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_J Immunol_ 2014; 192:5415-5425; Prepublished online 18 April 2014;
doi: 10.4049/jimmunol.1400256
http://www.jimmunol.org/content/192/11/5415

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/04/18/jimmunol.1400256.DCSupplemental

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CD8 T Cells Use IFN-γ To Protect against the Lethal Effects of a Respiratory Poxvirus Infection

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CD8 T cells are a key component of immunity to many viral infections. They achieve this through using an array of effector mechanisms, but precisely which component/s are required for protection against a respiratory orthopox virus infection remains unclear. Using a model of respiratory orthopox virus infection in mice, we could specifically determine the relative contribution of perforin, TRAIL, and IFN-γ-mediated pathways in protection against virus-induced morbidity and mortality. Unexpectedly, we observed that protection against death was mediated by IFN-γ without any involvement of the perforin or TRAIL-dependent pathways. IFN-γ mRNA and protein levels in the lung peaked between days 3 and 6 postinfection. This enhanced response coincided with the emergence of virus-specific CD8 T cells in the lung and the cessation of weight loss. Transfer experiments indicated that CD8 T cell–autonomous expression of IFN-γ restricts virus-induced lung pathology and dissemination to visceral tissues and is necessary for clearance of virus. Most significantly, we show that CD8 T cell–derived IFN-γ is sufficient to protect mice in the absence of CD4 and B-lymphocytes. Thus, our findings reveal a previously unappreciated mechanism by which effector CD8 T cells afford protection against a highly virulent respiratory orthopox virus infection. The Journal of Immunology, 2014, 192: 5415–5425.

Orthopox viruses (OPXV) are a family of large linear dsDNA viruses that replicate in the cytoplasm of cells and demonstrate a high degree of antigenic similarity (as reviewed in Ref. 1). Several OPXV can cause clinical disease in humans, such as variola virus (VARV) (2), monkeypox (3, 4), cowpox (5), and vaccinia virus (VACV) (6). VACV, the etiological agent of human smallpox, is highly infectious by aerosol or microdroplet transmission in a susceptible population, with a case mortality rate of up to 30–40% (2). Infected individuals generally experience a sudden onset of flu-like symptoms such as fever, malaise, headache, backache, and, in some cases, vomiting (2). Bronchopneumonia is considered the most frequent and serious complication of the disease and often the cause of death (2). The clinical features of human monkeypox virus are similar to those of VARV, but the disease is milder and produces a lower case mortality rate (2, 4). Many aspects of a respiratory OPXV infection remain unknown, and further research on the disease and immune correlates of protection are urgently needed.

In mice, intranasal (i.n.) infection with the highly virulent mouse-adapted Western Reserve strain of VACV (VACV-WR) causes striking local and systemic changes that in many respects resemble human VARV infection and has been used as a surrogate model for other highly pathogenic respiratory virus infections (7–12). Initial VACV-WR replication is thought to occur in the respiratory epithelium and alveolar macrophages before the development of a secondary viremia that disseminates the virus throughout the host (2, 13, 14). A sublethal respiratory infection with VACV-WR leads to marked increases in the number of cells recovered from lung and draining lymph nodes and a rapid accumulation of IFN-γ–producing CD8 and CD4 T cells in the lung parenchyma (10, 12). Previous studies of the immune response to a primary respiratory VACV-WR infection have established an important role for monocytes/macrophages (15) and dendritic cells (16) in protection. Our own studies have focused on T and B cells and have investigated their relative contribution to viral clearance and recovery from disease. Surprisingly, we found that absence of CD4 T cells does not substantially modify either survival or virus clearance from the respiratory tract (12). Likewise, virus-specific B lymphocytes can make neutralizing Abs against VACV-WR following a respiratory infection, but this occurs too late in the primary response to limit virus replication and dissemination. Instead, we found that VACV-specific CD8 T cells are needed for early clearance of virus from the lung and protection against death (12). However, exactly which effector mechanisms lung-infiltrating VACV-reactive CD8 T cells employ was not established.

VACV has evolved elaborate strategies that counteract many of the innate and adaptive immune responses of the host (reviewed in Ref. 17). Significantly, many VACV-encoded proteins target and disrupt components of the host's viral detection and response mechanisms, including the activity of host cytokines. In particular, VACV genes E3L and K3L encode intracellular proteins that block IFN-induced inhibition of protein synthesis by inhibiting both the activation and function of RNAse L (18, 19) and the dsRNA-activated kinase protein kinase R, respectively (19–21). In addition, the release of a VACV-encoded soluble receptor analog for the IFN-γ receptor (IFN-γR) B8R also inhibits host IFN-γ signaling (22, 23). B8R functions as a soluble decoy receptor by inhibiting the binding of IFN-γ to the cell surface–expressed IFN-γRs. Significantly, VACV deletion mutants that lack E3L (24), K3L (19), or B8R (22,
25–27) are variably attenuated in normal mice following respiratory infection with VACV, providing direct evidence for an important role for IFN signaling in anti-VACV immune responses. However, it remains unknown how IFN-γ alters the course and magnitude of the antiviral response in the lung and whether VACV-specific CD8 T cells use IFN-γ to protect against the lethal effects of respiratory infection with VACV.

