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Regulation of Cytokine Production by Virus-Specific CD8 T Cells in the Lungs▽

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Inflammation and the elimination of infected host cells during an immune response often cause local tissue injury and immunopathology, which can disrupt the normal functions of tissues such as the lung. Here, we show that both virus-induced inflammation and the host tissue environment combine to influence the capacity of virus-specific CD4 and CD8 T cells to produce cytokines in various tissues. Decreased production of cytokines, such as IFN-γ and TNF-α, by antigen-specific T cells is more pronounced in peripheral tissues, such as the lung and kidney, than in secondary lymphoid organs, such as the spleen or lymph nodes. We also demonstrate that tissues regulate cytokine production by memory T cells independently of virus infection, as memory T cells that traffic into the lungs of naïve animals exhibit a reduced ability to produce cytokines following direct ex vivo peptide stimulation. Furthermore, we show that cytokine production by antigen-specific memory CD4 and CD8 T cells isolated from the lung parenchyma can be rescued by stimulation with exogenous peptide-pulsed antigen-presenting cells. Our results suggest that the regulation of T-cell cytokine production by peripheral tissues may serve as an important mechanism to prevent immunopathology and preserve normal tissue function.

CD8 T cells control acute virus infections through the secretion of cytokines and the lysis of infected cells (16, 20, 41, 44). Acute infection of mice with lymphocytic choriomeningitis virus (LCMV) induces a massive activation and expansion of CD8 T cells (7, 25). Early studies suggested much of this expansion was not virus specific, because the primary technology at the time for quantifying virus-specific T cells, limiting-dilution analysis, could account for only approximately 10% of the activated T cells as being virus specific (17, 22, 26, 34, 39). The development of major histocompatibility complex (MHC) class I tetramers allowed for the first time the direct ex vivo visualization of antigen-specific CD8 T cells via flow cytometry and led to the landmark discovery that the majority of the activated T cells in the lymphoid organs following an acute LCMV infection were virus specific (25). Antigen-specific effector and memory CD8 T cells can also be identified by their production of gamma interferon (IFN-γ), the primary effector cytokine released by CD8 T cells. Thus, the surprising tetramer results were confirmed using functional assays, such as enzyme-linked immunospot or intracellular-cytokine staining (ICS) for IFN-γ, following short-term in vitro stimulation with virus-derived immunodominant peptides (7, 25). These three complementary approaches yielded similar, but not identical, numbers of virus-specific T cells in secondary lymphoid organs, such as the spleen (7, 25).

Recent evidence has suggested that virus-specific CD8 T cells in the lung may become impaired in their ability to secrete cytokines, such as IFN-γ or tumor necrosis factor alpha (TNF-α) following acute infection (1, 8, 10, 14, 40). Respiratory syncytial virus (RSV) infection of mice induces the expansion of a virus-specific CD8 T-cell population in the lung that can be readily identified via MHC class I tetramer staining (2, 8, 9, 28). Interestingly, significantly fewer RSV-specific T cells could be identified using ICS to detect IFN-γ production (8, 9, 40). These results suggested that RSV-specific cells were functionally impaired in their ability to make cytokines. Additional studies performed using either simian virus type 5 (SV5) or pneumonia virus of mice have yielded similar results and suggest that the reduced cytokine production by virus-specific T cells may occur only after infection by members of the *Paramyxoviridae* family of viruses (10, 14).

Here, we evaluated the virus-specific T-cell response following acute intranasal (i.n.) infection with three unrelated viruses: (i) RSV, a single-stranded RNA virus; (ii) LCMV, an ambisense single-stranded RNA virus; and (iii) vaccinia virus (VACV), a large double-stranded DNA virus. We show that decreased cytokine production by virus-specific pulmonary CD8 T cells occurs during the acute immune response to RSV and VACV but does not occur during acute LCMV infection. However, once the acute infection is resolved, pulmonary memory CD8 T cells exhibit decreased cytokine production following respiratory infection with each of these unrelated viruses. Surprisingly, using adoptive transfer of antigen-specific CD8 T cells into naïve recipients, we show that cells that enter the lung exhibit decreased ex vivo cytokine production, suggesting that the local tissue environment may suppress the production of cytokines in vivo. Importantly, we demonstrate that cytokine production by antigen-specific CD4 and CD8 T cells recovered from the lung parenchyma can be rescued by in vitro stimulation with exogenous peptide-pulsed antigen-presenting cells (APC). Analysis of multiple tissues revealed that regulation of cytokine production by antigen-specific T cells

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may serve as a potential mechanism to limit local tissue injury and prevent immunopathology.

MATERIALS AND METHODS

Mice. BALB/cAnNCr and C57BL/6NCr mice between 6 and 8 weeks of age were purchased from the National Cancer Institute (Frederick, MD). C57BL/6 P14 mice (31), which express a CD8 Vα2 Vβ8.1 T-cell-transgenic T-cell receptor (TCR) specific to the $GP_{33–41}$ epitope of LCMV, and C57BL/6 SMARTA mice (30), which express a CD4 Vα2 Vβ8.3 T-cell-transgenic TCR specific to the $GP_{61–80}$ epitope of LCMV, were gifts from Michael J. Bevan (University of Washington, Seattle, WA). Female mice were used in all experiments. All experimental procedures utilizing mice were approved by the University of Iowa's Animal Care and Use Committee.

Virus propagation and infection of mice. The Armstrong strain of LCMV was a gift from Raymond M. Welsh (University of Massachusetts Medical School, Worcester, MA) and was grown in BHK-21 cells (American Type Culture Collection, Manassas, VA). The RSV A2 strain was a gift from Barney S. Graham (National Institutes of Health, Bethesda, MD) and was propagated in HEp-2 cells (ATCC). The recombinant VACV containing the RSV protein M2 (VACVM2) (37) was a gift from Judy L. Beeler (U.S. Food and Drug Administration, Bethesda, MD) obtained via Thomas J. Braciale (University of Virginia, Charlottesville, VA) and was propagated in BSC-40 cells (ATCC). Mice were infected i.n. with 5×10^5 PFU of LCMV, 2.8×10^6 PFU of RSV, or 3×10^6 PFU of VACVM2.

Tissue isolation and preparation. The bronchoalveolar lavage (BAL) fluid was harvested from virus-infected mice by cannulation of the trachea and lavage with three successive washes with 1 ml of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10 U/ml penicillin G, 10 μg/ml streptomycin sulfate, 2 mM L-glutamine (Gibco), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO), and 10% fetal calf serum (FCS) (Atlanta Biologicals, Larwenceville, GA). After the BAL fluid was collected, the lungs were perfused by gently pushing 5 ml of RPMI 1640 medium supplemented as described above through the right ventricle of the heart. After the lungs were removed, the lobes were cut into small pieces prior to pressing the tissue through a wire mesh screen (Cellector; Bellco Glass, Inc., Vineland, NJ). To create a single-cell suspension, spleens and lymph nodes were harvested and pressed between the frosted ends of glass slides (Surgipath, Richmond, IL). All other organs were pressed through wire mesh screens to create single-cell suspensions. Lymphocytes from the liver and kidneys were isolated using Lympholyte-M (Cedarlane Laboratories, Ltd., Burlington, NC) as specified by the manufacturer.

Intracellular cytokine stain. Spleen and lung single-cell suspensions (1×10^6 to 2×10^6 cells) were incubated in 96-well round-bottom plates (Corning Inc., Corning, NY) for 5 h at 37°C with or without 1 μM of either $NP_{118–126}$ peptide or $M2_{82–90}$ peptide in the presence of 10 μg/ml brefeldin A (Sigma). All peptides were synthesized and purchased from Biosynthesis, Inc. (Lewisville, TX). Alternatively, virus-specific CD8 T cells were stimulated with EL-4 cells or irradiated (12,000 rads) P815 cells pulsed for 1 hour at 37°C with or without 1 μM of the appropriate peptide. EL-4 and P815 cells were washed twice with RPMI 1640 medium supplemented as described above and combined with lung and spleen cells at a 2:1 or a 3:1 effector/target cell (E:T) ratio, respectively. CHB3 cells were pulsed for 1 hour at 37°C with or without 1 μM of $GP_{61–80}$ peptide (Biosynthesis, Inc.) and combined with lung or spleen cells at a 1:1 E:T ratio. In some cases T cells were stimulated for 5 h at 37°C with 50 ng/ml phorbol myristate acetate (PMA) (Sigma) and 500 ng/ml of ionomycin (Sigma) in the presence of 10 μg/ml brefeldin A (Sigma). After incubation, the cells were washed with staining buffer (phosphate-buffered saline [PBS], 2% FCS, and 0.02% sodium azide), blocked with purified anti-FcγRII/III monoclonal antibody (MAb) (clone 93; eBioscience, San Diego, CA), and simultaneously stained with optimal concentrations of fluorescein isothiocyanate-conjugated anti-Thy1.2 MAb (clone 53-2.1; eBioscience) and either phycoerythrin-cyanine 7-conjugated anti-CD8 MAb (clone 53-6.7; eBioscience) or phycoerythrin-cyanine 7-conjugated anti-CD4 MAb (clone RM4.5; eBioscience) for 30 min at 4°C. In some experiments, cells were stained for activated caspase 3/7 using the Vybrant FAM caspase-3 and -7 Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The cells were then washed twice with staining buffer and fixed, and erythrocytes were lysed with fluorescence-activated cell sorter lysing solution (Becton Dickinson, San Jose, CA). The cells were then incubated in permeabilization buffer (staining buffer containing 0.5% saponin; Sigma) for 10 min and stained with an optimal concentration of allophycocyanin-conjugated anti-IFN-γ MAb (clone XMG1.2; eBioscience) and phycoerythrin-conjugated anti-TNF-α MAb (clone MP6-XT22; eBioscience). The cells were washed an additional time with permeabilization buffer and again with staining buffer prior to analysis on a Becton Dickinson FACsCanto flow cytometer. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Tetramer staining. Single-cell suspensions prepared from the spleen and lung were washed with staining buffer and stained with optimal concentrations of $M2_{82–90}$- or $NP_{118–126}$-specific allophycocyanin-conjugated tetramers (obtained from the NIH Tetramer Core Facility, Bethesda, MD) and simultaneously blocked with purified anti-FcγRII/III MAb for 30 min at 4°C. After the tetramer staining, the cells were washed twice with staining buffer and stained with optimal concentrations of fluorescein isothiocyanate-conjugated anti-Thy1.2 MAb and phycoerythrin-cyanine 7-conjugated anti-CD8 MAb. The cells were washed and fixed prior to analysis on a Becton-Dickinson FACsCanto flow cytometer. Data were analyzed using FlowJo software (Tree Star Inc.).

