Respiratory syncytial virus infection suppresses lung CD8+ T-cell effector activity and peripheral CD8+ T-cell memory in the respiratory tract

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Respiratory syncytial virus (RSV) is a major cause of morbidity from respiratory infection in infants, young children and the elderly. No effective vaccine against RSV is currently available and studies of the natural history of RSV infection suggest repeated infections with antigenically related virus strains are common throughout an individual's lifetime. We have studied the CD8+ T-cell response during experimental murine RSV infection and found that RSV inhibits the expression of effector activity by activated RSV-specific CD8+ T cells infiltrating the lung parenchyma and the development of pulmonary CD8+ T-cell memory by interfering with TCR-mediated signaling. These data suggest a possible mechanism to explain the limited duration of protective immunity in RSV infection.

Respiratory syncytial virus (RSV) is the most common cause of viral lower respiratory tract infection. RSV can cause severe disease in infants[1], in older immunodeficient children[2] and the elderly[3]. Reinfection by RSV is a frequent event in infants[4,5]. Adults also remain susceptible to repeated RSV infection, indicating that protective immunity to reinfection may be incomplete and of short duration[6]. In murine models, CD8+ T cells have been reported to play a pivotal role in recovery from RSV infection[7,8]. In BALB/c mice, a single immunodominant, H-2K^d-restricted peptide epitope in the matrix 2 (M2) protein of RSV ($M2_{82–90}$) is the major target of the CD8+ T cells responding to RSV infection; these CD8+ T cells appear to have a prominent role in both recovery from RSV infection and protective immunity[9,10]. However, the protective immunity conferred by M2-specific CD8+ T cells is short-lived and rapidly wanes[11], suggesting that the susceptibility to reinfection may be the result of immune dysregulation mediated by RSV.

To address the issue of possible immunosuppression mediated by RSV, we examined and characterized the dominant $M2_{82–90}$-specific CD8+ T-cell response in the lungs of BALB/c mice during RSV infection using tetramer staining and functional assays. We found that there is a large expansion of activated, $M2_{82–90}$-specific CD8+ T cells in the lungs during the course of primary and challenge RSV infection, indicating that the initial activation and proliferation of M2-specific CD8+ T-cell precursors is normal. However, the massively expanded and activated $M2_{82–90}$-specific CD8+ T-cell population that infiltrated the lungs showed significantly impaired expression of effector activity. Impaired processes included cytokine synthesis and *ex vivo* cytolytic activity, as well as development of memory/effector cells in the lung. This impaired effector phenotype was due to defective TCR signaling in the effector T cells and required pulmonary RSV infection. These results strongly suggest that RSV infection results in defective TCR-mediated signaling and expression of effector activity via activated lung CD8+ T cells and impaired development of CD8+ T-cell memory.

Lung CD8+ T cells in RSV are deficient in cytokines

We determined the magnitude and the kinetics of the primary CD8+ T-cell response in the lungs of BALB/c mice during experimental intranasal RSV infection. We stained CD8+ T cells infiltrating the lungs with RSV major histocompatibility complex (MHC) class I H-2K^d tetramer complexes loaded with $M2_{82–90}$ (designated as M2Tet). In parallel, we enumerated the frequency of M2-specific effector CD8+ T cells present in the lung by intracellular cytokine staining (ICS). Approximately 41% of the CD8+ T cells infiltrating the lungs of infected animals at the peak of the primary CD8+ response (day 8) stained specifically with the M2Tet (Fig. 1*a*). By contrast, about 20% of the lung CD8+ T cells produced interferon-γ (IFN-γ) in response to the $M2_{82–90}$ peptide. Therefore, only about 50% of the $M2_{82–90}$-specific CD8+ T cells present in the infected lungs (as defined by M2Tet staining) appeared to produce IFN-γ in response to antigen. A similar discrepancy between tetramer staining and cytokine production was also observed for antigen-dependent TNF-α production by lung-infiltrating CD8+ T cells (in this instance, <10% of the expected frequency; data not shown).

During the peak of the primary CD8+ T-cell response in the lungs (day 8–12; Table 1), only 40–60% of the expected number of $M2_{82–90}$-specific CD8+ T cells (as defined by M2Tet staining) secreted IFN-γ. This difference in tetramer staining and IFN-γ production was demonstrable as late as 60–90 days after infection when the frequency of M2Tet+CD8+ cells in the lungs had declined to unexpectedly low levels (2–4%). At these later time points, the frequency of residual M2Tet+CD8+ T cells in the lungs that secreted IFN-γ was even lower than the frequency of IFN-γ+CD8+ cells at the peak of the response. Throughout the course of primary RSV infection, more than 90% of the CD8+ T cells infiltrating the lungs expressed the

Table 1 Kinetics of $M2_{82-90}$-specific $CD8^+$ T-cell response in primary RSV infection[a]

	% $Tet^+/CD8^+$	% IFN-$\gamma^+/CD8^+$ (peptide)[c]	Ratio of % IFN-γ^+ to % $M2Tet^+$[d]	% $CD11a^{Hi}/CD8^+$	%$CD44^{Hi}/CD8^+$	%$CD62L^{Lo}/CD8^+$
Day 4	3.9 ± 0.5	4.8 ± 0.8	123.4 ± 11.0	91.0	95.1	91.5 ± 0.8
Day 5	14.6 ± 1.4	18.1 ± 0.1	124.7 ± 10.9	n.d.	n.d.	n.d.
Day 6	39.5 ± 2.5	28.6 ± 3.6	67.1 ± 3.2	94.0	94.2	94.4 ± 1.2
Day 8	42.2 ± 1.0	23.7 ± 4.2	56.4 ± 11.1	96.2	94.1	96.8 ± 0.5
Day 10	48.3 ± 4.2	26.5 ± 2.0	55.1 ± 2.9	95.6	94.2	96.1 ± 0.5
Day 11	47.7 ± 3.4	22.6 ± 2.4	47.0 ± 1.3	n.d.	n.d.	n.d.
Day 12	45.0 ± 2.7	20.9 ± 3.1	45.7 ± 4.2	94.7	94.2	95.6 ± 0.7
Day 14	41.5 ± 1.7	17.2 ± 1.6	41.8 ± 4.7	95.8	93.8	95.1 ± 0.6
Day 60	4.2 ± 0.5	1.7 ± 0.2	40.5 ± 8.1	n.d.	n.d.	n.d.
Day 90	2.9 ± 0.4	1.1 ± 0.1	38.4 ± 9.9	91.2	95.4	92.4 ± 0.4
Influenza[b]	12.1 ± 0.5	12.9 ± 0.3	105.0 ± 1.0	n.d.	n.d.	n.d.

