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The Number of Respiratory Syncytial Virus (RSV)-Specific Memory CD8 T Cells in the Lung Is Critical for Their Ability to Inhibit RSV Vaccine-Enhanced Pulmonary Eosinophilia

Matthew R. Olson,* Stacey M. Hartwig,* and Steven M. Varga2*†

Children that were administered a formalin-inactivated respiratory syncytial virus (FI-RSV) vaccine experienced enhanced respiratory disease, including pulmonary eosinophilia, after contracting a natural RSV infection. RSV vaccine-enhanced disease can be mimicked in BALB/c mice immunized with either FI-RSV or with a recombinant vaccinia virus (vacv) expressing the RSV attachment (G) protein. We have recently demonstrated that memory CD8 T cells directed against the RSV immunodominant M282–90 epitope inhibit the development of pulmonary eosinophilia in either vacvG- or FI-RSV-immunized mice by reducing the total number of Th2 cells in the lung after RSV challenge. In this study, we show that memory CD8 T cells specific to a subdominant epitope within the RSV fusion (F) protein fail to inhibit the development of pulmonary eosinophilia after RSV challenge of mice previously co-immunized with vacvF and with either vacvG or FI-RSV. We observed that the inability of RSV F85-specific memory CD8 T cells to inhibit the development of pulmonary eosinophilia was largely due to an inadequate total number of Th2 cells in the lung after RSV challenge. In this study, we show that increasing the number of F85-specific memory CD8 T cells after immunization grants these cells the ability to inhibit RSV vaccine-enhanced pulmonary eosinophilia. Moreover, we demonstrate that RSV-specific memory CD8 T cells, when present in sufficient numbers, inhibit the production of the Th2-associated chemokines CCL17 and CCL22. Taken together, these results indicate that RSV-specific memory CD8 T cells may alter the trafficking of Th2 cells and eosinophils into the lung.

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The successful establishment of a mouse model that mimics the RSV vaccine-enhanced disease observed in humans has greatly aided our understanding of the immune determinants that contribute to pathology. In this model, BALB/c mice are immunized with either FI-RSV or a recombinant vaccinia virus (vacv) that expresses the RSV attachment (G) protein (vacvG), both of which elicit an RSV-specific CD4 T cell response and do not prime a detectable RSV-specific CD8 T cell response (7–12). After subsequent RSV challenge, both FI-RSV- and vacvG-immunized mice develop robust RSV-specific memory CD4 T cell responses, pulmonary eosinophilia, and vaccine-enhanced disease that is similar to the disease observed in the children from the FI-RSV vaccine trials (9, 10, 12, 13). Previous work has demonstrated that both FI-RSV- and vacvG-immunized mice that also undergo a concurrent RSV-specific memory CD8 T cell response specific to the immunodominant RSV transcription anti-termination factor (M2)82–90 epitope have significantly reduced levels of pulmonary eosinophilia after RSV challenge as compared with controls (7, 14). These data suggest that RSV-specific memory CD8 T cells inhibit the Th2 response that is necessary for the development of pulmonary eosinophilia.

Besides the well-defined immunodominant M282–90-specific CD8 T cell response in BALB/c mice, several other subdominant CD8 T cell epitopes have been identified (15, 16). The RSV fusion (F) protein contains a subdominant CD8 T cell epitope at aa 85–93. CD8 T cells that recognize the F85 epitope comprise 2–5% of the CD8 T cells in the lung at the peak of the response after acute RSV infection (15). Previous work has demonstrated that vacvF-immunized mice develop a robust pulmonary CD4 and CD8 T cell response after RSV challenge (17–19). Interestingly, Ab-mediated depletion of either CD8 T cells or IFN-γ in vacvF-immunized mice results in the development of pulmonary eosinophilia after RSV challenge (19, 20). These data suggest that IFN-γ produced by F85-specific CD8 T cells prevents the development of pulmonary eosinophilia after RSV challenge of vacvF-immunized mice. However, it remains unclear whether F85-specific memory CD8 T cells can inhibit the development of pulmonary eosinophilia after RSV challenge of either vacvG- or FI-RSV-immunized mice.

Abbreviations used in this paper: RSV, respiratory syncytial virus; FI-RSV, formalin-inactivated respiratory syncytial virus; G, RSV attachment protein; F, RSV fusion protein; ICS, intracellular cytokine staining; BAL, bronchoalveolar lavage; DC, dendritic cell; β-gal, β-galactosidase; MFI, mean fluorescence intensity.

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We demonstrate in this study that only either vacvG- or FI-RSV-immunized mice that are also co-immunized with vacvM2, and not with vaccF, exhibit reduced levels of pulmonary eosinophilia after RSV challenge. Interestingly, there are a similar number of M282- and F85-specific CD8 T cells in the lungs of either vaccG plus vaccM2-immunized mice or vaccF plus vaccF-immunized mice at day 7 after RSV challenge. Although the total number of RSV-specific cells in the lung is similar in vaccG-immunized mice undergoing a concurrent M282- or F85-specific CD8 T cell response at day 7 after challenge, the total number of the F85-specific memory CD8 T cells is dramatically reduced compared with M282-specific CD8 T cells at early times (days 0–3) after RSV challenge. Importantly, decreasing the total number of M282-specific CD8 T cells early after RSV challenge leads to the development of pulmonary eosinophilia, and increasing the total number of F85-specific CD8 T cells early after RSV infection results in diminished eosinophilia. Furthermore, we go on to demonstrate that M282-specific memory CD8 T cells modulate the pulmonary protein levels of Th2-associated chemokines such as CCL17 and CCL22. Taken together, these data demonstrate that the total number of RSV-specific CD8 T cells in the lung early after infection dictates their ability to regulate the events that are required for the development of pulmonary eosinophilia.