Following a sublethal respiratory VACV-WR infection, we found that IFN-γ signaling restricts lung pathology and virus dissemination to visceral tissues and is necessary for early clearance of virus and protection against death. Interestingly, the accumulation of VACV-reactive CD8 T cells in the lung and their expression of cytolytic effector molecules (perforin, granzyme, FAS ligand [FASL], and TRAIL) or their ability to degranulate was not altered when IFN-γ activity was neutralized in vivo. Transfer experiments demonstrated that CD8 T cells primarily use IFN-γ to protect mice against the lethal effects of a respiratory infection with VACV-WR. These experiments provide compelling evidence in support of the idea that OPXV may have evolved strategies to target IFN-γ in part to subvert the primary effector mechanism of antiviral CD8 T cell responses in the respiratory tract.

Materials and Methods

Mice

The studies reported in this paper conform to the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in the biomedical research. All experiments were completed in compliance with the regulations of the University of Florida Animal Care Committee and in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Eight- to 12-wk-old female wild-type (WT) C57BL/6j, IFN-γ-deficient (IFN-γ−/−), IFN-γR-deficient (IFN-γR−/−), and RAG−/− mice were purchased from The Jackson Laboratory.

VACV and stock preparation

VACV-WR was purchased from the American Type Culture Collection, grown in HeLa cells, and subsequently titered on VeroE6 cells as described previously (28).

Respiratory VACV-WR infection model

Naive mice were anesthetized by isoflurane inhalation and infected i.n. with 1.25 × 107 PFU or i.p. with 2 × 105 PFU VACV-WR, with daily measurements of body weight, lung pathology, and viral titers [as described before (12, 29)]. No animals were allowed to die of natural causes; therefore, the time of death indicated on the survival curves is the time at which an animal was euthanized due to severe disease (weight loss of >25%). Body weight was calculated as percentage of the mean weight for each mouse on the day of challenge. Tissues were either used for single-cell isolation or weighed as percentage of the mean weight for each mouse on termination of the experiment.

VACV-WR titer assay

After VACV-WR infection, specific tissues from individual mice were homogenized and sonicated for 1 min with a pause every 10 s using an ultrasonic cleaner (1210 Branson). Serial dilutions were made, and virus titers were determined by plaque assay on confluent VeroE6 cells (31).

IFN-γ ELISA

Total lung protein was isolated and placed immediately into tissue protein extraction reagent (T-PER; Pierce) containing a mixture of protease inhibitors according to the manufacturer’s instructions (cOmplete; Roche). Following tissue disruption, lung tissue supernatant was serially diluted and analyzed according to the manufacturer's instructions (cOmplete; Roche). Following an enrichment of VACV-specific FACS buffer (PBS, and 2% FCS) and subsequent titration on VeroE6 cells, the supernatants were tested for the presence of VACV protein by plaque assay on confluent VeroE6 cells (31).

Flow cytometric analysis

All tissues were aseptically removed from euthanized mice, and single-cell suspensions were prepared by mechanically dispersing the tissues through 70-μm cell strainers (Falcon BD Labware) into HBSS. Lung tissue was treated for 1 h at 37°C with 250 μg Collagenase D (Roche) followed by treatments for 20 min at 4°C with 100 μM EDTA-supplemented media. Following RBC lysis (Sigma-Aldrich), cells were resuspended in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Omega Scientific), 1% l-glutamine (Invitrogen), 100 μg/ml streptomycin, 100 U/ml penicillin, and 25 mM HEPES and enumerated using a BD automated Vicell counter (BD Biosciences).

T cell subsets. Cells were washed with FACS buffer (PBS and 2% FCS) and stained with anti-Flc/II/III receptor mAb 2.4G2 for 15 min at 4°C. An additional 1% FACS buffer with the following Abs washed, incubated with the Abs above, and finally stained with the following Abs: CD45R0 (XM1.2; eBioscience) and anti-CD49d (BD Pharmingen) followed by fixation with Cytofix/Cytoperm (BD Biosciences). These were then added to the cultures according to the manufacturer’s instructions, and the incubation was continued for 2 h. Cells were then stained with anti-CD3 (53-6.7, BD Pharmingen) and anti-CD62L (ME-14, BD Pharmingen) followed by fixation with Cytofix/Cytoperm (BD Biosciences) for 20 min at 4°C. Fixed cells were subjected to intracellular cytokine staining in perm/wash buffer (BD Biosciences) for 30 min at 4°C. Cells were stained with anti-IFN-γ (XMG1.2; eBioscience) and anti–TNF-α (MP6-XT22; eBioscience) for 30 min at 4°C. Samples were analyzed for their proportion of cytoplasmic cytokines and surface expression of CD107a after gating on CD8 CD62L-low T cells by an FACSCanto II flow cytometer (BD Biosciences) using FlowJo software (Tree Star).