[a], Naive BALB/c mice were infected intranasally with 1 10^6 p.f.u. RSV. Lung lymphocytes were prepared at indicated time-points after infection and 1 10^6 cells were directly stained with anti-CD8 and M2Tet while the same batches were stimulated with the peptide for intracellular cytokine staining. The values are determined for 4 individual mice from each group. [b], Mice were infected intranasally with 0.5 LD_{50} (lethal dose in 50% of animals) of A/Jap/305/57 influenza virus. Lung lymphocytes were prepared 8 d after infection and assayed for tetramer staining with $HA_{204-212}$ tetramer, which recognize $HA_{204-212}$-specific $CD8^+$ T cells. [c], Cells were treated with 1 M peptide for intracellular cytokine staining. [d], Percentages of IFN-γ-producing cells among Tet^+ cells are calculated by dividing the percentage of peptide-stimulated IFN-$\gamma^+/CD8^+$ by the percentage of $Tet^+/CD8^+$ in each group. n.d., not determined.

$CD11^{Hi}CD44^{Hi}CD62L^{Lo}$ phenotype, which is characteristic of effector/memory cells throughout the course of primary RSV infection (Table 1). Thus, this deficiency in effector activity was exhibited at least by RSV M2-specific $CD8^+$ T cells localized to this peripheral pulmonary memory/effector T-cell compartment.

We recently identified and characterized a second subdominant MHC class I epitope within the RSV F protein[12]. F-specific $CD8^+$ T cells responding in the lung to RSV infection also displayed a comparable defect in cytokine synthesis[12], suggesting that this deficit in effector activity is not unique to the dominant M2-specific subpopulation of lung $CD8^+$ T cells and is characteristic of the host $CD8^+$ T-cell response to RSV.

This discrepancy between tetramer staining and intracellular IFN-γ production as measures of antigen-specific $CD8^+$ T-cell accumulation in the lung during primary RSV infection was unexpected; in other models of experimental pulmonary virus infection (for example, type A influenza), these two techniques for the quantification of antigen-specific $CD8^+$ cells in the lungs directly correlated[13]. To ensure that this deficit was not a general property of $CD8^+$ T cells infiltrating the lungs during respiratory virus infection, we examined, in parallel, tetramer staining and intracellular IFN-γ production by $CD8^+$ T cells infiltrating the lungs during primary infection of BALB/c mice with the A/Japan/305/57 influenza virus. At the peak of the primary response to influenza virus in the lungs (day 8), we found that the frequency of tetramer$^+$ and IFN-γ^+ lung $CD8^+$ T cells directed to the dominant hemagglutinin $(HA)_{204-212}$ epitope was comparable (Table 1). The frequency of lung $CD8^+$ T cells directed to the three other major influenza A $CD8^+$ T-cell epitopes[14], $HA_{210-219}$, $HA_{529-537}$ and nucleoprotein $(NP)_{147-155}$, at day 8 of infection also showed a close correspondence between tetramer staining and ICS ($HA_{210-219}$: 4% Tet^+, 4% IFN-γ^+; $HA_{529-537}$: 12% Tet^+, 13% IFN-γ^+; $NP_{147-155}$: 17% Tet^+, 22% IFN-γ^+). This was maintained throughout pulmonary influenza virus infection (data not shown).

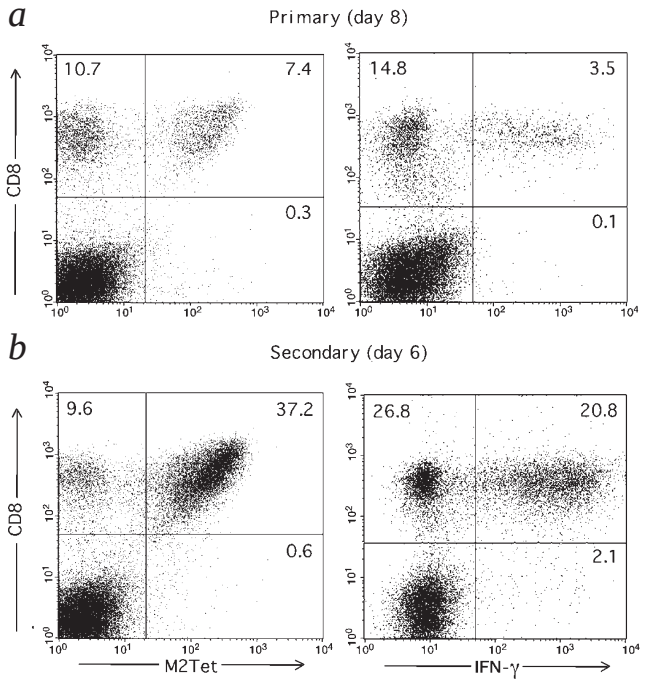

Fig. 1 Visualization and quantification of M2-specific $CD8^+$ T cells during RSV infection in BALB/c mice. ***a*** and ***b***, Lung-derived lymphocytes were prepared from infected animals at day 8 post-infection (*a*) or from vvM2-primed mice at day 6 post-challenge (*b*), stained for CD8 and M2Tet tetramer, and analyzed by flow cytometry. The same batches of cells were cultured in the presence of $M2_{82-90}$ peptide, then stained for CD8, followed by staining for intracellular IFN-γ. The numbers given show the percentages of $CD8^+$ T cells that are $M2Tet^+$ or IFN-γ^+. Results are representative of several independent experiments in which proportions of $M2Tet^+CD8^+$ T cells were consistent.