Materials and Methods

Viruses and infection of mice

The A2 strain of RSV was a gift from B. S. Graham (National Institutes of Health, Bethesda, MD) and was propagated in HEP-2 cells (American Type Culture Collections; ATCC). Recombinant vacc were a gift from 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). BALB/c mice were i.m. immunized with a 1:200 dilution of FI-RSV and were challenged intranasally with 3 × 10^6 PFU of recombinant vacc or a mixture of two different recombinant vacc and challenged with RSV 3 wk later as previously described (7). Alternatively, BALB/c mice were i.m. immunized with a 1/200 dilution of FI-RSV or formalin-inactivated mock preparation of HEP-2 cell supernatants (ATCC). BALB/cAnNcr mice between 6 and 10 wk of age were purchased from the National Cancer Institute (Bethesda, MD). IFN-γ-deficient C12957(B6)-Higgm11y/J mice were originally purchased from The Jackson Laboratory. Each mouse was scarified with 3 × 10^9 PFU of recombinant vacc or a mixture of two different recombinant vacc and challenged with RSV 3 wk later as previously described (7). Alternatively, BALB/c mice were i.m. immunized with a 1/200 dilution of FI-RSV or formalin-inactivated mock preparation of HEP-2 cell supernatants as previously described (21). Four weeks after mock or FI-RSV immunization, mice were scarified with 3 × 10^9 PFU of vaccM2 or vaccF and 4 wk later were challenged intranasally with 3 × 10^9 PFU of RSV. All mouse experiments have been evaluated and approved by the University of Iowa Institutional Animal Care and Use Committee.

Mononuclear cell isolation and intracellular cytokine staining (ICS)

Lung mononuclear cells and bronchoalveolar lavage (BAL) cells were harvested and prepared as previously described (7). Cytospin (Cytospin 2; Thermo 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 standard morphology and staining characteristics. Lungs were subsequently perfused with 5 ml of PBS before removal. Lung mononuclear cells were isolated by pressing the lung tissue through a 70-μm filter (Celllector; Belco Glass). Peptides corresponding to the CD4 T cell epitopes G183–195 and F51–66 and the CD8 T cell epitopes F85–93 and M282–90 were purchased from Biosynthesis. To enumerate the number of RSV-specific CD4 and CD8 T cells, lung mononuclear cells were stimulated in vitro in the presence of 1 μM peptide and 10 μg/ml brefeldin A (Sigma-Aldrich) for 5 h at 37°C. Cells were subsequently stained for cell surface CD4, CD8, Thy1.2 and intracellular IFN-γ as previously described (7). Peripheral blood was collected from isolu- rane-anesthetized mice by eye bleed into 4% (w/v) sodium citrate. Blood was subsequently lysed with ammonium chloride and washed with RPMI 1640. Enumeration of F85- or M282-specific CD8 T cells in the blood was performed by in vitro stimulation in either the presence or the absence of an optimized target to effector ratio (1:3) of P815 cells (ATCC) coated with 1 μM F85–93 or M282–90 peptide followed by ICS for IFN-γ. Lamp1/2 and granzyme B staining were done in combination with IFN-γ ICS. Lamp1 (clone 1D4B; BD Biosciences) and Lamp2 (clone M3/84; Biolegend) Abs were added before incubation of lung mononuclear cells with P815 cells coated with 1 μM F85–93 peptide in the presence of 2 μM monensin (Sigma-Aldrich). Granzyme B (Caltag Laboratories) staining was done after the 5-h ICS incubation in the presence of 1 μM F85–93 peptide and 10 μg/ml brefeldin A.

Plaque assays

Lungs were harvested from vacc-immunized mice on either day 4 or 7 after RSV challenge in 1 ml of serum-free RPMI 1640. Lung tissue was disrupted using a tissue homogenizer (Ultra-Turrax T25; IKA Works), and lung homogenates were subsequently centrifuged at 2000 rpm for 10 min. Cell-free supernatants were collected and frozen in liquid nitrogen. Frozen samples were thawed and then subjected to plaque assay on Vero cells as previously described (7).

ELISA

Lungs and BAL were harvested at day 3 after RSV challenge of vacc-immunized mice. Lungs were disrupted using a tissue homogenizer (Ultra-Turrax T25; IKA Works) in 10% FCS containing RPMI 1640 containing a 1/200 dilution of a protease inhibitor cocktail (Sigma-Aldrich). BAL samples were collected by lavage of the lung with 1 ml of RPMI 1640 containing 10% FCS. Lung homogenates and BAL samples were centrifuged at 2000 rpm for 10 min and chemokine protein content for CCL11, CCL17, and CCL22 in the supernatants was subsequently determined by ELISA as previously described (19). For CXCL10 (IP-10) and CCL5 (RANTES) ELISA analysis, flat-bottom 96-well plates (Nunc) were coated overnight at 4°C with 2 μg/ml purified anti-CXCL10 and anti-CCL5 (all Abs from R&D Systems). After extensive washing, samples were added to coated plates in 50 μl per well followed by incubation overnight at 4°C. Biotinylated detection Abs for CXCL10 and CCL5 (R&D Systems) at 0.1 μg/ml were added to plates for 2 h at room temperature. Following incubation with the biotinylated Abs, plates were washed and developed as previously described (19). ELISA plates were analyzed by an Elx800 reader (Bio-Tek Instruments) and data were analyzed using KC Junior software (Bio-Tek Instruments). Detection limits for chemokine protein levels were: 624 pg/ml CXCL10; 62.5 pg/ml CCL5; 31.2 pg/ml CCL11; 62.5 pg/ml CCL17; and 15.6 pg/ml CCL22.