Immunofluorescence studies

At various times p.i., VACV-infected lungs were inflated with 500 μl 50% OCT/PBS embedding solution and immediately snap frozen on dry ice. The 7-μm thick cryosections of OCT-embedded lung were cut by a Microtome HM 505E cryostat (Microm International) and prepared on super frost glass slides for immunofluorescence microscopy. Each cryosection slide was washed with 1 ml PBS, fixed with 4% paraformaldehyde for 15 min at 4°C, and subsequently permeabilized with 0.1% Triton X-100 for 5 min at 4°C. The sections were washed with cold PBS and incubated overnight in the dark at 4°C with rat anti-mouse CD8 allopolyocycin (clone 53-6.7, dilution 1:100; BD Pharmingen) and ActinGreen (Molecular Probes, as directed by provided protocol). Sections were washed three times with PBS, mounted with cyanoseal, and covered with glass coverslip. The stained sections were observed and analyzed at a wavelength of 488 nm for FITC (green) and 647 nm for allophycocyanin (magenta) labeling. Using an EVOS f1 Advanced Microscopy System (with inverted immunofluorescence microscope; Fisher Scientific), images were captured by ×20 and ×40 objectives, keeping all of the conditions of microscope and settings of software identical for all treatments and controls.

In vivo IFN-γ neutralization

Groups of VACV-WR–infected mice were neutralized of IFN-γ using an anti–IFN-γ Ab (clone XMG1.2; 200 μg/mouse) given in one i.v. injection 3 d before and i.p. injections on days −1 and every 3 d thereafter until the termination of the experiment.

In vivo naive CD8 T cell transfer

Naive CD8 T cells (CD3+CD8+CD44+low) were isolated from naïve WT C57BL/6j or IFN-γ−/− mice. Briefly, spleens were homogenized to a single-cell suspension as described above, and anti-CD8 (clone 2D10B4, Miltenyi Biotec) were subsequently added following the manufacturer’s instructions. Following CD8 T cell MACS column enrichment, the naive CD8 T cells were further purified using CD3+CD44+low populations and FACS
sorted with a BD Aria (BD Biosciences). Subsequently, $5 \times 10^6$ naive polyoma CD8 T cells/mouse was transferred into age-matched RAG$^-$/$-$, IFN-γ$^-/-$, and IFN-γR$^-/-$ mice via the retro-orbital plexus.

**RNA extraction and gene expression analysis**

**Inflammatory gene arrays.** Total lung RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA was subsequently treated with DNase I (Qiagen) and further purified using an RNeasy Mini Kit (Qiagen). A total of 1 μg high-quality total RNA (RNA integrity number >7) was then reverse transcribed using the First Strand Synthesis Kit (Qiagen) and subsequently loaded onto either an IFN and receptors or an inflammatory cytokine and receptor RT$^+$ profiler array according to the manufacturer’s instructions (Qiagen). Qiagen’s online Web analysis tool was used to produce comparative heat maps, and fold change was calculated by determining the ratio of mRNA levels to control values using the Δ threshold cycle (Ct) method ($2^{\Delta \text{Ct}}$). All data were normalized to an average of five housekeeping genes, Gusb, Hprt, Hsp90ab1, Gapdh, and Actb. PCR conditions used: hold for 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C.

**Real-time PCR analysis.** Total RNA from day 7 lung purified BSR tetramer$^+$ CD8 T cell (CD3+, CD8+, CD44$^+$) was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA was treated with DNase I (Qiagen) and further purified using an RNeasy Mini Kit (Qiagen) and was treated with DNase I (Qiagen) and further purified using an RNeasy Mini Kit (Qiagen). A total of 2 μg total lung or 200 ng CD8 T cell RNA was reverse transcribed using the Super Script III system (Invitrogen). Up to 1 ng cDNA was then amplified by real-time PCR using primers for Ifng (forward: 5′-AACGTCATACACTGATCCTTG-3′; reverse: 5′-GCGGTCGGTGTGGATCTCTCAAGG-3′), Fasl (forward: 5′-TCCCTGAGGTTCACACCCACCAACCAACAA-3′; reverse: 5′-GGGGGTCTCCCGTAAAAATGGGGT-3′), Trail (forward: 5′-ATGATGTTGGATCTCATGCTC-3′; reverse: 5′-AGCTGCTTCACCTGATGCTGGT-3′), granzyme B (forward: 5′-CCACTTCACCTCAGCTTACCAAGGG-3′; reverse: 5′-GGCTTCCCTGAGTACACCTGCTG-3′), perforin (forward: 5′-CAAGGTAGCCAATTTTGCAGC-3′; reverse: 5′-GTATCATGGGATCCCCAATTTTGCAGC-3′), or GAPDH (forward: 5′-AGGTCGGTGTGGATCTCTCAAGG-3′; reverse: 5′-TGTAGACCATCTGATGTTAGGAGG-3′) as internal housekeeper controls for normalization. Each sample was run in a 1:10 reaction using SYBR green PCR Master Mix (Roche). Reactions were performed in a Roche Light Cycler 480 (Roche). Ratios of mRNA levels to control values were calculated using the ΔCt method ($2^{\Delta \text{Ct}}$). All data were normalized to the housekeeper control genes L32 and GAPDH. PCR conditions used: hold for 5 min at 95°C, followed by 45 cycles of 10 s at 95°C, 10 s at 55°C, and 10 s at 72°C.