RSV-specific $CD8^+$ T cells show impaired effector activity

We next asked whether a similar defect in effector activity is detectable in the response of memory $CD8^+$ T cells accumulating in the lungs after a challenge RSV infection. To enrich for responding $M2_{82-90}$-specific T cells, we primed mice with a recombinant vaccinia virus expressing the RSV M2, followed by a challenge intranasal infection with RSV three to four weeks after priming. We[15] and others[16] have previously reported that this priming strategy leads to a robust *in vitro* secondary CTL response from immune splenocytes to infectious RSV. At day 6

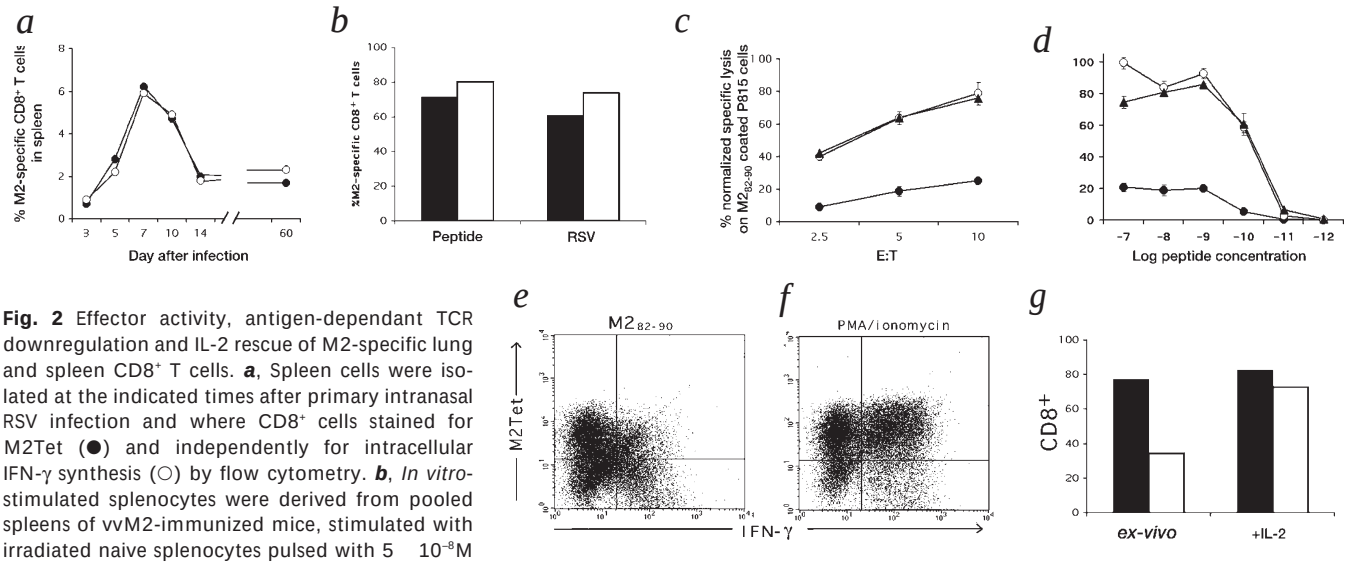

Fig. 2 Effector activity, antigen-dependant TCR downregulation and IL-2 rescue of M2-specific lung and spleen CD8+ T cells. ***a***, Spleen cells were isolated at the indicated times after primary intranasal RSV infection and where CD8+ cells stained for M2Tet (●) and independently for intracellular IFN-γ synthesis (○) by flow cytometry. ***b***, *In vitro*-stimulated splenocytes were derived from pooled spleens of vvM2-immunized mice, stimulated with irradiated naive splenocytes pulsed with 5 10^{-8}M $M2_{82-90}$ peptide or infected with RSV at MOI of 1. Cells were cultured for 6 d in the presence of IL-2 (10 U/ml), and analyzed for M2Tet staining (■) and intracellular IFN-γ synthesis (□). ***c*** and ***d***, Lung cells derived from vvM2-primed and RSV-challenged mice (●) and *in vitro*-cultured splenocytes (▲, peptide-stimulated; ○ RSV-stimulated) at the peak of antigen-specific response (day 6 post-challenge and post-stimulation, respectively) were assayed at the indicated E:T (*c*) ratio and peptide concentration (*d*) in a standard ^{51}Cr release assay. Specific lysis values were normalized to the actual number of M2Tet+ cells in each population. Results are representative of 4 independent experiments. ***e*** and ***f***, Down-modulation of TCR after cognate peptide stimulation. Lung lymphocytes derived from vaccinia M2-primed and RSV-challenged mice at day 6 post-infection were incubated in the presence of $M2_{82-90}$ peptide (*e*; 1 10^{-6}M) or PMA/ionomycin (*f*). Cells were stained for CD8 and M2Tet, permeabilized, and then stained for intracellular IFN-γ. Plots are gated on CD8+ cells, and numbers represent the percentage of gated cells in each quadrant. ***g***, Day 6 lung CD8+ T cells from RSV-challenged mice were purified using magnetic beads and cultured with IL-2 for 12–18 h. Cells were then analyzed for M2Tet staining (■) and IFN-γ production (□).

after challenge infection, approximately 80% of the CD8+ T cells stained with the M2Tet whereas only about 43% of the CD8+ cells produced IFN-γ in response to antigen (that is, ~53% of the expected percentage of M2-specific cells based on tetramer staining; Fig. 1*b*). Kinetic analysis revealed the expected, accelerated memory response to M2 as demonstrated by the rapid accumulation of the M2Tet+CD8+ T cells as early as day 4 after challenge (Table 2). The percentage of M2Tet+CD8+ cells in the lungs peaked at day 6 (~79%) and was maintained at a high level through post-challenge day 12. However, as in primary RSV infection, the frequency of cells producing IFN-γ in response to the $M2_{82-90}$ peptide during the peak of the memory response ranged from 52 to 66% of the expected percentage based on tetramer staining. Although the frequency of M2Tet+CD8+ T cells remained relatively high long after challenge RSV infection (day 45), the percentage of the M2-specific CD8+ T cells in the lungs producing IFN-γ in response to antigen dropped disproportionately. This again suggests that, as in the primary response, RSV infection alters the establishment and maintenance of peripheral CD8+ T-cell memory in the lung compartment.

We considered the possibility that these activated IFN-γ+CD8+ T cells infiltrating the lungs during primary and challenge infection belonged to the Type 2 cytokine subset[17]. The Type 2 cytokines interleukin-4 (IL-4), IL-5 and IL-13 were not detected by ELISPOT or ELISA in response to specific peptide stimulation (data not shown).