Adoptive transfer of TCR-transgenic T cells. The frequency of TCR-transgenic P14 CD8 T cells was determined by staining peripheral blood cells with allophycocyanin-conjugated anti-Thy1.1 MAb, phycoerythrin-cyanine 7-conjugated anti-CD8 MAb, phycoerythrin-conjugated anti-Vα2 MAb (Caltag Laboratories, Burlingame, CA), and fluorescein isothiocyanate-conjugated anti-Vβ8.1/8.2 MAb (clone MR5-2; BD Pharmingen). The frequency of TCR-transgenic SMARTA CD4 T cells was determined by staining peripheral blood cells with allophycocyanin-conjugated anti-Thy1.1 MAb, phycoerythrin-cyanine 7-conjugated anti-CD4 MAb, phycoerythrin-conjugated anti-Vα2 MAb (Caltag Laboratories, Burlingame, CA), and fluorescein isothiocyanate-conjugated anti-Vβ8.3 MAb (clone B3.3; BD Pharmingen). Peripheral blood Thy1.1 P14 CD8 T cells (1×10^3) or Thy1.1 SMARTA CD4 T cells (1×10^4) in a total volume of 100 μl sterile PBS were adoptively transferred intravenously (i.v.), via tail vein injection, into naïve Thy1.2 C57BL/6NCr recipient mice. Within 24 h posttransfer, the mice were infected i.n. with 5×10^5 PFU LCMV. In one set of experiments, 1×10^4 P14 CD8 T cells were adoptively transferred i.v. into naïve Thy1.2 C57BL/6NCr mice that were subsequently infected with 5×10^5 PFU LCMV i.n. At 15 days postinfection (p.i.), spleen and lung cells were isolated and pooled, and the frequency of P14 CD8 T cells was determined by flow cytometry as described above. Splenocytes were labeled for 10 min at 37°C with 25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) in PBS containing 0.1% bovine albumin (Sigma) and washed twice with supplemented RPMI 1640 medium containing 10% FCS. CFSE-labeled splenocytes and lung cells containing equal numbers of P14 CD8 T cells were mixed, and $\sim 2 \times 10^6$ to 3×10^6 total P14 CD8 T cells were adoptively transferred i.v. into naïve Thy1.2 C57BL/6NCr mice, which were allowed to rest for 7 days.

In vitro restimulation and cytokine ELISA. Spleens and lungs from C57BL/6NCr mice infected i.n. with 5×10^5 PFU LCMV between 40 and 60 days previously were harvested and prepared as single-cell suspensions as described above. Spleen and lung single-cell suspensions (1×10^6 cells) were incubated in 24-well plates (BD Falcon) in the presence or absence of 1 μM $GP_{33–41}$ peptide for 48 h at 37°C. Alternatively, the cells were stimulated with irradiated (6,000 rads) EL-4 cells pulsed with 1 μM $GP_{33–41}$ peptide as described above. The supernatants were then harvested and stored at −80°C prior to analysis by enzyme-linked immunosorbent assay (ELISA) to quantitate the amount of IFN-γ protein. ELISAs were performed as previously described (28).

Data analysis. Graphical and statistical analyses were performed using Prism software (Graphpad Software Inc., San Diego, CA). Unpaired Student *t* tests (two-tailed) and analyses of variance using the Dunnett multiple-comparison test were performed using InStat software (Graphpad Software, Inc.). *P* values were considered significant when *P* was <0.05.

RESULTS

Altered cytokine production by virus-specific CD8 T cells in the lung following acute pulmonary virus infection. Previous studies have shown that virus-specific pulmonary CD8 T cells are impaired in their ability to produce cytokines following acute respiratory infections with RSV (8, 9, 40), pneumonia virus of mice (10), or SV5 (14), all members of the *Paramyxoviridae* family of viruses. We first questioned if viruses unrelated to RSV would also induce the generation of virus-specific non-cytokine-producing CD8 T cells in the lung. Consistent with previous work (8, 9, 40), following acute i.n. RSV infection, a large proportion of RSV M2-specific CD8 T cells failed to produce IFN-γ following direct ex vivo peptide stimulation (Fig. 1A and 2A). As early as 8 days p.i., only ~50% of the

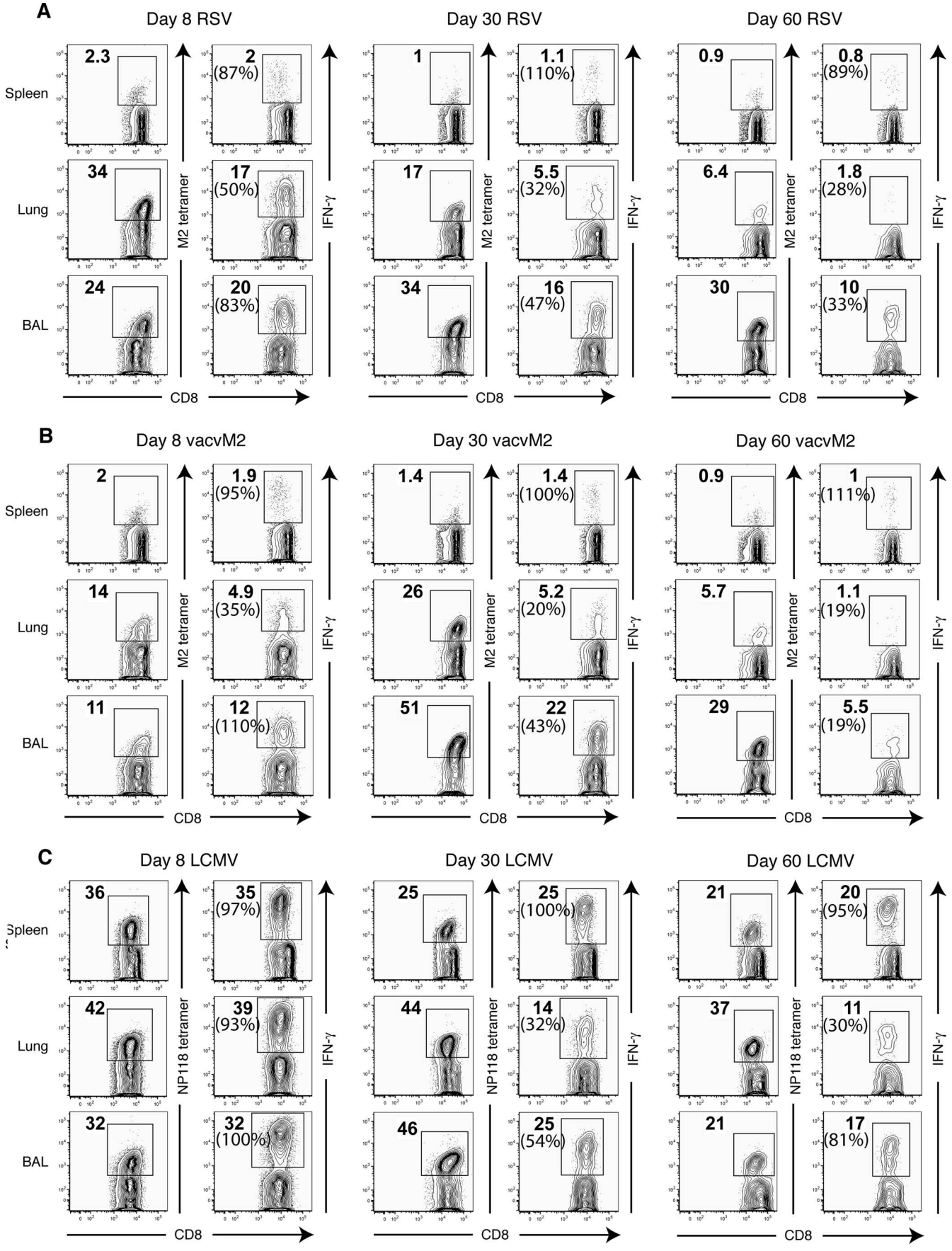
expected M2-specific CD8 T cells in the lung parenchyma, as determined by tetramer staining, produced IFN-γ following direct ex vivo peptide stimulation. In contrast, ~87% of the expected frequency of M2-specific splenic CD8 T cells produced IFN-γ, indicating that only a small proportion of the M2-specific CD8 T cells recovered from the spleen failed to produce IFN-γ upon direct ex vivo peptide stimulation. M2-specific CD8 T cells recovered from the lung airways (i.e., BAL fluid) and the spleen exhibited similar frequencies of non-cytokine-producing cells at day 8 p.i. By 30 and 60 days p.i., the proportion of M2-specific lung parenchymal and BAL fluid CD8 T cells capable of producing IFN-γ continued to decline, whereas the proportion in the spleen remained relatively constant (Fig. 1A and 2A). Additionally, in parallel with tetramer and IFN-γ staining, we tracked the production of TNF-α following direct ex vivo peptide stimulation. The frequency of M2-specific lung parenchyma CD8 T cells producing TNF-α was significantly ($P < 0.05$) lower relative to splenic CD8 T cells at all three time points (data not shown). The proportion of M2-specific CD8 T cells in the BAL that produced TNF-α was not significantly lower than in the spleen until ≥30 days p.i. (data not shown).