Dendritic cell (DC) generation and immunization

Flt3 ligand-secreting B16-melanoma cells were a gift from J. T. Harty (University of Iowa, Iowa City, IA) and were cultured in RPMI 1640 containing 10% FCS. A total of 1 × 10^6 Flt3 ligand-secreting B16 melanoma cells were i.p. injected into BALB/c mice in 200 μl of PBS. At 14 days post-injection, mice were i.p. injected with 2 μg of LPS (Sigma-Aldrich) in 200 μl of PBS. The following day, spleens of BALB/c mice were harvested in PBS and single cell suspensions were prepared. DC were collected by staining for CD11c (clone N418; eBioscience) and MHC class II (clone M5/114.15.2; eBioscience). Maturation of DC was monitored by expression of CD80 (clone 16–10A1; Bio-Tek Instruments) and CD86 (clone GL1; eBioscience). Over 90% of DC routinely expressed these costimulatory molecules (data not shown). The whole cell suspension was then incubated at 37°C for 1 h in either the presence or absence of 1 μM F85–93 peptide in PBS containing 2% normal rat serum (Jackson ImmunoResearch Laboratories). Cells were washed twice in PBS and 1 × 10^6 DC, as determined by flow cytometry, were i.v. injected into naïve recipients.

Data analysis and statistics

Statistical analyses were performed using Prism software (GraphPad). Data were analyzed using a Student’s t test or, where noted, by ANOVA followed by a Tukey post-test. Differences were considered significant when p < 0.05.

Results

RSV-specific T cell responses in vacc-immunized mice

Previous work has demonstrated that RSV M282-specific memory CD8 T cells inhibit the development of pulmonary eosinophilia after RSV challenge of either vaccG- or FI-RSV-immunized mice (7, 14). The F protein of RSV elicits a subdominant CD8 T cell response directed against aa 85–93 (15). It is currently unclear whether F85-specific memory CD8 T cells can inhibit vaccG-induced pulmonary eosinophilia. Mice immunized with a 1:1 ratio of either vaccG and vaccF (vaccG plus vaccF) or vaccG and vaccM2 (vaccG plus vaccM2) induced a
FIGURE 1. F85-specific memory CD8 T cells do not inhibit the development of pulmonary eosinophilia after RSV challenge of mice previously immunized with vacvG. BALB/c mice were immunized with vacvβ-gal, vacvG plus vacvβ-gal, vacvF, vacvG plus vacvF, or vacvG plus vacvM2. At least 21 days after vacv immunization mice were intranasally challenged with RSV. Seven days post-infection, the total number of F85-specific (A) and M282-specific (B) CD8 T cells was determined by IFN-γ ICS. The frequency (C) and total number (D) of eosinophils in the BAL were quantified by flow cytometry. Error bars represent the SEM from n = 3–4 mice per group. Data represent three independent experiments. *, p < 0.05. Levels at p > 0.05 were not significantly different (n.s.).

Similar total number of Ag-specific CD8 T cells at day 7 after RSV challenge (Fig. 1, A and B). With a similar total number of F85- and M282-specific CD8 T cells, we predicted that F85-specific CD8 T cells would inhibit the development of pulmonary eosinophilia in vacvG-immunized mice. Surprisingly, vacvG plus vacv expressing β-galactosidase (β-gal)-immunized and vacvG plus vacvF-immunized mice developed a similar frequency (Fig. 1C) and a total number (Fig. 1D) of eosinophils in the BAL at day 7 after RSV challenge, whereas mice immunized with vacvG plus vacvM2 exhibited a significant reduction (p < 0.05) in both the frequency and the total number of eosinophils in the BAL at this time (Fig. 1, C and D). As previously described (7), only vacvG plus vacvM2- and not vacvG plus vacvF-immunized mice demonstrated a significant decrease (p < 0.05) in the total number of G183-specific CD4 T cells (Fig. 1E). As expected only mice immunized with either vacvG plus vacvF or vacvF alone mounted an enhanced F85-specific CD4 and F85-specific CD8 T cell response (Fig. 1, A and F). Furthermore, only vacvG plus vacvM2-immunized mice exhibited an increased total number of M282-specific CD8 T cells over the acute (i.e., vacvβ-gal-immunized) RSV response (Fig. 1B).

M282-specific but not F85-specific CD8 T cells inhibit the Vβ14+ CD4 T cell response

Immunization of BALB/c mice with vacvG induces an oligoclonal Vβ14+ CD4 T cell population that is required for the development of pulmonary eosinophilia after RSV challenge (8, 22). Previously, we have demonstrated that RSV M282-specific CD8 T cells reduce the total number of CD4 as well as Vβ14+ CD4 T cells in the lung after RSV challenge of vacvG-immunized mice (7). Having shown that F85-specific CD8 T cells do not inhibit the development of pulmonary eosinophilia after RSV challenge of vacvG plus vacvF-immunized mice; we next asked whether these cells were also unable to inhibit the expansion of CD4 and Vβ14+ CD4 T cells in the lung after RSV challenge. Fig. 2 demonstrates that only mice immunized with vacvG plus vacvM2 and not vacvG plus vacvF exhibited a significantly reduced (p < 0.05) total number of CD4 (Fig. 2A) and Vβ14+ CD4 (Fig. 2B) T cells in the lung after RSV challenge.

Viral clearance in immunized mice

We analyzed virus clearance to determine its potential role in the differential development of pulmonary eosinophilia after RSV challenge of mice previously immunized with either vacvG plus vacvF or vacvG plus vacvM2. All immunized mice had significantly decreased (p < 0.05) viral titers at both day 4 and day 7 after RSV challenge as compared with vacvβ-gal-immunized mice that do not mount an RSV-specific memory T cell response (Fig. 3). Additionally, both vacvG plus vacvF- and vacvG plus vacvM2-immunized mice, which undergo memory CD4 and CD8 T cell
responses, had significantly reduced (p < 0.05) viral titers in their lungs as compared with vacvG plus vacvβ-gal-immunized mice at day 4 after RSV challenge. However, there was no significant difference (p > 0.05) in the viral titers in the lungs of vacvG plus vacvF- and vacvG plus vacvM2-immunized mice at day 4 after RSV challenge, suggesting that differential clearance of the virus does not account for the difference in pulmonary eosinophilia exhibited by these mice (see Fig. 1).