RSV selectively impairs CD8+ T-cell effector activity in lungs

The recruitment of many RSV M2-specific CD8+ T cells to the lung during both primary and challenge RSV infection suggested that the activation and proliferation of virus-specific CD8+ T cells in secondary lymphoid tissues were unaffected by RSV. To substantiate this point, we first compared the frequency of resident RSV-specific M2Tet+ and IFN-γ+CD8+ T cells found in the spleens of RSV-infected animals during primary infection. Although the frequencies of M2-specific CD8+ T cells present in the spleen defined by either tetramer staining or antigen-dependent cytokine production were low (typically <6% of total splenic CD8+ T-cell numbers), over the course of primary infection, the estimated frequency of M2-specific CD8+ T cells determined by either method was comparable; IFN-γ staining reproducibly yielded slightly higher frequency estimates (Fig. 2*a*). This result suggested that, in contrast to the lungs, there was no impairment in the response of CD8+ T effector cells or memory cells to RSV in this central lymphoid compartment.

To further explore this issue, we examined the IFN-γ response of immune M2-specific CD8+ T-cell effectors at day 6 after *in vitro* stimulation of splenocytes from M2-immune donors with

Table 2 Kinetics of $M2_{82-90}$-specific CD8+ T-cell response in secondary RSV infection

	% M2Tet+/CD8+	% IFN-γ+/CD8+ (peptide)	Ratio of % IFN-γ+ to % M2Tet+
Day 4	50.4 ± 0.7	26.4 ± 2.2	52.4 ± 3.8
Day 6	78.8 ± 0.1	42.3 ± 0.6	53.8 ± 0.6
Day 9	75.3 ± 4.6	50.0 ± 0.2	66.6 ± 4.2
Day 12	74.0 ± 2.0	40.1 ± 2.0	54.2 ± 4.1
Day 45	57.0 ± 1.2	12.5 ± 3.2	21.8 ± 5.1

BALB/c mice primed with vvM2 were infected intranasally with 1 10^{6} p.f.u. RSV and lung lymphocytes were prepared at indicated time point after infection.

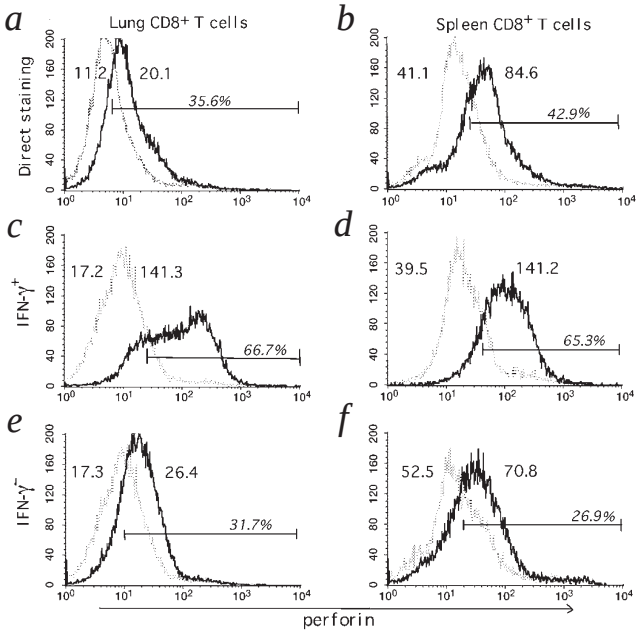

Fig. 3 Perforin expression of M2-specific cells derived from the lung and *in vitro* stimulated splenocytes culture. ***a*** and ***b***, Lung $CD8^+$ T cells at day 6 post-challenge with RSV and splenic $CD8^+$ T cells at day 6 after $M2_{82–90}$ peptide stimulation were directly stained with anti-perforin (solid line) or isotype control antibody (dotted line). ***c–f***, The same batches were treated as in intracellular IFN-γ assay, permeabilized, and then stained for CD8, intracellular perforin, and IFN-γ. Cells were gated on IFN-γ^+CD8^+ (*c* and *d*) or IFN-γ^- $CD8^+$ cells (*e* and *f*). The numbers given are mean fluorescence intensity for each staining and bar shows the percentage of stained cells above isotype control staining.

RSV-infected or $M2_{82–90}$-pulsed splenic antigen-presenting cells (APCs). We compared these responses with that of lung $CD8^+$ T cells isolated 6 days after challenge RSV infection of M2-primed donors. Cultures of M2-immune splenocytes stimulated with either RSV-infected cells or peptide were enriched for $M2Tet^+CD8^+$ T cells, which were present at frequencies comparable to those detected in the RSV-infected lungs (Fig. 2*b*). However, unlike lung-derived $CD8^+$ T cells, activated, M2-specific, spleen-derived effector $CD8^+$ T cells showed no deficit in IFN-γ synthesis. The cytokine response and cytolytic activity (see below) of effector $CD8^+$ T cells generated from immune spleens in response to RSV-infected APC were identical with or without IL-2 supplementation to the culture medium during effector T-cell generation *in vitro* (data not shown).

We next investigated whether the activated lung $CD8^+$ T cells also impaired *ex vivo* cytolytic activity. $CD8^+$ T cells were isolated from the lungs of M2-primed mice 6 days after challenge RSV infection and examined directly for *ex vivo* cytolytic activity on $M2_{82–90}$-pulsed targets cells. In parallel, we analyzed M2-specific cytolytic activity of activated $CD8^+$ T cells generated *in vitro* from the spleen of M2-immune donors 6 days after stimulation with RSV-infected or $M2_{82–90}$-pulsed APCs. Effector:target (E:T) ratios were normalized based on the frequency of $M2Tet^+CD8^+$ T cells in each effector population. As reported[10,15], effectors cells generated *in vitro* in response to infectious RSV or M2 peptide displayed potent cytolytic activity over a range of E:T ratios (Fig. 2*c*). By contrast, M2-specific $CD8^+$ T cells from the infected lungs routinely displayed less than 25% of the activity of spleen-derived effectors over the same E:T ratios (Fig. 2*c*). The peptide concentration for optimal lysis (1 10^{-7} to 1 10^{-9} M) was identical for lung- and spleen-derived effector cells (Fig. 2*d*), suggesting that there was not selective recruitment of low-avidity effector cells to the lungs during RSV infection. The peptide dose-dependence of IFN-γ synthesis by ICS for lung and spleen $CD8^+$ T-cell effectors directly paralleled the peptide dose dependence of *in vitro* cytolytic activity (data not shown).