We next examined the virus-specific CD8 T-cell responses following acute respiratory infections with the ambisense RNA arenavirus LCMV and the double-stranded DNA poxvirus VACV. Using a recombinant VACV expressing the RSV M2 protein (VACVM2), we analyzed the RSV M2-specific CD8 T-cell response. Similar to RSV, acute respiratory infection with VACVM2 resulted in a population of CD8 T cells in the lung parenchyma that failed to produce IFN-γ upon direct ex vivo peptide stimulation (Fig. 1B and 2B). Whereas >95% of M2-specific splenic CD8 T cells produced IFN-γ at day 8 p.i. with VACVM2, only ~35% of the expected M2-specific CD8 T cells recovered from the lung parenchyma produced IFN-γ at this time point. The proportion of M2-specific CD8 T cells that produced IFN-γ further decreased by 30 and 60 days p.i. with VACVM2. In contrast to the lung parenchyma, in the BAL fluid at day 8 p.i., the frequency of CD8 T cells that produced IFN-γ after direct ex vivo stimulation with $M2_{82–90}$ peptide was higher than the frequency of CD8 T cells that bound the $M2_{82–90}$ tetramer. As with RSV, we observed a significant ($P < 0.05$) decrease in the proportion of CD8 T cells from the BAL fluid that produced IFN-γ in response to direct ex vivo peptide stimulation at days 30 and 60 p.i. (Fig. 1B and 2B). Additionally, the proportion of M2-specific CD8 T cells in the lung parenchyma that produced TNF-α was significantly ($P < 0.05$) lower than that in the spleen (data not shown). We did not observe a significant decrease in the proportion of TNF-α-producing CD8 T cells in the BAL until ≥30 days p.i. with VACVM2.

The capacity of pulmonary CD8 T cells to produce IFN-γ following acute i.n. infection with LCMV differed slightly from that after acute i.n. infection with either RSV or VACVM2 (Fig. 1C and 2C). At day 8 p.i., similar frequencies of CD8 T cells in the lung and BAL could be identified as LCMV specific via tetramer staining versus ICS for IFN-γ production. However, we did observe a decrease in the proportion of IFN-γ-producing CD8 T cells recovered from the lung parenchyma and BAL at later time points. Because we observed similar results examining the $M2_{82–90}$-specific response after infection with RSV and after infection with a recombinant VACV expressing the RSV M2 protein, we questioned if the ability of pulmonary LCMV $NP_{118–126}$-specific CD8 T cells to produce IFN-γ during acute respiratory infection was epitope dependent. At day 8 after acute respiratory infection with a recombinant VACV expressing the $NP_{118–126}$ epitope, we noted a decrease in the proportion of IFN-γ-producing CD8 T cells from the lung parenchyma similar to that we previously observed after i.n. infection with either RSV or VACVM2 (data not shown). These data indicate that the ability of lung parenchymal $NP_{118–126}$-specific CD8 T cells to produce IFN-γ is a consequence of the acute LCMV respiratory infection. However, by 30 and 60 days p.i., only ~30% of the expected $NP_{118–126}$-specific CD8 T cells in the lung parenchyma produced IFN-γ. Interestingly, $NP_{118–126}$-specific CD8 T cells in the BAL fluid demonstrated a more sustained ability to produce IFN-γ than the cells in the lung parenchyma at days 30 and 60 p.i. Similar to the RSV and VACVM2 infection models, at 30 to 90 days p.i. with LCMV, the majority of lung parenchymal and BAL CD8 T cells failed to produce TNF-α following direct ex vivo peptide stimulation (data not shown). Thus, CD8 T cells recovered from the lung parenchyma more consistently failed to produce IFN-γ and TNF-α following direct ex vivo peptide stimulation than did cells isolated from the lung airways or the spleen.

We considered the possibility that the non-IFN-γ-producing T cells could be producing cytokines other than IFN-γ. In vitro studies have demonstrated that in the presence of IL-4, CD8 T cells can switch to a Th2-like pattern of cytokine production (24, 33). However, we did not detect a population of CD8 T cells producing interleukin 4 (IL-4), IL-5, or IL-10 following infection with LCMV, VACVM2, or RSV (data not shown). Thus, the decreased frequency of pulmonary virus-specific IFN-γ-producing CD8 T cells was not a result of switching to an alternative Tc2 phenotype. We also considered the possibility that we failed to liberate all of the APC from the lung parenchyma during tissue processing. To address this possibility, lungs harvested 8 and 30 days post-RSV infection were digested with a combination of collagenase, hyaluronidase, and DNase I prior to in vitro peptide stimulation. The proportion of IFN-γ-producing M2-specific CD8 T cells in the lung parenchyma did not significantly change compared to nondigested lung (data not shown). Thus, our results suggest that the failure of pulmonary-virus-specific CD8 T cells to produce IFN-γ and TNF-α following direct ex vivo peptide stimulation is not restricted to the *Paramyxoviridae* family of viruses. Instead, these data suggest that the failure of antigen-specific CD8 T cells in the lung parenchyma to produce IFN-γ and TNF-α may primarily be a result of the local tissue environment.

Exogenous peptide-pulsed APC are able to rescue cytokine production. MHC class I molecules are expressed on the surfaces of all nucleated cells in the body. Consequently, every host cell should be capable of presenting peptide to CD8 T cells and eliciting a proper effector response. However, recent work has suggested that CD8 T cells may require interaction with professional APC within tissues to elicit effector functions (3, 4, 43). Thus, one potential explanation for the reduced ability of virus-specific CD8 T cells isolated from the lung parenchyma to make cytokines is


A
Day 8 RSV
Day 30 RSV
Day 60 RSV
Spleen
Lung
BAL
2.3
2
(87%)
1
1.1
(110%)
0.9
0.8
(89%)
34
17
(50%)
17
5.5
(32%)
6.4
1.8
(28%)
24
20
(83%)
34
16
(47%)
30
10
(33%)
M2 tetramer
IFN-γ
CD8
B
Day 8 vacvM2
Day 30 vacvM2
Day 60 vacvM2
2
1.9
(95%)
1.4
1.4
(100%)
0.9
1
(111%)
14
4.9
(35%)
26
5.2
(20%)
5.7
1.1
(19%)
11
12
(110%)
51
22
(43%)
29
5.5
(19%)
C
Day 8 LCMV
Day 30 LCMV
Day 60 LCMV
Spleen
36
35
(97%)
25
25
(100%)
21
20
(95%)
42
39
(93%)
44
14
(32%)
37
11
(30%)
32
32
(100%)
46
25
(54%)
21
17
(81%)
NP118 tetramer

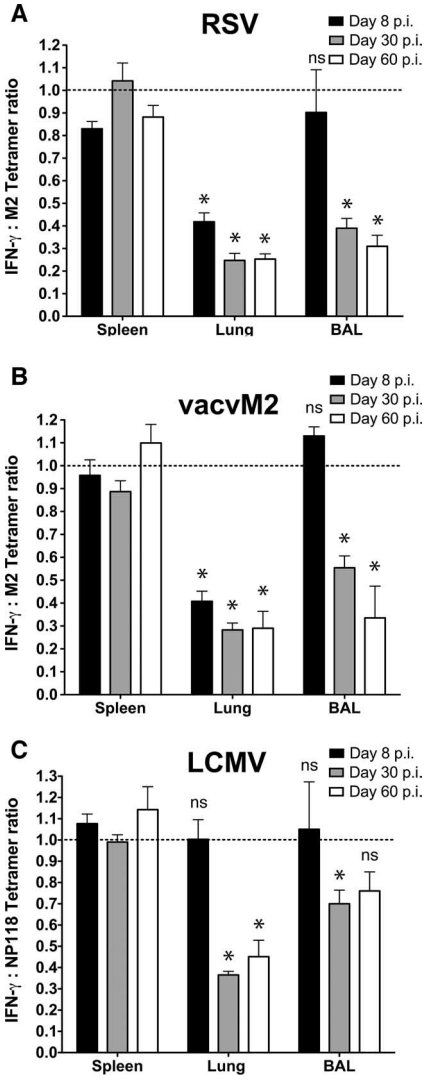


FIG. 2. Ratio of IFN-γ-secreting to tetramer binding cells following RSV, VACVM2, and LCMV infections. BALB/c mice were infected i.n. with RSV (A), VACVM2 (B), or LCMV (C). Cells from the spleen, lung parenchyma, and BAL fluid were stained with tetramer or for IFN-γ as described in the legend to Fig. 1. The data represent the mean plus standard error of the mean of at least three separate experiments with four mice per experiment. *P* values were calculated within each time point by comparing lung parenchyma and BAL fluid ratios to that of the spleen. *, $P < 0.05$; ns, not significant.

that they have not received proper stimulation by the endogenous tissue APC. Therefore, to test if the lung-resident APC were providing insufficient activation signals to stimulate IFN-γ or TNF-α, we used exogenous peptide-pulsed APC to stimulate splenic and pulmonary CD8 T cells (Fig. 3A and C). Stimulation with exogenous peptide-pulsed APC significantly ($P < 0.05$) increased IFN-γ production by CD8 T cells isolated from the spleen at 30 days p.i. with either RSV or LCMV (Fig. 3B and D). These data indicate that direct ex vivo peptide stimulation of the spleen fails to stimulate all of the virus-specific CD8 T cells to produce IFN-γ. Interestingly, stimulation with exogenous peptide-pulsed APC significantly ($P < 0.05$) recovered IFN-γ production by CD8 T cells isolated from the lung parenchyma, but not from the BAL fluid, at day 30 p.i. with either RSV or LCMV.