**Statistical analysis**

Tests were performed using Prism 5.0 (GraphPad, San Diego, CA). Statistics were done using an two-tailed, unpaired Student $t$ test with 95% confidence intervals unless otherwise indicated. Two-way ANOVA was used to determine differences in weight loss profiles, and the Mantel–Cox test was used for survival analysis. Unless otherwise indicated, data represent the mean ± 1 SEM, with $p < 0.05$ considered statistically significant.

**Results**

**IFN-γ restricts virus dissemination and promotes survival following a respiratory VACV infection**

Recently, we showed the ability of CD8 T cells to act in the first 3–6 d after a respiratory VACV-WR infection to reduce early viral titers in the lung and protect mice against death (12). We sought in this study to define the mechanism of this protection by focusing on IFN-γ. To determine whether a deficiency in IFN-γ signaling has a direct effect on recovery from a respiratory VACV-WR infection, cohorts of WT, IFN-γ$^-/-$, and IFN-γR$^-/-$ mice were infected with a sublethal i.n. dose of VACV-WR, and the rate of survival of these animals was observed over a period of 12 d. WT mice infected via this route and dose elicited a transient weight loss (Fig. 1A) with modest outward signs of illness that peaked 7 d postinfection (p.i.). All mice began to recover from disease starting at day 7, returning to their original mass by day 12 p.i. (Fig. 1A). The initial weight loss in IFN-γ$^-/-$ and IFN-γR$^-/-$ mice was comparable to WT mice (Fig. 1A, 1B); however, progressive weight loss and illness resulted in 100% mortality by day 8 p.i.

The severity of infection observed in IFN-γ$^-/-$ mice was also supported by histopathological analysis of lung sections. As shown, IFN-γ$^-/-$ mice developed a prominent inflammatory infiltrate, characterized by mononuclear inflammation in perivascular and peribronchial areas, along with extensive bronchial epithelial hyperplasia and necrosis. The lesion characteristics were consistent with a diagnosis of moderate to severe viral bronchopneumonia (Fig. 1C, bottom panel). In striking contrast, lung sections from WT mice had far fewer infiltrating cells around the bronchioles and blood vessels and relatively normal bronchial epithelium (Fig. 1C, top panel). IFN-γ$^-/-$ mice also failed to contain initial viral titers in the lung and displayed signs of viral dissemination, as determined by the significant levels of infectious virus in their heart, liver, and ovaries (Fig. 1D).

To determine if IFN-γ is required for survival in other less virulent VACV-WR infection models, WT and IFN-γ$^-/-$ mice were infected i.p. with $2 \times 10^7$ PFU of VACV-WR. As before, the extent of disease as measured by weight loss was monitored over a 2-wk period. Quite significantly, at the peak of infection, IFN-γ$^-/-$ mice experienced moderate weight loss (5–10%) but quickly recovered and went on to maintain normal weight after day 10 p.i. (Fig. 1E and data not shown). This was despite being infected with 16-fold higher inoculum of virus as compared with the i.n. model of infection. Eight days p.i., IFN-γ$^-/-$ mice had elevated titer of infectious virus in their ovaries as compared with WT controls, which was subsequently cleared by day 14 p.i. (Fig. 1F and data not shown). Collectively, these data suggest that IFN-γ plays a more prominent role in protection against disease and death following a respiratory VACV-WR infection.

**IFN-γ is highly upregulated in the lung between days 3 and 6 p.i. with VACV**

Next, we assessed the kinetics of IFN-γ gene expression in the WT lung and compared it to several key inflammatory mediators implicated in antiviral immunity. Total lung RNA was isolated from WT mice on days 3, 6, and 9 post–i.n. VACV-WR infection. Using a preformatted gene pathway array, we compared the expression of 84 inflammatory chemokine, cytokine, and IL receptor genes against naive levels. After disregarding unregulated, nondetectable gene products and presenting the relative levels of gene expression across all samples, we identified 26 inflammatory genes that categorized four temporally distinct groups (group [G] 1–4) (Fig. 2A). G1 consisted of seven inflammatory genes that were expressed during homeostasis before being downregulated over the course of the infection. These included the chemokine ligands Ccl2, Ccl12, and Cxcl5 and the chemokine receptor Cxcr5 (Fig. 2A). Collectively, these genes have been implicated in the recruitment of lymphocytes, dendritic cells, neutrophils, and monocytes into the lung parenchyma and in the initiation of acute inflammatory responses, G2 comprised a set of chemokines, inflammatory cytokines, and receptor genes that progressively increased in expression over the course of infection (Fig. 2A). A number of these genes, Ccl6, Il-1β, Tnf, and Cxcr2, have been associated with the potentiation of inflammatory responses by inducing the expression of additional chemokines, activating the endothelial microvasculature to assist neutrophil extravasation, and augmenting the production of several proteases that allow infiltrating cells to gain access to the site of infection. The inclusion of Il-2r and Il-2r$^-/-$ mice with VACV-WR infection.
mean percent survival (A) infected with 2 representative WT and IFN-R mice were i.n. infected with a sublethal inoculum of VACV-WR (1.25 × 10^6 PFU/mouse). The animals were weighed daily and euthanized if weight loss was >25% of their original body weight for 2 consecutive d. Percentage of initial body weight [(A), (B), left panels] and mean percent survival [(A), (B), right panels] from the indicated numbers of mice are presented. Weight loss data are presented as the mean ± SEM of three separate experiments containing four to eight mice per group and analyzed using a two-way ANOVA to determine statistical significance. Survival data use combined survival data across several experiments using the Mantel–Cox test. (C) Day 7 postinfection lung sections stained with H&E from representative WT and IFN-γ−/− mice (original magnification ×20). (D) On day 7 post-VACV-WR infection, viral titers (PFU) were determined in the indicated tissues. Students t test with Bonferroni correction was used to determine viral titer statistical significance. WT and IFN-γ−/− mice were also i.p. infected with 2 × 10^5 PFU VACV-WR and followed for weight loss (E) and titers for virus (F) on day 8 p.i. **p < 0.01.