Defective lung $CD8^+$ T cells are impaired in TCR signaling

To further explore the basis for the weak cytolytic activity of lung-derived M2-specific $CD8^+$ T cells, we analyzed the intracellular perforin content of lung- and spleen-derived effector T cells from M2-immune donors. Using flow cytometry with anti-perforin antibody, we found, in contrast to published observations[18], that the intracellular perforin content of both the *ex vivo* lung-derived (Fig. 3*a*) and *in vitro* spleen-derived (Fig. 3*b*) $CD8^+$ T-cell effectors was low. However, in companion studies, we observed that the short-term cocultivation of activated effector $CD8^+$ T cells with specific peptide under conditions resulting in IFN-γ synthesis, simultaneously resulted in the upregulation of perforin expression by the T cells (our unpublished observations).

We next determined if the weak cytolytic activity of lung-derived $CD8^+$ T-cell effectors might be linked to the inability of the IFN-γ^- fraction of M2-specific T cells to upregulate perforin expression in response to the antigen. We purified $CD8^+$ cells from the lungs of M2-primed mice (day 6 post-challenge RSV infection) and from cultures of M2-immune splenocytes 6 days after *in vitro* stimulation with RSV or $M2_{82–90}$ peptide. These $CD8^+$ T-cell populations were exposed to specific peptide in short-term culture (5 h at 37 °C) and then analyzed for IFN-γ production and perforin expression. The IFN-γ^+ and IFN-γ^-CD8^+ T cells were separately gated, and the intracellular perforin content of the gated cell populations was determined. Splenic, M2-specific, IFN-γ^+ T cells displayed uniform upregulation of perforin expression in response to peptide (Fig. 3*d*). The small fraction (<15%) of splenic $CD8^+$ T cells that were IFN-γ^- did not upregulate perforin (Fig. 3*f*) and likely represent residual irrelevant viable $CD8^+$ T cells. The IFN-γ^+CD8^+ T cells isolated from RSV-infected lungs also upregulated perforin expression (Fig. 3*c*). By contrast, lung IFN-γ^-CD8^+ T cells which consisted primarily of the $M2Tet^+$ cells (approximately 70% of the total IFN-γ^- $CD8^+$ T-cell population in the infected lungs) failed to upregulate perforin expression (Fig. 3*e*).

Activated $CD8^+$ T cells typically down-modulate cell-surface T-cell receptor (TCR) expression in response to antigen[19], resulting in a concomitant loss of specific tetramer staining. To directly assess if $CD8^+$ T cells responding to RSV infection in the lung were defective in antigen-dependent TCR down-mod-

Table 3 T-cell cytokine response to RSV and DNA challenge

	% $M2Tet^+/CD8^+$	% IFN-$\gamma^+/CD8^+$ (peptide)	Ratio of % IFN-γ^+ to %$M2Tet^+$
RSV challenge[a]	75.5 ± 0.3	43.6 ± 2.5	57.7 ± 3.1
DNA challenge[b]	13.8 ± 1.2	13.3 ± 1.4	96.6 ± 3.4

[a], Lung lymphocytes were prepared from lungs of vvM2-primed and RSV-infected mice 6 d after challenge. [b], vvM2-primed mice were inoculated intranasally with 100 g pCI-neo-M2 plasmid DNA and 10 g CpG ODN. Lung lymphocytes were prepared 6 d after challenge.

ulation, we analyzed M2Tet staining and IFN-γ production by activated lung CD8$^+$ T cells from M2-primed mice undergoing challenge RSV infection after short-term *in vitro* exposure to the M2 peptide. As Fig. 2*e* demonstrates, the majority (>60%) of the total lung M2-specific CD8$^+$ T cells (defined by either M2Tet staining or IFN-γ production) did not secrete IFN-γ in response to peptide and likewise showed no decrease in M2Tet staining. This impairment in expression of effector activity by lung CD8$^+$ T cells was not overcome even following *in vitro* exposure to phorbol 12-myristate 13-acetate (PMA)/ionomycin; that is, less than 60% of lung M2Tet$^+$ T cells secrete IFN-γ in response to this non-specific mitogenic stimulus (Fig. 2*f*).

The unresponsiveness of RSV-specific lung CD8$^+$ T cells to antigenic stimulation is reminiscent of the refractory state observed in anergic T cells[20]. Because T-cell anergy can be reversed by exposure of the cells to IL-2 (ref. 21), we questioned whether the defect in TCR signaling exhibited by RSV-specific lung CD8$^+$ T cells might likewise be reversed by IL-2 treatment. To test this possibility, CD8$^+$ T cells were purified from the lungs of M2-immune mice six days after challenge RSV infection. Approximately 80% of the purified T cells were M2-specific as judged by direct *ex vivo* tetramer staining and 40–50% of the lung CD8$^+$ T cells secreted IFN-γ in response to peptide stimulation. Purified lung CD8$^+$ T cells were cultured overnight (12–18 h at 37 °C) in medium with or without IL-2 supplementation (10 U/ml), and then tested for IFN-γ production by ICS in response to M2 peptide stimulation. Lung CD8$^+$ T cells incubated overnight in medium without IL-2 showed a 50% drop in overall cell viability and only 25–30% of the remaining viable T cells produced IFN-γ in response to the peptide (Fig. 2*g*). By contrast, lung CD8$^+$ T cells exposed to IL-2 showed no loss in cell viability and no increase in total cell counts (cell yields were ~100–105% of starting cell numbers). When IL-2-treated CD8$^+$ T cells were evaluated for IFN-γ production in response to M2 peptide, more than 80% of the predicted number of M2-specific CD8$^+$ T cells (based on tetramer staining) were IFN-γ$^+$ (Fig. 2*g*). These data suggest that the block in antigen-dependent TCR-mediated signaling had been reversed.