**F~85~**-specific CD8 T cells fail to inhibit FI-RSV-induced vaccine-enhanced pulmonary eosinophilia

We have previously demonstrated that induction of an M2~82~-specific memory CD8 T cell response in FI-RSV-immunized mice is independent of the oligoclonal Vβ14+ CD4 T cell expansion that is required for the development of pulmonary eosinophilia after RSV challenge of vacvG-immunized mice (11). We have shown (see Fig. 2) that only M2~82~-specific and not F~85~-specific memory CD8 T cells reduce the total number of Vβ14+ CD4 T cells in the lung after RSV challenge of vacvG plus vacvM2- and vacvG plus vacvF-immunized mice, respectively. Therefore it remained possible that F~85~-specific memory CD8 T cells could inhibit the development of pulmonary eosinophilia after RSV challenge of FI-RSV- but not vacvG-immunized mice. To determine whether F~85~-specific memory CD8 T cells can inhibit pulmonary eosinophilia after RSV challenge of FI-RSV-immunized mice, mice were either mock treated or i.m. immunized with FI-RSV. Four weeks after initial priming, mice were subsequently immunized with vacvβ-gal, vacvF, or vacvM2 before intranasal challenge with RSV after an additional 4 wk. Fig. 4 demonstrates that only FI-RSV-immunized mice co-immunized with vacvM2 and not vacvF have significantly reduced (p < 0.05) total numbers (Fig. 4A) of eosinophils in the BAL. Similar to what we have previously observed with vacvG plus vacvF- and vacvG plus vacvM2-immunized mice (see Fig. 1), there was not a significant difference (p > 0.05) in the total number of either F~85~- or M2~82~-specific CD8 T cells in FI-RSV/ vacvM2- and FI-RSV/vacvF-immunized mice (Fig. 4B).

**Function of F~85~**- and M2~82~-specific memory CD8 T cells

One possible reason F~85~-specific memory CD8 T cells are unable to inhibit the development of pulmonary eosinophilia after RSV challenge of either vacvG- or FI-RSV-immunized mice may be due to a defect in their effector function. To address this possibility, we compared the ability of F~85~ and M2~82~-specific CD8 T cells to produce common CD8 T cell effector cytokines (i.e., IFN-γ, TNF-α, or IL-2) by ICS. Interestingly, F~85~-specific CD8 T cells contained a higher frequency of cells capable of co-producing the cytokines IFN-γ and either TNF-α or IL-2 as compared with M2~82~-specific CD8 T cells (Fig. 5A and B). Furthermore, the relative amount of IFN-γ, TNF-α, and IL-2 produced, as measured by mean fluorescence intensity (MFI), was greater in F~85~-specific CD8 T cells as compared with M2~82~-specific CD8 T cells (Fig. 5C). Taken together, these data suggest that F~85~-specific CD8 T cells exhibit at least similar if not greater cytokine production capacity as compared with their M2~82~-specific counterparts.

**Kinetics of the F~85~**- and M2~82~-specific memory CD8 T cell responses

Although we found an equivalent number of F~85~ and M2~82~-specific CD8 T cells in the lung at day 7 after RSV challenge, it was unclear whether the early kinetics of the F~85~ and M2~82~-specific memory CD8 T cell responses were similar. Therefore, we examined the total number of F~85~ and M2~82~-specific CD8 T cells in the lung before and at various time points after RSV challenge. Fig. 6 demonstrates that before RSV challenge (21 days after vacv-immunization) there was a significantly greater (p < 0.05, 3–4 fold) total number of M2~82~-specific CD8 T cells in the spleen, blood, inguinal lymph nodes, and lungs of vacvG plus vacvM2-immunized mice (Fig. 6A) as compared with F~85~-specific CD8 T cells in vacvG plus vacvF-immunized mice. Furthermore, there are significantly (p < 0.05) more M2~82~-specific CD8 T cells in the lungs of vacvG plus vacvM2-immunized mice as compared with the number of F~85~-specific CD8 T cells in the lungs of vacvG plus vacvF-immunized mice at day 3 after RSV challenge (Fig. 6B). Consistent with the data presented in Fig. 1, the total number of F~85~ and M2~82~-specific CD8 T cells at day 7 after RSV challenge is not significantly different (p > 0.05) (Fig. 6B). These results suggest that the total number of RSV-specific memory CD8 T cells in the lungs at early time points after RSV challenge may be a critical determinant to the inhibition of pulmonary eosinophilia.

We next asked whether this discrepancy between the early kinetics of the F~85~ and M2~82~-specific memory CD8 T cell responses...
were identified by gating on CD8 and Thy1.2. Representative dot plots of costaining with IFN-γ and either IL-2 or TNF-α from gated lung mononuclear cells isolated from vacvG plus vacvF- and vacvG plus vacvM2-immunized mice. Numbers indicate frequency of cells in each quadrant. F85-specific CD8 T cells in vacvG plus vacvF-immunized mice after RSV challenge is 3- to 4-fold greater than the total number of M282-specific CD8 T cells in vacvG plus vacvM2-immunized mice at day 3 respectively (Fig. 7A). Mice immunized with 200 vacvG:vacvM2 had an equivalent total number of M282-specific cells in the lung as compared with the total number of F85-specific cells in vacvG plus vacvF-immunized mice at day 3 after RSV challenge (Fig. 7A). As discussed, at day 7 after RSV challenge, there was a similar total number of F85- and M282-specific CD8 T cells in the lungs of vacvG plus vacvF- and vacvG plus vacvM2-immunized mice, respectively (Fig. 7B). Importantly, there were substantially more M282-specific CD8 T cells in 200 vacvG:vacvM2-immunized mice could account for their differential ability to inhibit the development of pulmonary eosinophilia. We therefore altered the magnitude of the M282-specific memory response by immunizing mice with a ratio of 200:1 vacvG to vacvM2 (200 vacvG:vacvM2) in an attempt to match the early kinetics of the F85-specific CD8 T cell response. Fig. 7 demonstrates that the total number of M282-specific CD8 T cells in vacvG plus vacvM2-immunized mice at day 3 after RSV challenge is 3- to 4-fold greater than the total number of F85-specific CD8 T cells in vacvG plus vacvF-immunized mice (Fig. 7A). Mice immunized with 200 vacvG:vacvM2 had an equivalent total number of M282-specific cells in the lung as compared with the total number of F85-specific cells in vacvG plus vacvF-immunized mice at day 3 after RSV challenge (Fig. 7A). As discussed, at day 7 after RSV challenge, there was a similar total number of F85- and M282-specific CD8 T cells in the lungs of vacvG plus vacvF- and vacvG plus vacvM2-immunized mice, respectively (Fig. 7B). Importantly, there were substantially more M282-specific CD8 T cells in 200 vacvG:vacvM2-immunized mice could account for their differential ability to inhibit the development of pulmonary eosinophilia. We therefore altered the magnitude of the M282-specific memory response by immunizing mice with a ratio of 200:1 vacvG to vacvM2 (200 vacvG:vacvM2) in an attempt to match the early kinetics of the F85-specific CD8 T cell response. Fig. 7 demonstrates that the total number of M282-specific CD8 T cells in vacvG plus vacvM2-immunized mice at day 3 after RSV challenge is 3- to 4-fold greater than the total number of F85-specific CD8 T cells in vacvG plus vacvF-immunized mice (Fig. 7A). Mice immunized with 200 vacvG:vacvM2 had an equivalent total number of M282-specific cells in the lung as compared with the total number of F85-specific cells in vacvG plus vacvF-immunized mice at day 3 after RSV challenge (Fig. 7A). As discussed, at day 7 after RSV challenge, there was a similar total number of F85- and M282-specific CD8 T cells in the lungs of vacvG plus vacvF- and vacvG plus vacvM2-immunized mice, respectively (Fig. 7B). Importantly, there were substantially more M282-specific CD8 T cells in 200 vacvG:vacvM2-immunized mice.