monocyte and T lymphocyte populations at sites of infection. Similar to that observed in G2, the expression of Cxcr3 and Itgb2, two receptors important for T cell migration, and Ifng, an inflammatory cytokine, correlated with the large numbers of CD8 T cells present in the lung between days 6 and 9 following an i.n. VACV-WR infection. Finally, G4 described two genes, namely Ccl11 and Cxcl13, which were transiently expressed early in the infection before returning to homeostatic levels by day 6 p.i. (Fig. 2A). These two chemokines play important roles in the recruitment of T lymphocytes, monocytes, and B cells as well as possibly reflecting a germinal center origin of T cells.

To further interrogate the array data and determine the genes most highly regulated, we measured the fold change in gene expression between days 3 and day 0 (naive), days 6 and 3, and day 9 and 6 p.i. (Fig. 2B). This data representation revealed that IFN-γ was the most highly regulated gene across all time periods and was upregulated >10-fold between days 3 and 6 p.i. (Fig. 2B, middle panel). Notable other genes that demonstrated high fold changes include: Cxcl13 between days 0 and 3; Il2rb, Ccl5, and Cxcr3 between days 3 and 6; and Ccl2, Ccl7, and Cxcr3 between days 6 and 9 p.i. (Fig. 2B, left, middle, and right panels, respectively). To further validate these findings, we measured both the mRNA and total lung protein levels of IFN-γ at several time points in a separate experiment (Fig. 2C, 2D, respectively). Analogous to the array data, the relative expression of IFN-γ mRNA increased dramatically after day 3 and peaked between days 6 and 10 p.i. before returning to near baseline values by day 14 (Fig. 2C). In order to determine that the mRNA profile reflected protein expression, an ELISA for IFN-γ was performed on supernatants of homogenized lung tissue. Consistent with the mRNA data, levels of IFN-γ protein followed a similar kinetic, with the highest concentration detected between days 3 and 10 p.i. (Fig. 2D). Therefore, by using several independent methods, we have identified IFN-γ as one of the most highly upregulated genes in the lung during a respiratory VACV-WR infection. Most significantly, the kinetics of IFN-γ production paralleled the clearance of virus from the lungs of infected mice and the cessation of weight loss.

An early IFN-γ response is necessary for protection against a respiratory VACV infection

We next wanted to determine the precise time at which IFN-γ signaling provides its protective function. To achieve this, we performed kinetic blocking experiments in which WT mice received anti–IFN-γ neutralizing Ab starting on day −1, 3, or 6 p.i. (Fig. 3A). Mice depleted of IFN-γ throughout the course of infection or from day 3 onward failed to control VACV-WR infection, which resulted in 100% mortality by day 8 p.i. (Fig. 3A, right panel). Both groups of mice presented with similar lung pathology (not shown) and viral dissemination to that observed in IFN-γ−/− and IFN-γR−/− mice (compare Figs. 3B and 1D). In contrast, mice depleted of IFN-γ from day 6 p.i. displayed comparable weight loss recovery and survival profile to those observed in isotype-treated (Ig) control mice (Fig. 3A, left and right panels).
IFN-γ−/− mice develop normal VACV-specific CD8 T cell responses in the lung

Because both CD8 T cells (12) and IFN-γ are required during the same phase of the anti-VACV-WR response in the lung, we reasoned that early IFN-γ production might be important for priming, differentiation, and/or recruitment of virus-specific CD8 T cells.