Defective CD8$^+$ T-cell response requires pulmonary infection

Our results show expansion and accumulation of M2-specific CD8$^+$ T cells in the lungs during primary and challenge RSV infection, as well as normal effector activity displayed by resident spleen CD8$^+$ T cells during RSV infection. These data suggest that the response to RSV infection was unimpaired in central lymphoid tissue and that pulmonary RSV infection might be critical for the development of impaired effector activity displayed by lung-infiltrating CD8$^+$ T cells. If so, then M2-specific CD8$^+$ T cells recruited to the lungs in response to M2 antigen without concomitant RSV infection might display a normal effector T-cell phenotype. To test this, we made use of the recent finding that intranasal introduction of plasmid DNA encoding specific antigens under control of a strong promoter stimulates the development of an antigen-specific cellular immune response in the lungs[22,23]. We therefore introduced plasmid DNA encoding the full-length RSV M2 protein intranasally into M2-primed mice; we did this in conjunction with CpG oligodeoxynucleotides (ODNs), which stimulate local inflammation and enhance CD8$^+$ T-cell recruitment to the lungs[24]. At day 6 after DNA immunization, the frequency of M2Tet$^+$CD8$^+$ T cells infiltrating the lungs of plasmid recipients (~13%) was lower than that of mice undergoing challenge RSV infection. However, about 96% of the expected number of M2-specific CD8$^+$ T cells in the lungs of DNA challenged mice scored positive for IFN-γ production by ICS (Table 3).

Discussion

Here we analyzed the CD8$^+$ T-cell responses in the lungs to the immunodominant $M2_{82–90}$ epitope during primary and challenge RSV infection. Pulmonary RSV infection induces a massive expansion and accumulation of $M2_{82–90}$-specific CD8$^+$ T cells in the lungs. However, although these M2Tet$^+$ T cells displayed the phenotype of activated effectors and peripheral memory/effector cells, they were deficient in both pro-inflammatory cytokine secretion and *ex vivo* cytolytic activity. Furthermore, the frequency of RSV M2-specific memory CD8$^+$ T cells in the lung compartment rapidly declined after recovery from primary RSV infection, and the cytokine response of the RSV-specific memory CD8$^+$ T cells was significantly reduced late after challenge infection.

This selective impairment in lung CD8$^+$ T-cell responsiveness induced by RSV likely reflects a defect in antigen-dependent TCR-mediated signaling. Along with the deficiency in cytokine synthesis and *ex vivo* cytolytic activity, M2Tet$^+$IFN-γ$^-$ lung CD8$^+$ T-cell effectors also failed to upregulate perforin and downmodulate cell-surface TCRs in response to antigen. Reversal of the block in antigen-dependent cytokine synthesis by IL-2 is noteworthy and directly parallels the restoration of antigen responsiveness observed in anergic T cells after IL-2 treatment[21,25]; in those cells, alterations or deficiencies in intracellular signaling intermediates involved in TCR-dependent signal transduction have been implicated in the defective antigen responsiveness of anergic T cells[21,26]. The refractory RSV-specific lung CD8$^+$ T-cell effectors may likewise have an alteration in one or more intracellular signaling intermediates necessary for TCR-dependant triggering of effector activity which can be overcome by IL-2 receptor engagement[21].

Although pulmonary RSV infection does alter peripheral (lung) CD8$^+$ effector activity and memory T cells, it does not affect the initial activation and expansion of primary or memory CD8$^+$ T-cell precursors or the recruitment of activated CD8$^+$ T cells from central lymphoid tissues to the lungs. Nor does RSV infection affect the effector activity, frequency or function of central (splenic) effector/memory CD8$^+$ T cells. Given that lung M2-specific CD8$^+$ T cells responding to intranasal plasmid DNA immunization likewise show no deficit in effector activity, RSV replication in the respiratory tract may be required for the development of the impaired CD8$^+$ T-cell effectors and memory response. Respiratory epithelial cells are the primary targets of productive RSV replication[27], whereas macrophages and dendritic cells are abortively infected[28]. Productive RSV infection in the respiratory tract could lead to the selective inhibition of lung CD8$^+$ T-cell function, either directly through the accumulation of one or more RSV gene products capable of suppressing host immune/inflammatory responses (for example, RSV G and/or SH)[29] or indirectly through the releases of inhibitory factors from RSV-infected respiratory epithelial cells[30–32]. In either case, RSV-induced inhibition of CD8$^+$ T-cell effector activity and memory development and maintenance would be restricted to a respiratory tract where RSV gene products and productively infected epithelial cells are abundant.

An intriguing feature of RSV infection is the susceptibility of previously infected individuals to reinfection with antigenically closely related viruses or the identical virus strain. In in-

fants, reinfection with RSV has been reported to occur within weeks of recovery from primary infection[33]. In adults with high levels of circulatory neutralizing antibodies, upwards of 25% immune individuals could be re-infected with RSV of the same serotype within two months of natural infection[6]. These and related observations suggest that the duration of protective immunity to challenge RSV infection is short-lived. The results reported here suggest one possible mechanism for the absence of durable long-lived immunity to RSV infection: Perhaps through its effect on TCR-dependent signaling, RSV suppresses the development of peripheral $CD8^+$ T-cell memory in the lungs. If RSV also inhibits the function of virus-specific $CD4^+$ T-cell effectors in the lungs, then RSV infection could impact on the strength and durability of both cellular and humoral immunologic memory in this compartment. Finally, our results suggest that a localized respiratory infection with an immunosuppressive virus like RSV can inhibit the function of effector T cells and local immunologic memory development at that peripheral site without impacting on virus-specific T-cell activation or memory development in central lymphoid tissues.

Methods

Mice. 6–8-wk-old female BALB/c mice ($H\text{-}2^d$) were purchased from Taconic Farms Inc. (Germantown, New York). Mice were housed in a pathogen-free environment in an AAALAC-approved vivarium at the University of Virginia.

Viruses and infection of mice. The A2 strain of RSV was a gift from P.L. Collins. RSV stock was grown on HEp-2 cells (ATCC, Manassas, Virginia) and titered for infectivity. vvM2 was a gift from J.L. Beeler. Mice were infected with 5 10^6 plaque-forming units (p.f.u.) of vvM2 by scarification at the base of tail. 3–4 wk after priming, mice were lightly anesthetized with 2:1 mixture of ether and chloroform, and intranasally inoculated with 1 10^6 p.f.u. of RSV in 50 l. At various times after infection, infected mice were killed by cervical dislocation.