Exogenous peptide-pulsed APC stimulation of pulmonary CD8 T cells had similar effects between the infection models. In RSV, exogenous peptide-pulsed APC stimulated ~71% of lung parenchymal M2-specific CD8 T cells to produce IFN-γ, which represented a significant ($P < 0.05$) 2.2-fold increase in comparison to direct ex vivo peptide stimulation with endogenous APC (Fig. 3A and B). Following infection with LCMV, exogenous peptide-pulsed APC stimulated ~80% of LCMV NP118-specific CD8 T cells recovered from the lung parenchyma to produce IFN-γ, which was a significant ($P < 0.05$) 2.5-fold increase compared to direct ex vivo peptide stimulation alone (Fig. 3C and D). Interestingly, stimulation with exogenous peptide-pulsed APC had no significant effect on either RSV- or LCMV-specific BAL CD8 T cells. This suggests that the lack of cytokine production by virus-specific CD8 T cells in the BAL fluid is not a consequence of insufficient activation by APC in the airways. We observed similar results in the spleen, lung parenchyma, and BAL fluid following VACVM2 infection (data not shown). Importantly, these data indicate that virus-specific CD8 T cells in the spleen and lung parenchyma are not irreversibly impaired. Compared to the spleen, endogenous APC in the lung parenchyma appear to be much less efficient at stimulating maximal cytokine production by virus-specific CD8 T cells.

Failure of pulmonary LCMV-specific TCR-transgenic CD8 T cells to produce cytokines following direct ex vivo peptide stimulation. Downregulation of the TCR following stimulation and reorganization of the TCR and CD8 on the cell surface can both lead to decreased tetramer binding (11, 12). Consequently, we were unable to stain with tetramer and examine effector functions of virus-specific CD8 T cells within the same assay. In order to trace an antigen-specific T-cell population independently of tetramer staining, we utilized an adoptive-transfer system using D^b-restricted LCMV GP_{33-44}-specific TCR-transgenic P14 CD8 T cells. We adoptively transferred 1×10^3 Thy1.1 P14 CD8 T cells i.v. into naïve Thy1.2 C57BL/6 mice and challenged them i.n. with LCMV. At day 30 p.i., ~93% of P14 CD8 T cells in the spleen produced IFN-γ, whereas only ~35% in the lung parenchyma and ~63% in the BAL fluid produced IFN-γ (Fig. 4A). These data are consis-

FIG. 1. Diminished IFN-γ production by pulmonary CD8 T cells following acute respiratory-virus infection. BALB/c mice were infected i.n. with RSV (A), VACVM2 (B), or LCMV (C). At 8, 30, and 60 days p.i., cells from the spleen, lung parenchyma, and BAL fluid were stained with tetramer or stimulated with or without peptide in the presence of brefeldin A for 5 h at 37°C and subsequently stained for IFN-γ. The cells were gated on $CD8^+$ $Thy1.2^+$ cells. The boldface numbers represent the percentages of $tetramer^+$ or IFN-γ^+ CD8 T cells; the numbers in parentheses are the percentages of IFN-γ^+/$tetramer^+$ CD8 T cells. Representative staining from one of four mice is shown. Similar results were obtained in three separate experiments.

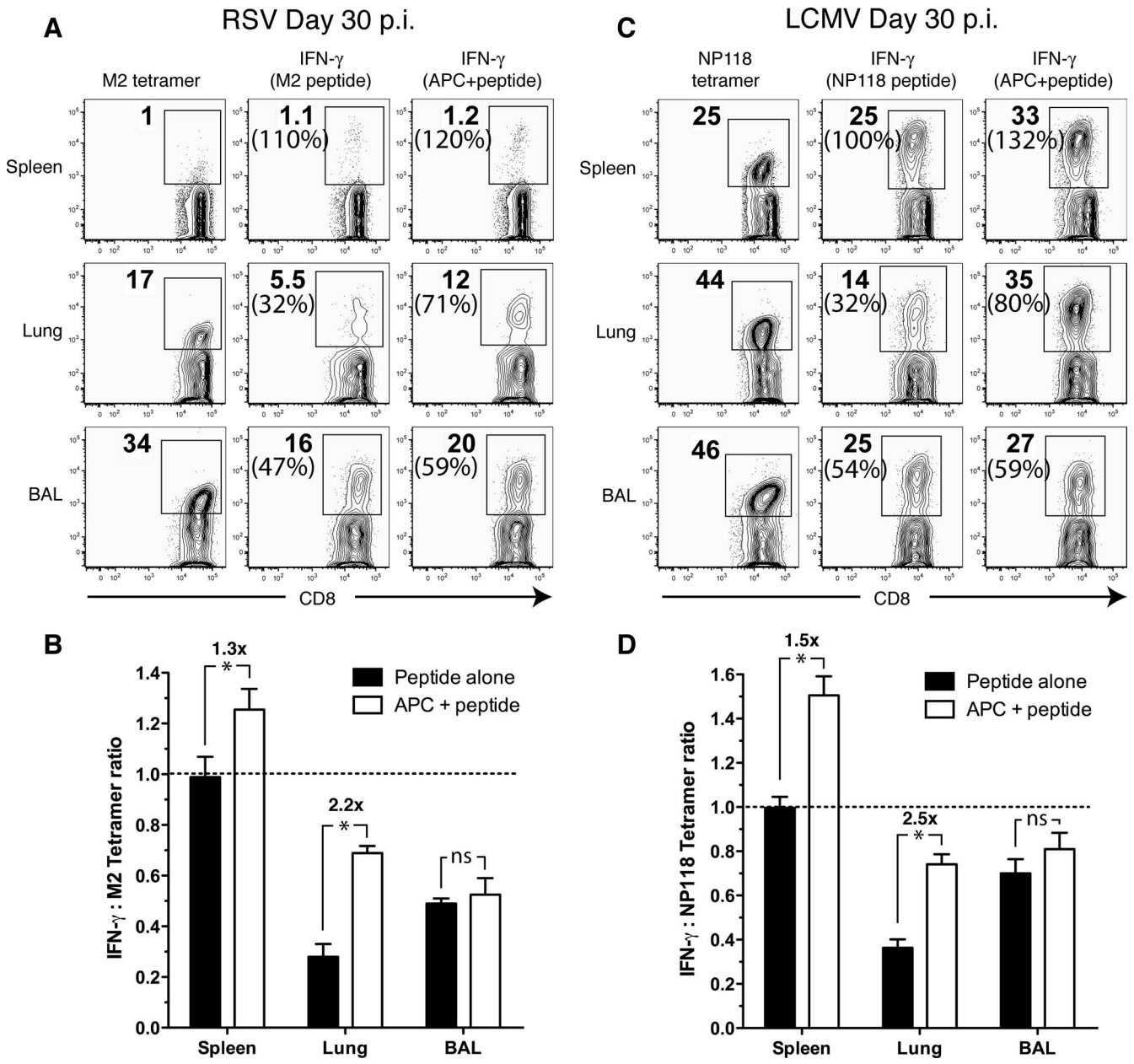


FIG. 3. Presentation of peptide by exogenous APC rescues IFN-γ production by CD8 T cells recovered from the lung. BALB/c mice were infected with RSV (A and B) or LCMV (C and D), and tissues were harvested 30 days p.i. Irradiated P815 cells incubated with or without peptide were mixed with spleen or lung single-cell suspensions at a 1:3 E:T ratio, stimulated for 5 h, and stained for IFN-γ. The cells were gated on $CD8^+$ $Thy1.2^+$ cells. The boldface numbers in panels A and C represent the percentages of $tetramer^+$ or IFN-γ^+ CD8 T cells; the numbers in parentheses are the percentages of IFN-γ^+/$tetramer^+$ CD8 T cells. The data in panels B and D represent the mean plus standard error of the mean from two separate experiments with four mice per experiment. The boldface numbers represent the increase in the means between treatment groups. The flow plots and the corresponding summary graphs are representative of two separate experiments with four mice per experiment. *, $P < 0.05$; ns, not significant.