We thus performed a number of phenotypic and functional analyses to quantitate the extent of CD8 T cell priming and recruitment in the absence of functional IFN-γ signaling. Analyses of virus-induced total (CD8) and activated (CD44high) CD8 T cells in IFN-γ−/− (Fig. 4A) and IFN-γR−/− (Supplemental Fig. 1A) mice showed no significant difference compared with WT controls. To assess induction of virus-specific CD8 T cells, the immunodominant VACV-reactive population was tracked with a tetramer containing the B8R (20–27; TSYKFESV) peptide (32, 33) or through the production of IFN-γ and TNF following stimulation with the B8R peptide epitope. Again, at the peak of the acute infection, the total number, percentage, and proliferative capacity (Ki67+) of B8R-tetramer–reactive CD8 T cells in the lung and draining lymph nodes (not shown) were comparable among WT, IFN-γ−/− (Fig. 4A, 4B, and 4C, left panels), and IFN-γR−/− (Supplemental Fig. 1B, left panel) mice. The majority of WT B8R-specific CD8 T cells produced IFN-γ, with a minor population that produced TNF only (Fig. 4B, Supplemental Fig. 1B, right panel). The B8R-specific CD8 T cells that produced IFN-γ could be divided into two subsets based on the TNF cytokine pattern: IFN-γ− only (single producers) and IFN-γ− TNF-high (double producers). As expected, IFN-γ−/− mice were completely devoid of B8R-specific CD8 T cells that were capable of producing IFN-γ, whereas IFN-γR−/− mice contained comparable frequency of IFN-γ− CD8 T cells to WT (Supplemental Fig. 1B, right panel).

FIGURE 2. Inflammatory gene expression profile of VACV-WR–infected lung tissue. WT C57BL/6J mice were i.n. infected with VACV-WR (1.25 × 106 PFU/mouse). Total lung mRNA transcript levels of 26 inflammatory genes were measured at days 0 (naïve), 3, 6, and 9 p.i. and presented as a heat map (A) representing the relative value of the gene expression across all samples. G1–G4 denote four temporally distinct groups of genes. (B) Fold change in gene expression between day 3 and naïve, days 6 and 3, and days 9 and 6 p.i. is also represented. All array data are presented as the average gene expression level of four combined mice per group at each specified time point analyzed. At the indicated time points p.i. with VACV-WR, lungs were collected and assayed independently for total IFN-γ mRNA levels (C) and IFN-γ protein levels (D) using ELISA. Data are presented as the mean ± 1 SEM of two independent experiments with three to five mice per group. Students t test with Bonferroni correction was used to determine statistical significance compared with day 0 levels. **p < 0.01.

FIGURE 3. Early IFN-γ signaling is required for survival following a respiratory VACV-WR infection. WT C57BL/6J mice were i.n. infected with VACV-WR (1.25 × 106 PFU/mouse) and survival (A, left panel) and viral titers (A, right panel) were monitored in WT mice that were continuously treated with a neutralizing IFN-γ Ab, or isotype control, starting 1 d before infection (day −1) or 3 or 6 d post–i.n. infection with VACV-WR. (B) On day 8 p.i., viral titers were measured in mice in which IFN-γ had been neutralized from the outset of infection. Data are presented as the mean ± 1 SEM of two independent experiments with three to five mice per group. Survival data use combined survival data across combined experiments using the Mantel–Cox test. **p < 0.01.
localization of CD8 T cells around the peribronchial regions in WT, IFN-\(\gamma\)-/-, and IFN-\(\gamma\)R-/- mice. In naive WT and IFN-\(\gamma\)-/- mice, CD8 T cells were sporadically dispersed in the lung parenchyma. Notably, only a few cells were seen in close proximity to the airways in the peribronchial areas of the lung (Fig. 4D–F, top two panels). In striking contrast, during the acute phase of infection, high numbers of CD8 T cells could be readily visualized around the large airways, many of which were in direct contact with airway epithelial cells (Fig. 4D, 4E, bottom two panels). This was particularly evident in IFN-\(\gamma\)-/- (Fig. 4D, 4E, bottom two panels) and IFN-\(\gamma\)R-/- mice (Supplemental Fig. 1D). Therefore, absence of IFN-\(\gamma\) signaling does not impact the localization of virus-specific CD8 T cells to the peribronchial regions of the lung.

**A limited role for perforin- and TRAIL-mediated cytotoxicity in resistance to respiratory VACV infection**

Differential of CD8 T cells in response to recognition of viral Ags also leads to the expression of effector molecules perforin and GrzB, which can be used by CD8 T cells to kill virus-infected cells (35, 36). In addition, previous studies have identified a role for perforin (37), FAS/FASL (37) and TRAIL (38, 39) expressing CD8 T cells in antiviral immunity in the lung. Therefore, to study further the relationship between maturation and cytolytic function, we assessed whether expression of cytolytic effector molecules by VACV-specific CD8 T cells was impacted by a lack of IFN-\(\gamma\) signaling. On day 7 post–VACV-WR infection, cell-surface (Fig. 5A, 5B) or mRNA levels (Fig. 5C) of FASL, TRAIL, CD107\(\alpha\) (a marker of degranulation), GrzB, and perforin were analyzed on FACS-sorted lung-resident CD3\(^+\)CD8\(^+\)CD44\(^{low}\)B8R-tetramer\(^+\) cells. Naive, CD3\(^+\)CD8\(^+\)CD44\(^{high}\)B8R-tetramer\(^+\) cells were used as control. Consistent with our TNF expression data (Fig. 4C), we found that in the absence of IFN-\(\gamma\) signaling, virus-specific CD8 T cells expressed higher surface levels of FASL and TRAIL (Fig. 5A), but other cytolytic indicators including CD107\(\alpha\) (Fig. 5A, 5B), perforin, and GrzB were comparable to WT cells (Fig. 5C). This showed that several direct cytolytic pathways of CD8 T cells were intact in the absence of functional IFN-\(\gamma\) signaling.