Preparation and culture of lymphocytes. Lungs were perfused with 5 ml of PBS containing 10 U/ml heparin (Sigma) through the right ventricle using a syringe fitted with 25-gauge needle. The lungs and spleens were removed and placed into Iscove's modified Dulbecco's medium (IMDM) supplemented with glutamine, gentamicin, penicillin G and 10% FBS. The tissue was processed through a steel screen to obtain single-cell suspension and particulate matter was removed by quick centrifugation at 1000*g*. Cells were counted and resuspended at the given cell concentrations for the appropriate *in vitro* assay. Pooled spleen cells from vvM2-immunized mice were resuspended in complete IMDM supplemented with recombinant human IL-2 (20 U/ml), and 2.5 10^7 cells were added to each well of a 6-well plate. Syngeneic splenocyte stimulators were prepared from naive mice by irradiation (2000 rad) and pulsing for 0.5 h at room temperature with the peptide or infecting with RSV for 1 h at 37 °C at multiplicity of infection (MOI) of 1. Thereafter, cells were extensively washed to remove free unbound peptide or virus, and 5 10^6 cells were added to the each well of responder cells. In companion experiments, immune splenocytes were cultured with irradiated, RSV-infected splenocytes in medium without supplemental IL-2. The effector T cells that were generated exhibited the same cytokine response and cytolytic activity as did effector $CD8^+$ T cells that were generated in the presence of IL-2 in culture medium. To purify $CD8^+$ cells, cell suspensions were positively selected with mouse CD8 microbeads and MACS separation column (Miltenyi Biotec, Auburn, California) according to manufacturer's instructions. The typical purification results in 80–90% $CD8^+$ cell purity.

Flow cytometry and tetramer staining. MHC class I–peptide tetramers were produced as described[34]. Freshly explanted lung lymphocytes or *in vitro* cultured splenocytes (1 10^6) were purified by density-gradient centrifugation and stained in PBS:3% (wt:vol), FBS:0.09% (wt:vol) NaN_3 using fluorochrome-conjugated antibodies and MHC class I tetramers. Antibodies used were anti-CD8 (clone 53-6.7), anti-CD11a (clone 2D7), anti-CD44 (clone IM7) and anti-CD62L (clone MEL-14). All antibodies were purchased from PharMingen (San Diego, California). After staining, cells were fixed in PBS:2% (wt:vol) paraformaldehyde, and events acquired using a FACSCalibur flow cytometer (Becton Dickinson, San Diego, California). Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed using CELLQuest (Becton Dickinson).

Intracellular staining. To enumerate the number of cytokine-producing cells, intracellular cytokine staining was performed as previously[35]. In brief, 1 10^6 freshly explanted lung lymphocytes were cultured in culture tube. Cells were left untreated, stimulated with $M2_{82-90}$ peptide, or treated with PMA (50 ng/ml) and ionomycin (500 ng/ml). In certain experiments using MACS-purified lung $CD8^+$ cells or *in vitro* cultured splenocytes, 5 10^5 P815 cells were added as APCs. In all cases cells were incubated for 5 h at 37 °C in 7% CO_2. Brefeldin A (10 g/ml; Sigma) was added for the duration of the culture period to facilitate intracellular cytokine accumulation. The antibodies used were anti-IFN-γ (clone XMG1.2) or its control isotype antibody (rat IgG1). Intracellular perforin staining was performed as described[18].

CTL assay. A standard chromium release assay was performed as described[15]. Effectors from lung-derived lymphocytes or *in vitro*-cultured splenocytes were prepared by density gradient centrifugation. For peptide titration, assays were performed at a constant E:T ratio of approximately 2.5:1. Percent specific lysis was calculated as follows: [(^{51}Cr release with effector cells – spontaneous ^{51}Cr release) (total ^{51}Cr release with 1% Triton X-100 – spontaneous ^{51}Cr release)] 100. The percent specific-lysis values represent the mean values of quadruplicate wells.

Intranasal plasmid DNA injection. The plasmid, pCI-neo-M2, containing M2 gene of RSV under the control of cytomegalovirus immediate early promoter was constructed by inserting M2 gene fragment into *Xho*I and *Not*I sites of pCI-neo (Promega, Madison, Wisconsin). LPS-free DNA was prepared using EndoFree Plasmid Mega kit (Qiagen, Chatsworth, California) according to the manufacturer's instruction, and 100 g of plasmid with 10 g CpG ODN was injected intranasally to the left nostril of mice in a volume of 100 l. pCI-neo parental vector plasmid was used as a control.

Acknowledgments

We wish to acknowledge the expert and dedicated support of S. Gill in the completion of this work. The work was supported by USPHS grants to T.J.B.

RECEIVED 4 SEPTEMBER; ACCEPTED 3 DECEMBER 2001

1. Glezen, P. & Denny, F.W. Epidemiology of acute lower respiratory disease in children. *N. Engl. J. Med.* **288**, 498–505 (1973).
2. Chanock, R.M., Parrott, R.H., Connors, M., Collins, P.L. & Murphy, B.R. Serious respiratory tract disease caused by respiratory syncytial virus: prospects for improved therapy and effective immunization. *Pediatrics* **90**, 137–143 (1992).
3. Falsey, A.R. & Walsh, E.E. Relationship of serum antibody to risk of respiratory syncytial virus infection in elderly adults. *J. Infect. Dis.* **177**, 463–466 (1998).
4. Glezen, W.P., Taber, L.H., Frank, A.L. & Kasel, J.A. Risk of primary infection and reinfection with respiratory syncytial virus. *Am. J. Dis. Child* **140**, 543–546 (1986).
5. Beem, M. Repeated infections with respiratory syncytial virus. *J. Immunol.* **98**, 1115–1122 (1967).
6. Hall, C.B., Walsh, E.E., Long, C.E. & Schnabel, K.C. Immunity to and frequency of reinfection with respiratory syncytial virus. *J. Infect. Dis.* **163**, 693–698 (1991).
7. Alwan, W.H., Record, F.M. & Openshaw, P.J. CD4+ T-cells clear virus but augment disease in mice infected with respiratory syncytial virus. Comparison with the effects of CD8+ T-cells. *Clin. Exp. Immunol.* **88**, 527–536 (1992).
8. Cannon, M.J., Openshaw, P.J. & Askonas, B.A. Cytotoxic T-cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J. Exp. Med.* **168**, 1163–1168 (1988).
9. Kulkarni, A.B. *et al.* Cytotoxic T-cells specific for a single peptide on the M2 protein of respiratory syncytial virus are the sole mediators of resistance induced by immunization with M2 encoded by a recombinant vaccinia virus. *J. Virol.* **69**, 1261–1264 (1995).
10. Openshaw, P.J., Anderson, K., Wertz, G.W. & Askonas, B.A. The 22,000-kilodalton protein of respiratory syncytial virus is a major target for Kd-restricted cyto-