**FIGURE 6.** Kinetics of the F85- and M282-specific memory CD8 T cell responses in vacv-immunized mice. BALB/c mice were immunized with recombinant vacv and subsequently intranasally challenged with RSV at least 21 days after vacv immunization as described in Fig. 1. A, Spleens, lungs, inguinal lymph nodes (ILN), and peripheral blood were harvested 21 days post-immunization with vacv. The total number of F85-specific (■) and M282-specific (□) were determined by IFN-γ ICS. Error bar represents the SEM from 3 mice per group, and inguinal lymph nodes were pooled from 3 mice. B, Lungs were harvested at 0, 3, or 7 days post-challenge with RSV and the total number of RSV-specific CD8 T cells was quantified by IFN-γ ICS. Error bar represents the SEM from 3–4 mice per group. Data represent three independent experiments. *p < 0.05, significantly different as compared with vacvG plus vacvF-immunized controls.
as compared with vacβ-gal-immunized mice, indicating that we successfully primed a M282-specific memory CD8 T cell response (Fig. 7B). However, the M282-specific memory CD8 T cell response in 200 vacG:vacM2-immunized mice is significantly reduced (2- to 3-fold, \( p < 0.05 \)) as compared with vacG plus vacM2-immunized mice at day 7 after RSV challenge (Fig. 7B). Mice immunized with vacG plus vacβ-gal have a robust RSV G183-specific CD4 T cell response at day 7 after RSV challenge (Fig. 7C). This G183-specific CD4 T cell response is significantly reduced (\( p < 0.05 \)) in mice undergoing a concurrent M282-specific, but not a concurrent F85-specific, memory CD8 T cell response (Fig. 7C). Interestingly, when we equalized the M282- and F85-specific response at day 3 after RSV challenge by immunizing mice with 200 vacG:vacM2 (Fig. 7A) we observed a significant increase (\( p < 0.05 \)) in the total number of G183-specific CD4 T cells at day 7 after RSV challenge. As demonstrated, mice immunized with vacG plus vacβ-gal and vacG plus vacF have a similar total number (Fig. 7D) of eosinophils and this is significantly reduced (\( p < 0.05 \)) in vacG plus vacM2-immunized mice after RSV challenge (Fig. 7D). Interestingly, 200 vacG:vacM2-immunized mice have similar levels of pulmonary eosinophilia as compared with vacG plus vacβ-gal- and vacG plus vacF-immunized mice (Fig. 7D). To ensure that M282-specific CD8 T cells induced by vacG plus vacM2 and 200 vacG:vacM2 immunization behave similarly after RSV challenge we compared the frequency of IFN-γ-producing CD8 T cells (Fig. 7E) and the MFI of IFN-γ produced by these cells by IFN-γ ICS (Fig. 7F). Fig. 7E demonstrates that there is a slight decrease in the frequency of IFN-γ-producing cells that is consistent with the 2- to 3-fold decrease in the total number of M282-specific CD8 T cells noted earlier (Fig. 7B). Additionally, M282-specific CD8 T cells in vacG plus vacM2- and 200 vacG:vacM2-immunized mice produce IFN-γ at a similar per cell capacity as measured by the MFI of IFN-γ, which is significantly greater than the nonstimulated controls (\( p < 0.05 \)). These data indicate that the early kinetics of entry and accumulation in the lung by RSV-specific memory CD8 T cells is a major determinant of their ability to inhibit the development of pulmonary eosinophilia after RSV challenge of vacG-immunized mice.

### FIGURE 7

Reducing the early M282-specific CD8 T cell response results in pulmonary eosinophilia. BALB/c mice were immunized with recombiant vacv and challenged with RSV as described in Figs. 1 and 6. An additional group of mice were immunized with 200 vacG:vacM2, a 1:200 dilution of vacvM2 mixed 1:1 with vacvG. A Lungs were harvested 3 days after RSV challenge and the total number of F85-specific (□) and M282-specific (■) CD8 T cells were quantified by IFN-γ ICS. B The total number of F85-specific (□) and M282-specific (■) CD8 T cells 7 days after RSV challenge as quantified by IFN-γ ICS. C The total number of G183-specific CD4 T cells 7 days after RSV challenge as quantified by IFN-γ ICS. D The total number of eosinophils in the BAL of vacv-immunized mice at day 7 after RSV challenge was quantified by quantitative morphometry. Detection limits at \(<1 \times 10^5 \) cells were below limit of detection (B.L.D.). E Representative dot plots of IFN-γ production by CD8 T cells in vacvβ-gal-, vacG plus vacM2-, or 200 vacG:vacM2-immunized mice isolated from the lungs at day 7 after RSV challenge. Frequency of IFN-γ-producing CD8 T cells is shown for each gate. F Quantification of IFN-γ-MFI from mice represented in E. Error bar represents the SEM from \( n = 3–4 \) mice per group. Data represent three independent experiments. *, \( p < 0.05 \).