Previous work has demonstrated that mice that have inactivating mutations in FAS (B6-lpr) are resistant to respiratory virus infections (40, 41). Therefore, to address the role of perforin- and TRAIL-mediated cytotoxicity, we i.n. infected perforin-/- (Fig. 5D) and TRAIL-/- (Fig. 5E) mice with VACV-WR and monitored their weight loss over time. In three independent experiments, perforin-/- and TRAIL-/- mice displayed comparable weight loss and recovery profiles to those observed in WT mice. The lack of requirement for FAS-, TRAIL-, and perforin-dependent pathways in resistant to respiratory VACV-WR infection, combined with the normal expression of these molecules on VACV-specific IFN-\(\gamma\)-/- CD8 T cells.

**FIGURE 4.** CD8 T cell priming is quantitatively and qualitatively analogous in WT and IFN-\(\gamma\)-/- mice. WT C57BL/6J and IFN-\(\gamma\)-/- mice were i.n. infected with 1.25 \(\times\) 10\(^4\) PFU of VACV-WR. On day 8 p.i., total lung cells were stained with CD3\(^+\), CD8\(^+\), CD44\(^{low}\) B8R tetramer, intracellular Ki67, or stimulated with B8R peptide and subsequently stained for intracellular IFN-\(\gamma\) and TNF-\(\alpha\). Total numbers of CD3\(^+\)CD8\(^+\), CD3\(^+\)CD8\(^+\)(CD44\(^{high}\), B8R tetramer–positive T cells (A), relative frequency of CD3\(^+\)CD8\(^+\)(CD44\(^{high}\) B8R tetramer– and Ki67-positive cells (B), left and middle panels, respectively), and representative plots for cytokine staining (B, right panel), gated on CD8\(^+\)(CD62L\(^{low}\) cells, are indicated. Total numbers of day 8 lung CD8\(^+\) TNF-producing CD8 T cells (C) are also quantified. Quadrant settings were based on controls using infected cells that were not stimulated with peptide or stained with B8R tetramer and uninfected lung cells stimulated with B8R peptide (data not shown). Results are mean number + SEM (\(n = 4\) mice/group) from one experiment. Similar results were obtained in three separate experiments. In situ CD8 T cell location (peribronchial and parenchymal) was also determined in naive and day 8 infected OCT-embedded lung sections fluorescently stained for actin (green) and CD8\(^+\) (magenta) (D–F).
implies that CD8 T cells likely use IFN-γ to protect against the lethal effects of infection.

CD8 T cell–derived IFN-γ is necessary and sufficient for protection against a respiratory VACV infection

To seek direct evidence on whether CD8 T cell–derived IFN-γ was important for viral protection, we employed three separate and complimentary strategies. In our first approach, highly purified naive polyclonal WT or IFN-γ−/− CD8 T cells were adaptively transferred into IFN-γ−/− mice deficient in T and B cells (RAG−/−) and compared with nontransferred WT controls (Fig. 6A). We used RAG−/− as recipients to prevent any endogenous T cell response. An increased number of CD8 T cells were transferred to limit homeostatic proliferation that is common in lymphopenic environments, allowing us to examine the VACV-induced response in a nontransgenic system. Twenty-four hours later, these mice, along with two groups of control mice (RAG−/− and WT mice with no CD8 T cell transfer), were infected i.n. with VACV-WR and monitored for signs of illness and survival (Fig. 6B). As demonstrated previously (12), RAG−/− mice exhibit weight loss and death within 12 d. Adoptive transfer of naive WT CD8 T cells into RAG−/− recipients can afford protection against death in this model (Fig. 6B) (12), closely mirroring the illness and recovery observed in WT mice. In marked contrast, RAG−/− mice that received naïve IFN-γ−/− CD8 T cells failed to control the virus, developed clinical symptoms similar to those observed in IFN-γ−/− and IFN-γR−/− mice, and all succumbed to infection by day 11 (Fig. 6B). The presence of IFN-γ–producing VACV-specific effector CD8 T cells in the lungs of RAG−/− mice that received WT CD8 T cells confirmed that CD8 T cells were primed, able to migrate to the site of infection, and functional in the absence of CD4 T lymphocytes (Fig. 6C). These results demonstrate that CD8 T cell–derived IFN-γ is crucial for the control of a respiratory VACV-WR infection. Importantly, they implied that IFN-γ could not be functionally compensated for by other immune mechanisms.

To address this more directly, we transferred naive polyclonal WT CD8 T cells into IFN-γR−/− mice (Fig. 6D). In this model, the absence of functional IFN-γ signaling would facilitate the detection of any compensatory effector mechanism/s capable of providing protection during a respiratory VACV-WR infection. As before, WT and IFN-γR−/− mice with no CD8 T cells transferred were used as controls. The following day after transfer, all mice were infected i.n. with VACV-WR, and weight loss and survival was monitored for 3 wk (Fig. 6E). In contrast to our previous transfer experiments, IFN-γR−/− mice that received WT CD8 T cells failed to control the virus, and all animals succumbed to the infection (Fig. 6E). This was evident despite the presence of a robust virus-specific CD8 T cell response in the lungs of IFN-γR−/− mice at day 8 p.i. (Fig. 6F). These results show that perforin-, granzyme-, TRAIL-, and FASL-dependent cytotoxicity, which are clearly induced by VACV infection, are not measurably involved in clearance of VACV from the respiratory tract.