tent with our LCMV data for BALB/c mice. Additionally, by day 30 p.i., ~89 ± 6% standard deviation ($n = 11$ mice; three separate experiments) of splenic P14 CD8 T cells became capable of coproducing TNF-α and IFN-γ. In contrast, only ~64 ± 11% standard deviation ($n = 11$ mice; three separate experiment) of P14 CD8 T cells in the lung parenchyma produced both TNF-α and IFN-γ. Stimulation with exogenous peptide-pulsed APC resulted in equivalent IFN-γ production by splenic P14 CD8 T cells and a significant ($P < 0.05$) increase in IFN-γ production by P14 CD8 T cells in the lung parenchyma and BAL (Fig. 4B). PMA and ionomycin stimulation of P14 CD8 T cells recovered from the spleen, lung parenchyma, and BAL fluid between 40 and 60 days p.i. with LCMV resulted in frequencies of IFN-γ production similar to those resulting from stimulation with exogenous peptide-pulsed APC (Table 1). Importantly, these data indicate that the antigen-specific T cells that enter the lung parenchyma remain capable of producing cytokines when stimulated with exogenous pep-

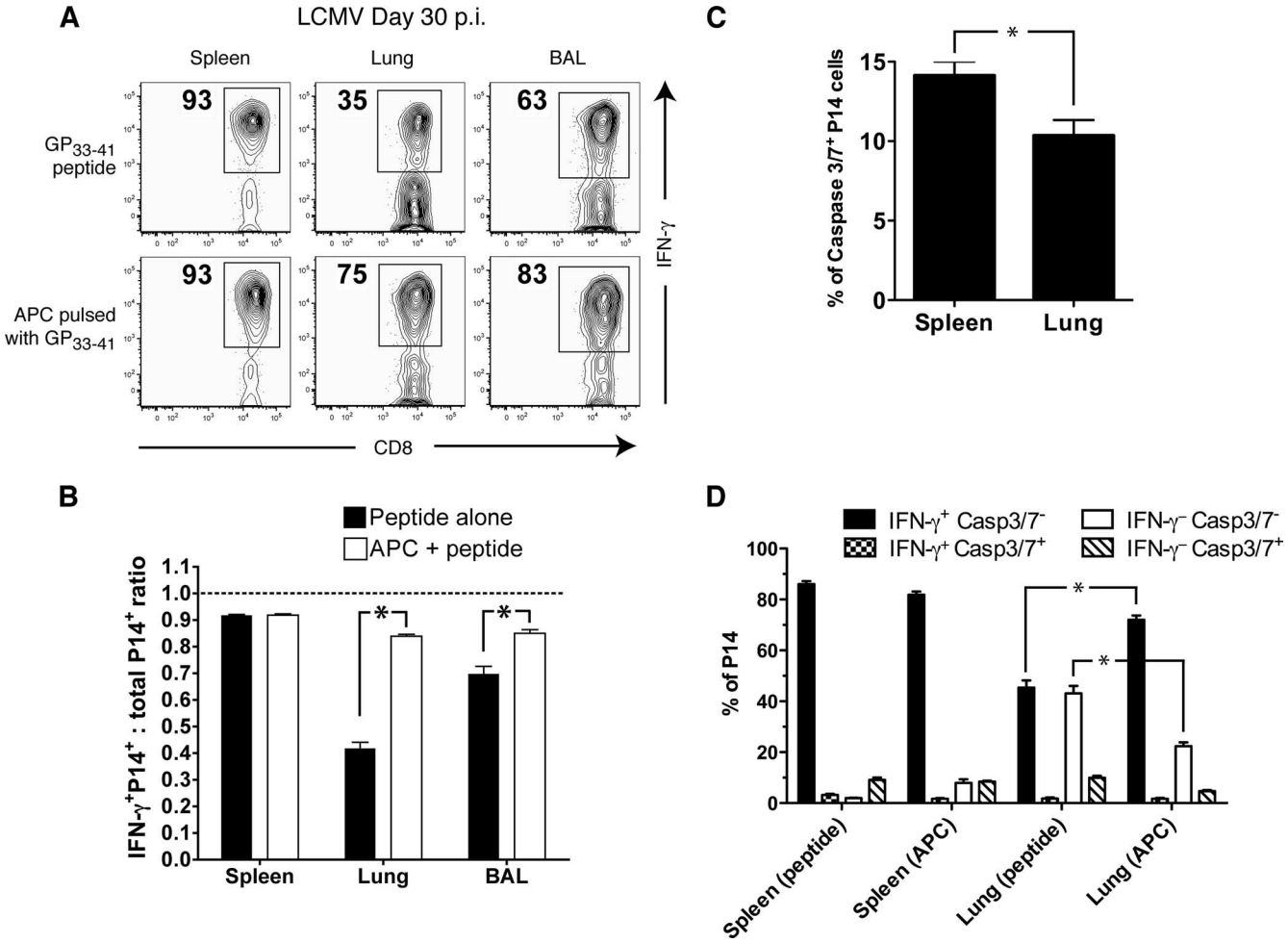


FIG. 4. LCMV TCR-transgenic CD8 T cells exhibit decreased cytokine production. P14 TCR-transgenic Thy1.1$^+$ CD8 T cells (1×10^3) were transferred i.v. into naïve Thy1.2$^+$ C57BL/6 mice. At 30 days p.i., spleens and lungs were harvested. The cells were stimulated with or without peptide or peptide-pulsed EL-4 cells and stained for IFN-γ. (A) Cells were gated on donor CD8$^+$ Thy1.1$^+$ P14 T cells. The boldface numbers represent the percentages of IFN-γ$^+$ CD8 P14 T cells. (B) Summary graph showing the mean plus standard error of the mean (SEM) from a total of four separate experiments with four mice per experiment. (C) Cells were stained for caspase 3/7 directly ex vivo without peptide stimulation. The graph represents the mean plus SEM from a total of two separate experiments with four mice per experiment. (D) Cells were stimulated ex vivo with peptide alone or with peptide-pulsed EL-4 cells. The cells were stained for IFN-γ and caspase 3/7. The summary graph shows the mean plus SEM from a total of two separate experiments with four mice per experiment. *, $P < 0.05$.

tide-pulsed APC, suggesting that these cells are not anergic or irreversibly impaired.

Because we could never stimulate all of the P14 CD8 T cells recovered from the spleen or the lung to produce IFN-γ, we next assessed the proportion of apoptotic cells among the non-IFN-γ-producing P14 CD8 T cells. Directly ex vivo, ~14% of P14 CD8 T cells recovered from the spleen and ~10% of P14 CD8 T cells obtained from the lung parenchyma stained positive for activated caspase 3/7 (Fig. 4C). Following direct ex vivo peptide stimulation, the majority of non-IFN-γ-producing P14 CD8 T cells recovered from the spleen stained positive for activated caspase 3/7 (Fig. 4D). These data indicate that at least some of the non-IFN-γ-producing P14 CD8 T cells obtained from the spleen are apoptotic and not a result of inadequate stimulation. In contrast to splenic P14 CD8 T cells, only a small proportion of non-IFN-γ-producing P14 CD8 T cells recovered from the lung parenchyma stained positive for acti-

TABLE 1. Production of IFN-γ by splenic and pulmonary CD8 T cells following ex vivo stimulation[a]

Tissue	% IFN-γ		
	Peptide only	APC + peptide	PMA + ionomycin
Spleen	91 ± 1	88 ± 2	88 ± 2
Lung	29 ± 2	69 ± 4	71 ± 2
BAL	37 ± 16	77 ± 1	76 ± 4

[a] P14 CD8 T cells were adoptively transferred i.v. into naïve C57BL/6 mice prior to i.n. infection with LCMV. Cells were harvested from the indicated tissues between 40 and 60 days p.i. and stained for IFN-γ following ex vivo stimulation as described in the legend to Fig. 4. The numbers represent the mean percentages of IFN-γ$^+$ CD8 T cells ± standard deviations. Spleen and lung results represent data from three separate experiments with four mice per experiment. BAL results represent a pool of four mice per experiment from two separate experiments.

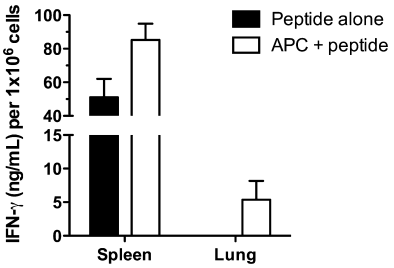


FIG. 5. Pulmonary CD8 T cells produce less IFN-γ protein than splenic CD8 T cells. Lungs and spleens were harvested from C57BL/6 mice infected with LCMV 40 to 60 days previously, and single-cell suspensions were stimulated with $GP_{33–41}$ peptide or peptide-pulsed irradiated EL-4 cells for 48 h. The supernatants were then assayed for total IFN-γ protein by ELISA. The data represent the mean plus standard error of the mean from one of two separate experiments with three mice per experiment.

vated caspase 3/7. Importantly, the frequency of P14 CD8 T cells obtained from the spleen and lung that expressed activated caspase 3/7 did not increase after direct ex vivo peptide stimulation, indicating that we did not induce apoptosis during stimulation. When P14 CD8 T cells from the spleen were stimulated with exogenous peptide-pulsed APC, the frequency of cells expressing the activated form of caspase 3/7 did not change. Similarly, stimulation of P14 CD8 T cells from the lung parenchyma with exogenous peptide-pulsed APC increased the frequency of IFN-γ-producing P14 CD8 T cells but did not significantly alter the frequency of non-IFN-γ-producing P14 CD8 T cells that stained positive for activated caspase 3/7. These data demonstrate that the inability of LCMV-specific CD8 T cells in the lung parenchyma to produce cytokines following direct ex vivo peptide stimulation could be rescued by stimulation with exogenous peptide-pulsed APC.