toxic T lymphocytes from mice primed by infection. *J. Virol.* **64**, 1683–1689 (1990).
11. Kulkarni, A.B., Connors, M., Firestone, C.Y., Morse, H.C. & Murphy, B.R. The cytolytic activity of pulmonary CD8+ lymphocytes, induced by infection with a vaccinia virus recombinant expressing the M2 protein of respiratory syncytial virus (RSV), correlates with resistance to RSV infection in mice. *J. Virol.* **67**, 1044–1049 (1993).
12. Chang, J., Srikiatkhachorn, A. & Braciale, T.J. Visualization and characterization of respiratory syncytial virus F-specific CD8(+) T-cells during experimental virus infection. *J. Immunol.* **167**, 4254–4260. (2001).
13. Flynn, K.J. *et al.* Virus-specific CD8+ T-cells in primary and secondary influenza pneumonia. *Immunity* **8**, 683–691 (1998).
14. Sweetser, M.T., Braciale, V.L. & Braciale, T.J. Class I major histocompatibility complex-restricted T lymphocyte recognition of the influenza hemagglutinin. Overlap between class I cytotoxic T lymphocytes and antibody sites. *J. Exp. Med.* **170**, 1357–1368 (1989).
15. Srikiatkhachorn, A. & Braciale, T.J. Virus-specific CD8+ T lymphocytes downregulate T helper cell type 2 cytokine secretion and pulmonary eosinophilia during experimental murine respiratory syncytial virus infection. *J. Exp. Med.* **186**, 421–432 (1997).
16. Alwan, W.H., Kozlowska, W.J. & Openshaw, P.J. Distinct types of lung disease caused by functional subsets of antiviral T-cells. *J. Exp. Med.* **179**, 81–89 (1994).
17. Sad, S., Marcotte, R. & Mosmann, T.R. Cytokine-induced differentiation of precursor mouse CD8+ T-cells into cytotoxic CD8+ T-cells secreting Th1 or Th2 cytokines. *Immunity* **2**, 271–279 (1995).
18. Slifka, M.K., Rodriguez, F. & Whitton, J.L. Rapid on/off cycling of cytokine production by virus-specific CD8+ T-cells. *Nature* **401**, 76–79 (1999).
19. Valitutti, S., Muller, S., Cella, M., Padovan, E. & Lanzavecchia, A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375**, 148–1451 (1995).
20. Zajac, A.J. *et al.* Viral immune evasion due to persistence of activated T-cells without effector function. *J. Exp. Med.* **188**, 2205–2213 (1998).
21. Madrenas, J., Schwartz, R.H. & Germain, R.N. Interleukin 2 production, not the pattern of early T-cell antigen receptor-dependent tyrosine phosphorylation, controls anergy induction by both agonists and partial agonists. *Proc. Natl. Acad. Sci. USA* **93**, 9736–9741 (1996).
22. McCluskie, M.J. *et al.* Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA. *Antisense Nucleic Acid Drug Dev.* **8**, 401–414 (1998).
23. McCluskie, M.J. *et al.* Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. *Mol. Med.* **5**, 287–300 (1999).
24. Schwartz, D.A. *et al.* CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J. Clin. Invest.* **100**, 68–73 (1997).
25. Boussiotis, V.A. *et al.* Prevention of T-cell anergy by signaling through the γc chain of the IL-2 receptor. *Science* **266**, 1039–1042 (1994).
26. Sloan-Lancaster, J., Shaw, A.S., Rothbard, J.B. & Allen, P.M. Partial T-cell signaling: Altered phospho-ζ and lack of zap70 recruitment in APL-induced T-cell anergy. *Cell* **79**, 913–922 (1994).
27. Hall, C.B., Douglas, R.G., Jr., Schnabel, K.C. & Geiman, J.M. Infectivity of respiratory syncytial virus by various routes of inoculation. *Infect. Immun.* **33**, 779–783 (1981).
28. Becker, S., Quay, J. & Soukup, J. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. *J. Immunol.* **147**, 4307–4312 (1991).
29. Tripp, R.A. *et al.* Respiratory syncytial virus G and/or SH protein alters Th1 cytokines, natural killer cells, and neutrophils responding to pulmonary infection in BALB/c mice. *J. Virol.* **73**, 7099–7107 (1999).
30. Thomas, L.H., Wickremasinghe, M.I., Sharland, M. & Friedland, J.S. Synergistic upregulation of interleukin-8 secretion from pulmonary epithelial cells by direct and monocyte-dependent effects of respiratory syncytial virus infection. *J. Virol.* **74**, 8425–8433 (2000).
31. Preston, F.M., Beier, P.L. & Pope, J.H. Identification of the respiratory syncytial virus-induced immunosuppressive factor produced by human peripheral blood mononuclear cells in vitro as interferon-α. *J. Infect. Dis.* **172**, 919–926 (1995).
32. Olszewska-Pazdrak, B. *et al.* Cell-specific expression of RANTES, MCP-1, and MIP-1α by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. *J. Virol.* **72**, 4756–4764 (1998).
33. Henderson, F.W., Collier, A.M., Clyde, W.A. & Denny, F.W. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. *N. Engl. J. Med.* **300**, 530–534 (1979).
34. Altman, J.D. *et al.* Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**, 94–6 (1996).
35. Murali-Krishna, K. *et al.* Counting antigen-specific CD8 T-cells: A reevaluation of bystander activation during viral infection. *Immunity* **8**, 177–187 (1998).