Th2-associated chemokines are important in the development of RSV vaccine-enhanced pulmonary eosinophilia (19, 23–25). The production of several Th2-associated chemokines peaks early after RSV challenge of vaccG-immunized mice (26). Therefore, we asked whether either a concurrent F85- or M282-specific memory CD8 T cell response would reduce the early Th2-associated chemokine production after RSV challenge of vaccG-immunized mice. Lungs and BAL were harvested at day 3 after RSV challenge and subsequently analyzed for protein levels of either Th1-associated (CXCL10, CCL5) or Th2-associated (CCL11, CCL17, CCL22) chemokines by ELISA. Mice immunized with vaccG, vaccG plus vaccF, vaccG plus vaccM2, or 200 vacG:vacM2 showed similar enhanced protein levels of all Th1-associated chemokines and interestingly the Th2-associated chemokine CCL11 in both the lung and BAL (Fig. 8). Our data also demonstrates that only M282-specific, but not F85-specific, memory CD8 T cells are able to significantly decrease (\( p < 0.05 \)) protein levels of CCL17 and CCL22 in both the lung and BAL (Fig. 8). Importantly, mice immunized with 200 vacG:vacM2 had increased levels (\( p < 0.05 \)) of CCL17 and CCL22 as compared with mice immunized with vaccG plus vaccM2, suggesting that a reduced number of M282-specific memory CD8 T cells are unable to alter the chemokine environment (Fig. 8). These data indicate that M282-specific memory CD8 T cells prevent the trafficking of Th2 cells, and possibly eosinophils, into the lung by inhibiting the early production of the Th2-associated chemokines CCL17 and CCL22.
Boosting the F<sub>85</sub>-specific memory CD8 T cell response results in the inhibition of pulmonary eosinophilia

Having established that reducing the total number of M<sub>2</sub>-specific CD8 T cells that enter the lung early after RSV infection results in the development of pulmonary eosinophilia (Fig. 7), we next asked whether increasing the number of memory F<sub>85</sub>-specific CD8 T cells early after RSV challenge would result in the abrogation of pulmonary eosinophilia. Mice were immunized with 1 × 10<sup>6</sup> DC that were either left untreated or coated with F<sub>85-93</sub>-peptide. After 2–3 wk, DC-immunized mice were subsequently immunized with vacc<sub>β</sub>-gal, vaccG plus vacc<sub>β</sub>-gal, or vaccG plus vaccF and challenged with RSV after an additional 3 wk of rest. We were unable to detect F<sub>85</sub>-specific CD8 T cells in either the peripheral blood or lungs of mice immunized with DC-F<sub>85-93</sub> and vacc<sub>β</sub>-gal, whereas we were able to detect a low number of F<sub>85</sub>-specific CD8 T cells after DC-no peptide and vaccG plus vaccF immunization (Fig. 9, A and B). However, mice immunized with DC-F<sub>85-93</sub> and vaccG plus vaccF exhibited a significantly greater (p < 0.05) number of F<sub>85</sub>-specific CD8 T cells in both the peripheral blood and lung 21 days after vacc immunization as compared with mice immunized with DC-no peptide and vaccG plus vaccF (Fig. 9, A and B), demonstrating that we were able to boost the number of F<sub>85</sub>-specific memory CD8 T cells in the peripheral blood and lung 5- to 10-fold. At day 3 after RSV challenge of DC- and vacc-immunized mice there was a significantly (p < 0.05) greater total number of F<sub>85</sub>-specific CD8 T cells in the lungs of DC-F<sub>85-93</sub> and vaccG plus vaccF-immunized mice as compared with their DC-no peptide-immunized counterparts (Fig. 9B). After RSV challenge only DC-F<sub>85-93</sub> and vaccG plus vaccF-immunized mice had a significantly decreased (p < 0.05) total number of eosinophils in the lungs as compared with vaccG plus vacc<sub>β</sub>-gal-immunized mice (Fig. 9C).

Interestingly, there was only a 2- to 3-fold increase (p < 0.05) in the total number of F<sub>85</sub>-specific CD8 T cells at day 7 after RSV challenge in the lungs of DC-F<sub>85-93</sub> and vaccG plus vaccF-immunized mice as compared with DC-no peptide- and vaccG plus vaccF-immunized mice (Fig. 9D). These data demonstrate that F<sub>85</sub>-specific memory CD8 T cells are capable of inhibiting the development of pulmonary eosinophilia if present in high enough numbers at the time of or early after RSV challenge. Furthermore, these data provide strong evidence that both the total number and early kinetics of entry into the lung of RSV-specific memory CD8 T cells are critical for the control of RSV vaccine-enhanced pulmonary eosinophilia.