Pulmonary CD8 T cells produce less total IFN-γ than splenic CD8 T cells. To verify that the reduced capacity of pulmonary CD8 T cells to produce IFN-γ would also impact the total amount of cytokine released, we measured total IFN-γ protein production by ELISA. Equivalent numbers of cells from spleens and lungs of C57BL/6 mice that were infected i.n. with LCMV 40 to 60 days earlier were restimulated in vitro for 48 h with either $GP_{33–41}$ peptide or exogenous peptide-pulsed APC. There were comparable total numbers of $GP_{33–41}$-specific CD8 T cells within the spleen and lung populations, as determined by ICS following stimulation with exogenous peptide-pulsed APC (data not shown). Stimulation directly ex vivo with peptide induced high levels of IFN-γ protein production by $GP_{33–41}$-specific CD8 T cells in the spleen that was further enhanced by stimulation with exogenous peptide-pulsed APC (Fig. 5). In contrast to splenic CD8 T cells, in vitro peptide stimulation did not result in detectable IFN-γ production by pulmonary CD8 T cells (Fig. 5). Stimulation with exogenous peptide-pulsed APC resulted in detectable levels of IFN-γ protein. These results are consistent with our ICS data showing that virus-specific memory CD8 T cells that enter the lung exhibit a greatly diminished capacity to produce IFN-γ following direct ex vivo peptide stimulation.

Reduced cytokine production by pulmonary CD4 T cells. We reasoned that if respiratory viruses and/or the tissue environment had broad effects on dampening the immune response, then virus-specific CD4 T cells would also exhibit a similar failure to produce cytokines. We adoptively transferred 1×10^4 LCMV $GP_{61–80}$-specific Thy1.1 SMARTA CD4 T cells into naïve Thy1.2 C57BL/6 mice, followed by i.n. infection with LCMV. Similar to CD8 T cells, a higher proportion of SMARTA CD4 T cells recovered from the spleen produced IFN-γ upon direct ex vivo peptide stimulation relative to SMARTA CD4 T cells obtained from the lung. At day 30 p.i., ~74% of SMARTA CD4 T cells recovered from the spleen produced IFN-γ, whereas only ~14% from the lung parenchyma and ~21% from the BAL fluid produced IFN-γ (Fig. 6A and B). Consistent with published data (42), at day 30 p.i. >90% of splenic SMARTA CD4 T cells produced both IFN-γ and TNF-α following stimulation (data not shown). In contrast, only ~66% of SMARTA CD4 T cells isolated from the lung parenchyma produced both IFN-γ and TNF-α (data not shown). Stimulation with exogenous peptide-pulsed APC resulted in equivalent frequencies of IFN-γ-secreting SMARTA CD4 T cells in the spleen and significantly ($P < 0.05$) increased the proportion of IFN-γ-producing SMARTA CD4 T cells in the lung parenchyma (Fig. 6B). PMA and ionomycin stimulation of spleen, lung, and BAL fluid cells resulted in frequencies of IFN-γ-producing SMARTA CD4 T cells similar to those with exogenous peptide-pulsed APC (Table 2). Directly ex vivo, ~11% of SMARTA CD4 T cells from the spleen and ~9% of SMARTA CD4 T cells from the lung parenchyma stained positive for activated caspase 3/7 (Fig. 6C). These proportions did not significantly change following direct ex vivo peptide stimulation, indicating that stimulation did not induce apoptosis (Fig. 6D). Similar to our results with P14 CD8 T cells, only a small proportion of SMARTA CD4 T cells from the lung parenchyma stained positive for activated caspase 3/7 (Fig. 6D). These data suggest that, like CD8 T cells, the majority of CD4 T cells recovered from the lung parenchyma can be rescued to produce IFN-γ following stimulation with exogenous peptide-pulsed APC.

The lung environment inhibits cytokine production by CD8 T cells. Our results suggest that tissue endogenous APC isolated from the lung parenchyma fail to stimulate cytokine production by virus-specific memory T cells directly ex vivo regardless of the initial infecting virus. Because all three virus systems we examined demonstrated similar reductions in the capacity of virus-specific CD8 T cells to produce IFN-γ following direct ex vivo peptide stimulation at day 30 p.i., we hypothesized that the lung environment in a naïve animal may also fail to optimally stimulate cytokine production by virus-specific T cells. To directly test this possibility, we adoptively transferred 1×10^4 Thy1.1 P14 CD8 T cells into naïve Thy1.2 C57BL/6 mice that were subsequently infected i.n. with LCMV. At day 15 p.i., ~95% of P14 CD8 T cells isolated from the spleen produced IFN-γ following direct ex vivo peptide stimulation (Fig. 7A). Consistent with our LCMV data for BALB/c mice (Fig. 1C and 2C), only ~69% of P14 CD8 T cells in the lung parenchyma produced IFN-γ following direct ex vivo peptide stimulation. Spleen and lung cells from multiple mice were then pooled, and the splenocytes were labeled with CFSE (Fig. 7B). Splenocytes and lung cells containing equal numbers of P14 CD8 T cells were mixed at a 1:1 ratio, and ~2×10^6 to 3×10^6 total P14 CD8 T cells were adoptively transferred i.v. into naïve Thy1.2 C57BL/6 recipients. To control for

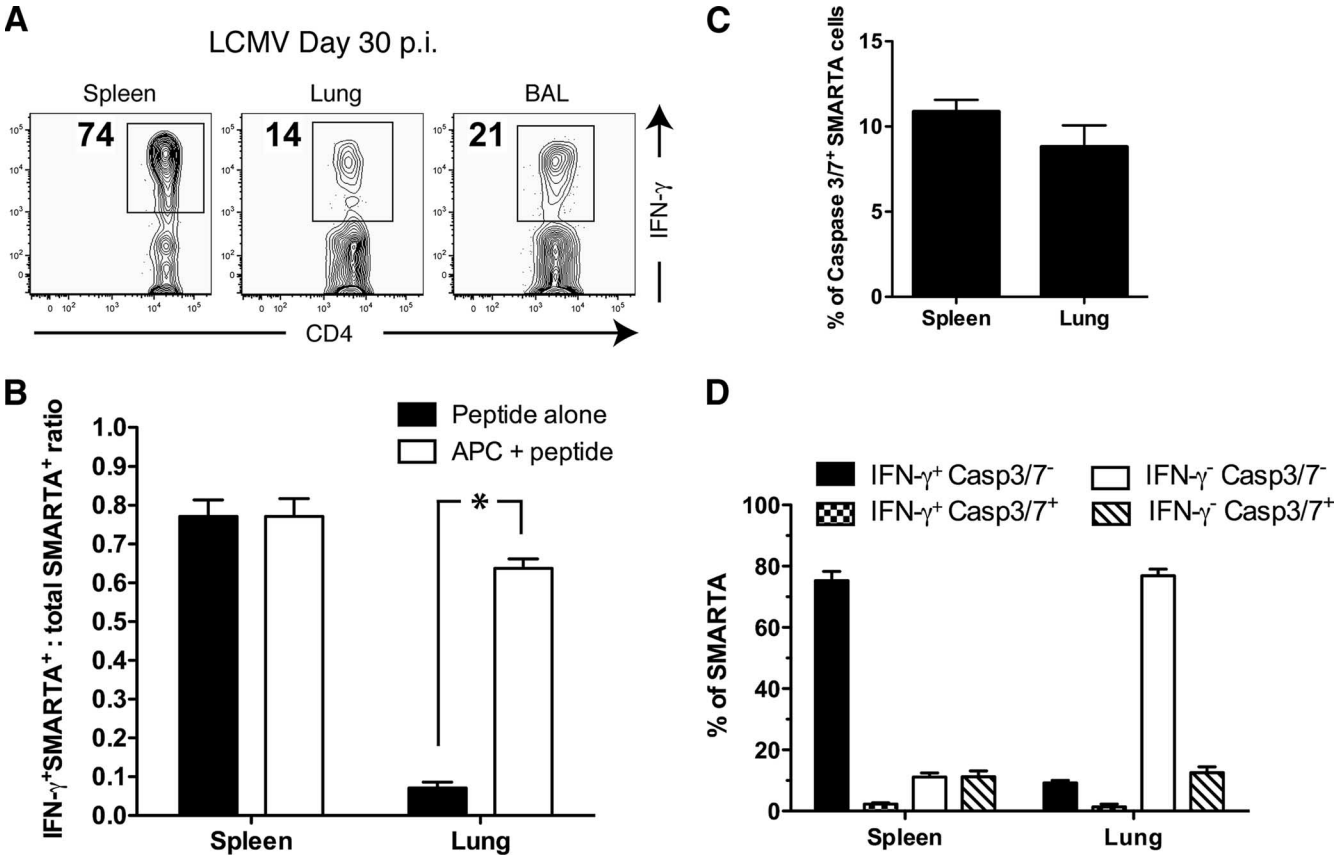


FIG. 6. CD4 T cells also exhibit decreased cytokine production following pulmonary-virus infection. TCR-transgenic Thy1.1$^+$ CD4 SMARTA T cells (1×10^4) were transferred i.v. into naïve Thy1.2$^+$ C57BL/6 mice. At 30 days p.i., spleens and lungs were harvested, stimulated with or without peptide, and stained for IFN-γ. (A) Cells were gated on donor CD4$^+$ Thy1.1$^+$ T cells. The boldface numbers in panel A represent the percentages of IFN-γ$^+$ SMARTA CD4 T cells. (B) Summary graph showing the mean plus standard error of the mean (SEM) from a total of two separate experiments with four mice per experiment. (C) Cells were stained for caspase 3/7 directly ex vivo without peptide stimulation. The graph represents the mean plus SEM from one of two separate experiments with four mice per experiment. (D) Cells were stimulated ex vivo with peptide alone or with peptide-pulsed CHB3 cells. The cells were then stained for IFN-γ and caspase 3/7. The data show the mean plus SEM from a total of two separate experiments with four mice per experiment. *, $P < 0.05$.

