified CD4 T cells from LCMV-infected, β_2m-deficient mice can induce wasting disease in naive β_2m-deficient recipient mice (29). However, adoptive transfer of these same CD4 T cells into β_2m-sufficient mice does not result in wasting disease (29). These data provide another example of CD8 T cells that play a key role in the inhibition of an aberrant CD4 T cell population that induces systemic pathology.

We questioned the role of IFN-γ in the inhibition of RSV vaccine-enhanced pulmonary eosinophilia by M2-specific CD8 T cells due to the fact that IFN-γ can inhibit Th2 CD4 T cell development in vitro (30) and IFN-γ inhibits Th2-driven immunopathology in models of allergy/asthma (20, 31). In addition, it has been previously demonstrated that RSV F-specific CD8 T cells may require IFN-γ to suppress pulmonary eosinophilia in vvF-immunized mice (15). However, our data clearly demonstrate that RSV M2-specific CD8 T cells do not require IFN-γ to inhibit vvG-induced, vaccine-enhanced pulmonary eosinophilia (Fig. 9, *A* and *B*). These data suggest that F- and M2-specific CD8 T cells may qualitatively differ in their ability to inhibit pulmonary eosinophilia. It is possible that a proportion of M2-specific CD8 T cells are of a regulatory phenotype and do not require IFN-γ to abrogate pulmonary eosinophilia in this model system. We were unable to detect the production of IL-10, a key regulatory cytokine, by M2-specific CD8 T cells via ICS (data not shown). However, it is still possible that a subset of these M2-specific CD8 T cells are regulatory cells. We are currently examining the expression of regulatory cell markers (e.g., FoxP3, CD25, and TGF-β) on M2-specific CD8 T cells.

Although CD8 T cells are known to be important for reducing pulmonary eosinophilia in vvG-immunized mice, it was unknown whether CD8 T cells played the same role in FI-RSV-immunized

mice. Furthermore, the effect of formalin fixation of RSV on the RSV-specific CD8 T cell response was unclear. We demonstrated in this study that FI-RSV does not prime an $M2_{82}$- or F_{85}-specific CD8 T cell response (Fig. 10, *A* and *B*) and that RSV challenge of FI-RSV-immunized mice does not result in an increased number of $M2_{82}$- or F_{85}-specific CD8 T cells compared with mock-immunized controls (Fig. 10*C*). These data are further supported by a recent study showing an inability of FI-bovine RSV to induce a virus-specific CD8 T cell response after bovine RSV challenge above that of control cows receiving a mock treatment (32). Furthermore, we show that the induction of an $M2_{82}$-specific CD8 T cell response after FI-RSV immunization results in abrogation of pulmonary eosinophilia (Fig. 11, *A* and *B*). Aldehyde-based fixatives (e.g., formaldehyde, formalin, and paraformaldehyde) are useful for the preservation of tissues and morphological structure; however, these fixatives have the potential to destroy protein structure and induce the loss of antigenic epitopes due to protein crosslinking (33). Taken together, these data suggest that formalin inactivation of RSV may lead to the masking or destruction of CD8 T cell epitopes and that it is the lack of a CD8 T cell response that contributes to RSV vaccine-enhanced pulmonary eosinophilia.

Interestingly, recent work has demonstrated that the introduction of carbonyl groups during the process of formaldehyde inactivation of the RSV vaccine may, in part, have been responsible for enhancing the Th2 response occurring after subsequent RSV challenge in FI-RSV-immunized mice (34). Although chemical reduction of the carbonyl groups resulted in an increased Th1 response in FI-RSV-immunized mice, it was not clear that eliminating the carbonyl modifications resulted in a restored CD8 T cell response. Thus, it remains unknown whether the introduction of carbonyl groups by formaldehyde fixation of RSV is solely responsible for the inability of FI-RSV immunization to elicit an RSV-specific CD8 T cell response.

Previous work by several laboratories has shown that increased eosinophilia in vvG- and FI-RSV-immunized mice often correlates with enhanced systemic disease as measured by weight loss and illness scores (35–38). Our data indicate that M2-specific CD8 T cells inhibit RSV vaccine-enhanced pulmonary eosinophilia; however it was unknown whether this reduction of pulmonary eosinophilia would correlate with reduced systemic illness. We demonstrated that either vvG- or FI-RSV-immunized mice undergoing a concurrent RSV-specific CD8 T cell response exhibit increased systemic illness (Fig. 11*D* and data not shown) despite having little to no pulmonary eosinophilia. These data demonstrate a clear disconnect between RSV vaccine-enhanced pulmonary eosinophilia and vaccine-enhanced systemic illness, indicating that these two phenomena are not unequivocally correlated. In addition, these data suggest that simply restoring a RSV-specific CD8 T cell response can have both a beneficial (i.e., inhibition of pulmonary eosinophilia) and a detrimental (i.e., enhanced systemic disease) consequence.

M2-specific CD8 T cells may increase systemic illness by a number of mechanisms. Several sepsis models have indicated that both TNF-α and/or IFN-γ play a role in mediating sepsis-induced weight loss and clinical illness. Additionally, TNF-α depletion abrogates systemic disease in vvG-immunized mice that generate a robust CD4 T cell response after RSV challenge (39). It is currently unclear which cytokines may play a role in CD8 T cell-induced systemic illness, but TNF-α and IFN-γ are promising molecules because they are both produced by a large frequency of RSV-specific CD8 T cells (24, 40, 41).

Mounting evidence supports the rationale for the development of vaccines that induce a balanced CD4 and CD8 T cell response upon immunization. The mouse model for RSV vaccine-enhanced disease is a prime example of how an unbalanced CD4 T cell response can lead to enhanced lung pathology and systemic disease. However, CD8 T cells can also induce potent systemic disease. Data presented here suggest that novel vaccines designed against RSV should incorporate both a CD4 and CD8 T cell epitope to inhibit Th2-driven immunopathology, but great care must be taken to limit CD8 T cell-driven systemic disease.

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Disclosures

The authors have no financial conflict of interest.

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