Characteristics of F<sub>85</sub>-specific CD8 T cells in DC- and vacc-immunized mice

One drawback to using the DC prime and boost methodology described in this study to increase the total number of F<sub>85</sub>-specific memory CD8 T cells in the lung is that the resultant cells are now
secondary memory cells, as compared with primary memory cells generated after vacvG plus vacvF immunization. Consequently, the F85-specific secondary memory CD8 T cells may differ in their phenotypic or functional characteristics. To address this issue, we examined the functional and phenotypic characteristics of F85-specific memory CD8 T cells at day 21 after vacvG plus vacvF immunization of mice previously immunized with DC that were either left untreated or coated with F85–93 peptide. DC-no peptide-immunized mice exhibited a greater frequency of cells expressing CD62L but not TNF-α or IL-2 as compared with mice immunized with DC-F85–93 (Table I). However, after RSV challenge of mice previously immunized with either DC-no peptide and vacvG plus vacvF or DC-F85–93 and vacvG plus vacvF, F85-specific primary and secondary memory CD8 T cells exhibited similar functional avidity (Fig. 10A), degranulation, as determined by Lamp1/2 staining (Fig. 10B) and similar expression of intracellular granzyme B (Fig. 10C). However, there was a difference in the ability of these cells to produce the cytokines TNF-α and IL-2 (Fig. 10, D and E). These data suggest that although there are some phenotypical and functional differences in F85-specific cells in DC-no peptide- and DC-F85–93-immunized mice, these differences are relatively small and are unlikely to greatly alter their ability to inhibit the development of pulmonary eosinophilia.

F85-specific CD8 T cells, when present at sufficient numbers early after RSV challenge, alter the pulmonary chemokine environment

As demonstrated, M282-specific memory CD8 T cells in vacvG plus vacvM2-immunized mice are present at higher numbers early after RSV challenge as compared with F85-specific CD8 T cells in vacvG plus vacvF-immunized mice (Figs. 6 and 7). Additionally, these M282-specific CD8 T cells are able to alter the pulmonary chemokine environment, whereas the F85-specific CD8 T cells, that are present at a lower number, are not (Fig. 8). To determine whether increasing the total number of F85-specific CD8 T cells by DC-F85–93 and vacvG plus vacF immunization (see Fig. 9) would also alter the pulmonary chemokine environment, DC- and vacvF-immunized mice were harvested at day 3 after RSV challenge and subsequently analyzed for the protein levels of the chemokines CXCL10, CCL11, CCL17, and CCL22 by ELISA. Fig. 11 shows that, like before, a low number of F85-specific CD8 T cells in DC-no peptide- and vacvG plus vacF-immunized mice were unable to decrease the protein levels of CCL17 and CCL22 in the lung as compared with DC-no peptide- and vacvG plus vacvM2-gal-immunized mice. However, in DC-F85–93 and vacvG plus vacF-immunized mice, where there are a higher total number of F85-specific CD8 T cells, there are significant decreases (p < 0.05) in the levels of CCL17 and CCL22 protein in the lung (Fig. 11). Interestingly, as observed with M282-specific CD8 T cells in vacvG plus vacvM2-immunized mice (see Fig. 8), there are no significant decreases (p > 0.05) in protein levels of CXCL10 or CCL11 in DC-F85–93 and vacvG plus vacF-immunized mice (Fig. 11). These data strongly suggest that a critical number of RSV-specific memory CD8 T cells are required to alter the pulmonary chemokine environment and prevent the development of pulmonary eosinophilia.

Table I. Phenotype of F85-specific CD8 T cells in DC-no peptide- and DC-F85–93-immunized mice after subsequent vacvG plus vacvF immunization

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Marker</th>
<th>Percentage of Positive Cells of F85-Specific CD8 T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-no peptide → vacvG</td>
<td>IL-2</td>
<td>8.0 ± 4.9</td>
</tr>
<tr>
<td>plus vacvF</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>59.8 ± 13.2</td>
</tr>
<tr>
<td></td>
<td>CD62L</td>
<td>32 ± 5.3</td>
</tr>
<tr>
<td>DC-F85–93 → vacvG</td>
<td>IL-2</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td>plus vacvF</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>64.5 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>CD62L</td>
<td>15.5 ± 1.3</td>
</tr>
</tbody>
</table>

* Mice were immunized with untreated DC (DC-no peptide) or DC coated with F85–93 peptide (DC-F85–93). Two weeks after DC immunization, mice were subsequently immunized with vacvG plus vacvF.
+ F85-specific CD8 T cells were identified in the peripheral blood 21 days after vacv immunization by IFN-γ ICS.
+ These data represent two individual experiments with n = 4 mice per group. Frequency of marker positive cells is expressed as the mean ± SE of the mean.
+ Significantly different (p < 0.05) than DC-F85–93 → vacvG plus vacF-immunized mice.
CD8 T CELLS INHIBIT RSV VACCINE-ENHANCED AIRWAY EOSINOPHILIA

FIGURE 11. F₈₅₉-specific memory CD8 T cells after the pulmonary chemokine environment if present at sufficient numbers early after RSV challenge. BALB/c mice were immunized with 1 × 10⁶ DC that were either left untreated or coated with F₉₅₉₉₉ peptide as described in Fig. 9. Two weeks after DC immunization, mice were subsequently immunized with vacvβ-gal, vacvG plus vacvβ-gal, and vacvG plus vacvF and were challenged with RSV 3 wk after vacv immunization. Protein levels of CXCL10, CCL11, CCL17, and CCL22 were measured by ELISA from lung homogenates at day 3 after RSV challenge. These data represent pooled data from three individual experiments for n = 9–12 mice per group. Error bar represents the SEM. *, p < 0.05 comparing vacvG plus vacvβ-gal-immunized and vacvG plus vacvF-immunized mice as determined by ANOVA.