the effects of CFSE on P14 T cells, CFSE-labeled splenocytes were mixed 1:1 with unlabeled splenocytes and adoptively transferred into naïve recipients. At 7 days posttransfer, spleens and lungs were harvested and cells were stimulated directly ex vivo with peptide or exogenous peptide-pulsed APC. In naïve mice that received unlabeled or CFSE-labeled spleen-derived P14 CD8 T cells, we observed equivalent survival and trafficking to the spleen, lung, and blood in both groups (data not shown). Importantly, the same proportion of CFSE$^-$ and CFSE$^+$ P14 CD8 T cells that had trafficked into the spleen and lung produced IFN-γ following direct ex vivo peptide stimulation (data not shown). As expected, ~96% of spleen-derived P14 CD8 T cells that had trafficked back into the spleen produced IFN-γ upon direct ex vivo peptide stimulation (Fig. 7C and D). An equivalent frequency of P14 CD8 T cells produced IFN-γ when stimulated with exogenous peptide-pulsed APC. Interestingly, ~96% of lung-derived P14 CD8 T cells that had trafficked into the spleen produced IFN-γ upon direct ex vivo stimulation with peptide or upon stimulation with exogenous peptide-pulsed APC (Fig. 7C and D). In contrast, only ~48% of spleen- and lung-derived P14 CD8 T cells that had trafficked into the lung parenchyma produced IFN-γ following direct ex vivo peptide stimulation. Stimulation with exogenous peptide-pulsed APC resulted in frequencies of IFN-γ-producing P14 CD8 T cells similar to those for the spleen. Importantly, these data demonstrate that the decreased capacity of antigen-specific CD8 T cells isolated from the lung

TABLE 2. Production of IFN-γ by splenic and pulmonary CD4 T cells following ex vivo stimulation[a]

Tissue	% IFN-γ		
	Peptide only	APC + peptide	PMA + ionomycin
Spleen	87 ± 3	89 ± 2	96 ± 2
Lung	9 ± 3	77 ± 6	88 ± 5
BAL	9 ± 0.5	61 ± 2	91 ± 2

[a] SMARTA CD4 T cells were adoptively transferred i.v. into naïve C57BL/6 mice prior to i.n. infection with LCMV. Cells were harvested from the indicated tissues between 30 and 40 days p.i. and stained for IFN-γ following ex vivo stimulation as described in the legend to Fig. 6. The numbers represent the percentages of IFN-γ$^+$ CD4 T cells ± standard deviations. Spleen and lung results represent data from two separate experiments with three mice per experiment. BAL results represent a pool of three mice per experiment from two separate experiments.

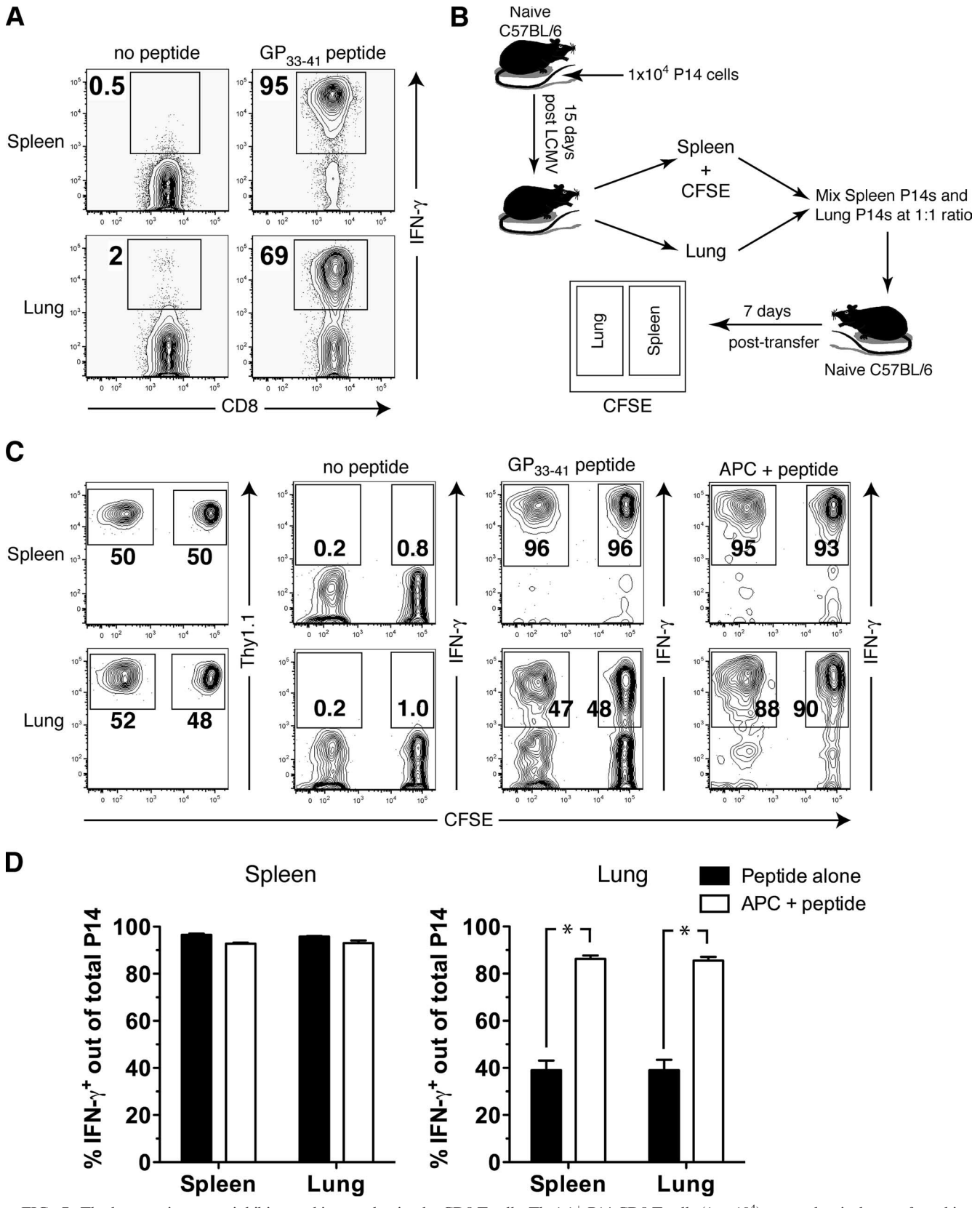


FIG. 7. The lung environment inhibits cytokine production by CD8 T cells. Thy1.1$^+$ P14 CD8 T cells (1×10^4) were adoptively transferred i.v. into naïve Thy1.2$^+$ C57BL/6 mice that were subsequently infected i.n. with LCMV. (A) At 15 days p.i., spleens and lungs from multiple mice were harvested, pooled, stimulated with or without peptide, and stained for IFN-γ. (B) Diagram representing the overall experimental design. The pooled splenocytes were labeled with CFSE and mixed with lung cells containing similar numbers of P14 cells. The mixture of cells was adoptively

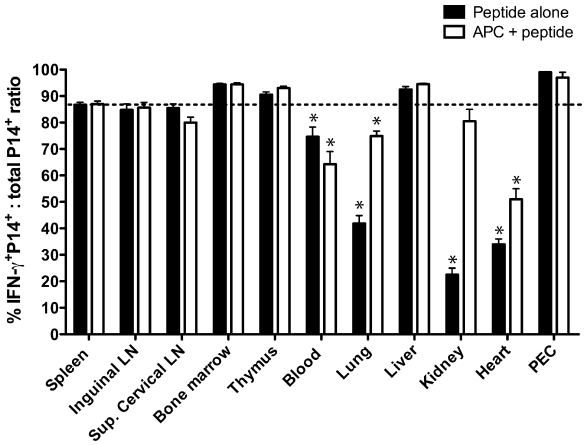


FIG. 8. Reduced cytokine production by LCMV-specific CD8 T cells in peripheral tissues following ex vivo peptide stimulation. P14 TCR-transgenic Thy1.1$^+$ CD8 T cells (1×10^3) were transferred i.v. into naïve Thy1.2$^+$ C57BL/6 mice prior to infection with LCMV. At 30 days p.i., cells were harvested from various tissues and stained for IFN-γ as described in the legend to Fig. 1. The data showing the mean plus standard error of the mean from a total of two separate experiments with four mice per experiment. The kidneys, heart, and peritoneal exudate cells (PEC) from four mice were pooled. LN, lymph node; sup., superficial. *P* values were calculated using peptide-stimulated splenic P14 T cells as the control group. *, $P < 0.05$.

parenchyma to produce cytokines following direct ex vivo peptide stimulation occurs in the absence of virus infection and is instead a consequence of the lung tissue environment. In addition, these results demonstrated for the first time that virus-specific CD8 T cells that were isolated from the lung and adoptively transferred into naïve recipients regained their capacity to produce IFN-γ after entry into the spleen.

Regulation of cytokine production by LCMV-specific CD8 T cells in various tissues. To determine if other peripheral tissues also regulate cytokine production by antigen-specific CD8 T cells, we examined P14 CD8 T-cell cytokine production in multiple tissues following acute LCMV infection. In addition to the lung parenchyma, P14 CD8 T cells exhibited significantly ($P < 0.05$) reduced IFN-γ production in peripheral tissues, such as the peripheral blood, kidneys, and heart, at day 30 p.i. (Fig. 8). Interestingly, reduced cytokine production was not ubiquitous in peripheral sites; P14 CD8 T cells recovered from the inguinal and superficial cervical lymph nodes, bone marrow, thymus, liver, and peritoneal cavity exhibited a high proportion of antigen-specific CD8 T cells capable of producing IFN-γ. Similar to the lung parenchyma, stimulation with exogenous peptide-pulsed APC significantly ($P < 0.05$) increased IFN-γ production by P14 CD8 T cells obtained from the kidney. In contrast, stimulation with exogenous peptide-pulsed APC did not significantly rescue IFN-γ production by P14 CD8 T cells recovered from the peripheral blood and heart. Importantly, these data indicate that tissues differentially regulate IFN-γ production by antigen-specific CD8 T cells.

DISCUSSION

Increasing evidence indicates that peripheral tissues can regulate multiple facets of innate and adaptive immunity (8, 10, 14, 15, 32, 36, 38, 40). Previous studies have suggested that acute pulmonary infection with any one of several related viruses, including RSV, SV5, and pneumonia virus of mice, results in the functional impairment of virus-specific CD8 T cells obtained from either the lung parenchyma or the airways (8–10, 14, 29, 40). More recent studies have indicated that the lung environment may directly regulate the capacity of antigen-specific T cells to produce cytokines. For example, RSV challenge of LCMV-immune mice results in the diminished capacity of both RSV-specific and nonspecifically recruited LCMV-specific memory CD8 T cells to produce IFN-γ and TNF-α (40). In addition, more recent work has shown that antigen-specific effector CD8 T cells that have been adoptively transferred into naïve recipients and traffic into the lung parenchyma exhibit within 48 h a decreased capacity to produce IFN-γ following direct ex vivo stimulation (1). This interesting result suggests that the CD8 T cells that enter the lung are conditioned by the lung environment and may potentially enter an altered state of differentiation. Thus, it appears that the lung environment is directly responsible for regulating the ability of virus-specific T cells to produce cytokines.

Here, we have demonstrated that acute respiratory infection with VACVM2 and LCMV, two viruses unrelated to RSV, also reduce the capacity of pulmonary memory CD8 T cells to produce IFN-γ and TNF-α following direct ex vivo peptide stimulation. Interestingly, we did not observe a decrease in IFN-γ production by LCMV-specific CD8 T cells recovered from the lung parenchyma during the peak of the acute CD8 T-cell response at day 8 p.i. These data suggest that acute LCMV infection may alter the lung inflammatory environment or modulate the capacity of pulmonary APC to stimulate CD8 T cells. LCMV infection may recruit or preferentially activate an APC population that is not present early after either RSV or VACVM2 infection. Thus, virus-induced inflammation can override the regulatory lung environment to stimulate the production of cytokines by pulmonary T cells. However, the effect of acute LCMV infection is transient, because by day 30 p.i., LCMV-specific CD8 T cells recovered from the lung fail to produce IFN-γ following direct ex vivo peptide stimulation. This suggests that, following resolution of the virus infection, the lung environment returns to its preinfection state with regard to regulating the capacity of antigen-specific T cells to produce cytokines.

Using SMARTA CD4 T cells, we demonstrated that, like CD8 T cells, CD4 T cells that enter the lung parenchyma also exhibit a diminished capacity to produce cytokines following

transferred i.v. into naïve Thy1.2$^+$ C57BL/6 mice, which were then allowed to rest for 7 days. (C and D) Spleens and lungs were then harvested, stimulated with or without peptide, and stained for IFN-γ. The cells in panels A and C were gated on donor CD8$^+$ Thy1.1$^+$ P14 T cells. The boldface numbers represent the percentages of IFN-γ$^+$ CD8$^+$ P14 T cells. The data are representative of two separate experiments with two mice per experiment. *, $P < 0.05$. The data in panel D represent the mean plus standard error of the mean from two separate experiments.

direct ex vivo peptide stimulation. The inability of virus-specific memory T cells to produce cytokines after entry into the lung could be due to active suppression or to a lack of optimal stimulation. To address the possibility that pulmonary APC were not supplying sufficient activation signals during direct ex vivo peptide stimulation, we used exogenous peptide-pulsed APC to stimulate virus-specific T cells. We were largely able to rescue cytokine production by virus-specific memory T cells located in the lung parenchyma at 30 days after infection with each of the viruses we examined (Fig. 3 and data not shown). Importantly, these results demonstrate for the first time that T cells in the lung parenchyma are not irreversibly impaired. Moreover, these data suggest that endogenous peptide-pulsed pulmonary APC are not able to stimulate ex vivo cytokine production by virus-specific T cells as adequately as the endogenous APC present in the spleen. In contrast to the lung parenchyma, stimulation with exogenous peptide-pulsed APC had little effect on cytokine production by virus-specific T cells obtained from BAL fluid, suggesting that T cells in the BAL fluid might be more functionally impaired than T cells in the lung parenchyma. This concept would be consistent with previous work demonstrating that cells that have migrated into the airways progressively lose various effector functions over time (40). Interestingly, when we examined IFN-γ protein production 48 h following in vitro stimulation with either exogenous peptide or peptide-pulsed exogenous APC, we observed an even more profound deficiency in cytokine production compared to our earlier results obtained using short-term ICS assays (Fig. 5 versus Figure 4A). These results may further support the concept that the capacity of pulmonary-virus-specific T cells to produce cytokines following antigenic stimulation is proportional to the amount of time that they have been exposed to the lung environment (1). The regulation of cytokine production by antigen-specific T cells is not limited to the lung, as our analysis of a number of peripheral tissues 30 days post-LCMV infection revealed that virus-specific CD8 T cells that had migrated into other tissues also failed to produce cytokines following direct ex vivo peptide stimulation (Fig. 8). We believe our findings have important consequences for the measurement of virus-specific T-cell responses using only functional readouts because the true virus-specific frequency will be underestimated. More importantly, our results demonstrate that the amounts of underestimation of the antigen-specific T-cell response will differ greatly depending on the tissue being analyzed.

It is currently unclear how the lung parenchyma and lung airways differentially regulate the effector functions of antigen-specific T cells. Previous studies have demonstrated that as virus-specific memory CD8 T cells traffic into the lung airspace (i.e., BAL fluid), they decrease their cell surface expression of CD62L (21) and LFA-1 (13), two proteins that have been shown to be important in both T-cell trafficking and activation (18). For example, LFA-1 is important in the formation of immunological synapses between T cells and APC or target cells (35). Thus, it is possible that the reduced expression of integrins and/or costimulatory molecules on CD8 T cells in the BAL fluid may further limit the ability of these cells to be efficiently stimulated to produce cytokines. In contrast to the BAL fluid, it appears to be easier to rescue cytokine production by antigen-specific CD8 T cells in the lung parenchyma. Importantly, stimulation with exogenous APC did not fully rescue IFN-γ production by all of the antigen-specific CD8 T cells isolated from the lung parenchyma (Fig. 3). Thus, the failure of CD8 T cells recovered from the lung parenchyma to produce cytokines following direct ex vivo peptide stimulation does not appear to be solely a consequence of inadequate stimulation. In contrast, stimulation of TCR-transgenic P14 CD8 T cells with exogenous peptide-pulsed APC significantly rescued IFN-γ production by cells recovered from the BAL fluid and lung parenchyma (Fig. 4). These disparities may be a result of tetramer staining that did not detect all of the antigen-specific CD8 T cells or a consequence of using an oligoclonal TCR-transgenic T cell population compared to a polyclonal endogenous T-cell population.

Active suppression of T cells by regulatory cytokines or through the actions of regulatory cells, such a T-regulatory cells or alveolar macrophages, may also potentially explain our results (5, 6, 19, 23, 27). To further support the regulatory role of local tissue environments in immune responses, we showed, using an adoptive-transfer system, that in the absence of respiratory-virus infection, functional spleen-derived P14 CD8 T cells were reduced in their ability to produce cytokines when they entered the lung parenchyma of naïve mice but not when they migrated back into the spleen (Fig. 7). Importantly, we also showed within the same experiment that P14 CD8 T cells isolated from the lung parenchyma that were reduced in their ability to produce cytokines following direct ex vivo peptide stimulation regained their capacity to produce cytokines when they trafficked into the spleen. These results demonstrate a direct role for tissue-specific regulation of cytokine production by CD8 T cells in the absence of infection.

Only recently has research begun to suggest that tissue microenvironments play an important role in regulating the adaptive immune response. One clear benefit to regulating the adaptive immune response in peripheral tissues, such as the lung, would be to prevent tissue injury and preserve normal tissue function during an inflammatory immune response. Understanding how tissues regulate adaptive T-cell responses will provide us with novel therapeutic approaches to modulate immune responses and to prevent tissue damage caused by immunopathology.

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