M₂₈₂₉-specific CD8 T cells inhibit vacvF-induced pulmonary eosinophilia via an IFN-γ-independent mechanism

BALB/c mice immunized with vacvF do not develop pulmonary eosinophilia after RSV challenge (19, 20). However, IFN-γ-deficient mice immunized with vacvF develop pulmonary eosinophilia after RSV challenge (19, 20). These data suggest that F₈₅₉₉₉-specific CD8 T cells inhibit vacvF-induced pulmonary eosinophilia by an IFN-γ-dependent mechanism. We have previously demonstrated that M₂₈₂₉₉-specific memory CD8 T cells inhibit vacvG-induced pulmonary eosinophilia via an IFN-γ-independent mechanism (7). Thus, it appears that there are differences in the requirements for IFN-γ based on the CD8 T cell population responding to the challenge as well as the immunization (i.e., either vacvG or vacvF). Therefore, we asked whether M₂₈₂₉₉-specific memory CD8 T cells could inhibit vacvF-induced pulmonary eosinophilia in the absence of IFN-γ. BALB/c IFN-γ-deficient mice were immunized with vacvβ-gal, vacvF plus vacvβ-gal, vacvM2 plus vacvβ-gal, and vacvF plus vacvM2. At least 21 days later mice were intranasally challenged with RSV. The total number of eosinophils (A) in the BAL at day 7 after RSV challenge was enumerated by quantitative morphometry. B, The total number of F₈₅₉₉₉ and M₂₈₂₉₉-specific CD8 T cells were quantified by ICS for TNF-α. Error bar represents the SEM from n = 3–4 mice per group. These data represent three independent experiments. Detection limits at <1 × 10⁶ cells were below limit of detection (B.L.D.). *, p < 0.05.

Discussion

CD8 T cells play a prominent role in the clearance of intracellular pathogens. In addition, CD8 T cells also have been shown to inhibit pathological CD4 T cell responses (7, 14, 27, 28). Previous work has established that RSV-specific CD8 T cells can inhibit the development of pulmonary eosinophilia during RSV challenge of previously immunized mice (14, 20). We have recently demonstrated that induction of an RSV M₂₈₂₉₉-specific memory CD8 T cell response inhibits both the total number of Th2 cells and eosinophils in the lung after RSV challenge of mice previously immunized with either vacvG- or FI-RSV (7). Because the presence of Th2 cells in the lung is required to induce pulmonary eosinophilia in this model (29), these results suggest that RSV-specific memory CD8 T cells inhibit the trafficking or expansion of Th2 cells. In this study, we show that the ability of RSV-specific memory CD8 T cells to inhibit RSV vaccine-enhanced pulmonary eosinophilia is critically dependent on the total number of virus-specific memory CD8 T cells in the lung early after infection. Simply decreasing the number of M₂₈₂₉₉-specific CD8 T cells present in the lungs early after RSV challenge (day 3) results in the development of pulmonary eosinophilia (Fig. 7). Moreover, we demonstrate that memory CD8 T cells specific to the subdominant F₉₅₉₉₉₉ epitope, if present in high enough numbers early after RSV challenge, also inhibit the development of pulmonary eosinophilia in vacvG plus vacvF-immunized mice (Fig. 9). However, the underlying mechanisms of how these early memory CD8 T cell responses prevent the Th2-driven eosinophilic response remain unclear.

Chemokines are critical for trafficking of immune cells to sites of inflammation. Murine eosinophils and CD4 T cells have been shown to express different chemokine receptors based on their localization or functional phenotype (30). Our data indicates that the presence of a high number of RSV-specific memory CD8 T cells in the lung early after RSV challenge alters the chemokine environment in the lung (Figs. 8 and 11). Interestingly, we observed a preferential decrease in the protein levels of CCL17 and CCL22 and no change in the levels of CCL11 (Figs. 8 and 11). CCL11, CCL17, and CCL22 have all been shown to play a role in the recruitment of Th2 cells and eosinophils that express their cognate receptors (CCR4 for CCL17 and CCL22, CCR3 for CCL11) (30–33). Matthews et al. (23) demonstrated that depletion of CCL11 in vacvG-immunized mice reduces the total number of eosinophils in the lung after RSV challenge. Importantly, the reduction of eosinophils in CCL11-depleted mice is ~2-fold as compared with mock-treated mice, suggesting that other Th2-associated chemokines may compensate and facilitate the trafficking of eosinophils into the lung. These data are similar to allergic models of airway disease in which CCL11-deficient mice display only a partially decreased number of eosinophils in the lung, again suggesting roles for other Th2-associated chemokines (i.e., CCL17, CCL22) in the onset of eosinophilic disease (34, 35).
Interestingly, whereas the absence of CCL11 in asthma models only partially reduces airway eosinophilia, depletion of either CCL17 or CCL22 completely abrogates pulmonary eosinophilia induced by repeated allergen stimulation (34–37). Further studies concluded that depletion of CCL22 dramatically reduced the trafficking of Th2 cells into the lung of allergen challenged mice (32). These data are consistent with our previous findings that vacvG-immunized mice undergoing a concurrent M282-specific memory CD8 T cell response have a similar number of RSV-specific Th2 cells at day 5 after RSV challenge but have a significantly decreased total number of RSV-specific Th2 cells at the same time point (7). Taken together, these data suggest that the modulation of CCL17 and CCL22 chemokine levels by RSV-specific memory CD8 T cells may alter the trafficking of both Th2 cells and eosinophils into the lungs after RSV challenge of vacvG-immunized mice.

It is currently unclear how memory CD8 T cells inhibit the production of CCL17 and CCL22 in the lungs early after RSV challenge of vacvG-immunized mice. It is unlikely that accelerating viral clearance is responsible for this early decrease in chemokine production as the kinetics of viral clearance is similar in vacvG plus vaccF- and vaccG plus vaccm2-immunized mice (Fig. 3). Russell et al. (20) demonstrated that vaccF-immunized mice develop pulmonary eosinophilia after Ab-mediated depletion of either CD8 T cells or IFN-γ, suggesting that IFN-γ may be required for CD8 T cell inhibition of RSV vaccine-enhanced pulmonary eosinophilia. This concept is attractive because of the known inhibitory effects of IFN-γ on Th2 cells (38–40). Furthermore, IFN-γ has been previously demonstrated to inhibit the production of CCL17 or CCL22 (41–43). However, we have previously shown that RSV M282-specific memory CD8 T cells inhibit the development of pulmonary eosinophilia after RSV challenge of vaccG plus vaccm2-immunized mice in the absence of IFN-γ (7). We have also demonstrated in this study that M282-specific memory CD8 T cells inhibit the development of pulmonary eosinophilia after RSV challenge of vaccG plus vaccm2-immunized mice in the absence of IFN-γ (7).

References