In our last approach, we explored the relative importance of host-derived IFN-γ to recovery from infection with VACV-WR by adoptively transferring naive polyclonal WT CD8 T cells into IFN-γ−/− recipients (Fig. 7A). WT and IFN-γ−/− mice with no CD8 T cell transfer were used as controls (Fig. 7A). The following day, all mice were infected i.n. with VACV-WR, and weight loss and survival was monitored for 3 wk (Fig. 7B, both panels). Despite a small delay in recovery (Fig. 7B, left panel), all IFN-γ−/− mice that received WT CD8 T cells survived the infection (Fig. 7B, right panel). Far fewer B8R-specific adoptively transferred WT CD8 T cells (transferred cells) were present in the lungs of IFN-γ−/− mice after VACV-WR infection as compared with endogenous cells in the same host or WT controls (Fig. 7C), suggesting the extent of recovery is related to the number of IFN-γ–producing VACV-specific CD8 T cells. Together, these data provide compelling evidence that CD8 T cell–derived IFN-γ is both necessary and sufficient to confer resistance to a respiratory VACV-WR infection.

Discussion

Precisely how CD8 T cells control a primary poxvirus infection has been the subject of intense debate for the past 25 years. A collection of early studies indicated that the dominant effector mechanism employed by CD8 T cells during an intradermal ectromelia virus (mousepox) infection involves the cytotoxic granule exocytosis pathway (36, 42–44). The combined release of these effector molecules, such as perforin and the serine proteases GrzA and GrzB, culminate in virus-infected target cell death. Paradoxically, subsequent studies revealed that perforin-mediated cytolysis was
not required for the elimination of i.p. administered cowpox or VACV-WR infections (40, 43), which are closely related to the mousepox virus. This outcome could be rationalized by more recent findings demonstrating that CD4 T cell–dependent Ab responses, rather than CD8 T cell–mediated cytotoxicity, are the dominant immune mechanism responsible for clearing virus when VACV-WR

**FIGURE 6.** IFN-γ-competent CD8 T cells can protect lymphopenic but not IFN-γR−/− mice against mortality following a respiratory VACV-WR infection. (A) A total of 5 × 10⁶ naive (CD3⁺CD8⁺CD44low) WT or IFN-γ−/− CD8 T cells were transferred i.v. into RAG−/− mice that were infected i.n. 24 h later with 1.25 × 10⁵ PFU VACV-WR. WT and RAG−/− mice with no CD8 T cells transferred were used as controls. The animals were weighed daily and euthanized if weight loss was ≥25% of their original body weight for 2 consecutive d. Percentage of initial body weight [(B), left panel] and mean percent survival [(B), right panel] from the indicated numbers of mice are presented. (C) On day 21 p.i., total lung cells from WT and RAG−/− mice that received WT CD8 T cells were stimulated with B8R peptide and assessed for intracellular IFN-γ production. To determine frequency of cytokine-producing cells, CD8 T cells were gated on CD8⁺CD62Llow cells, and a representative FACS plot from each surviving experimental group was shown. (D) In a separate experiment, 5 × 10⁶ naive (CD3⁺CD8⁺CD44low) WT CD8 T cells were transferred i.v. into IFN-γ−/− mice that were infected i.n. 24 h later with VACV-WR. Percentage of initial body weight [(E), left panel] and mean percent survival [(E), right panel] from the indicated numbers of mice are presented. (F) On day 8 p.i., total lung cells were isolated and stimulated with B8R peptide to determine the frequency of cytokine-producing CD8 T cells. Weight loss data are presented as the mean ± SEM of three separate experiments containing three to five mice per group and analyzed using a two-way ANOVA to determine statistical significance. Survival data use combined survival data across combined experiments using the Mantel-Cox test. **p < 0.01.

**FIGURE 7.** CD8 T cell–derived IFN-γ is sufficient for protection in the absence of host-derived IFN-γ. (A) A total of 5 × 10⁶ naive (CD3⁺CD8⁺CD44low) WT CD8 T cells were transferred i.v. into IFN-γ−/− mice that were infected i.n. 24 h later with VACV-WR. The animals were weighed daily and euthanized if weight loss was ≥25% of their original body weight for 2 consecutive d. Percentage of initial body weight [(B), left panel] and mean percent survival [(B), right panel] from the indicated numbers of mice are presented. WT and IFN-γ−/− mice with no T cell transfer were used as control. (C) On day 21 p.i., lungs were harvested and stimulated overnight with B8R peptide for intracellular IFN-γ and TNF staining. Representative plots for cytokine staining, gating on CD8⁺CD62Llow cells, are shown, and the percentages that stained positive for IFN-γ alone or TNF and IFN-γ/TNF are indicated as well as the transferred cells (IFN-γ+) in IFN-γ−/− mice. Data are presented as the mean ± SEM of three separate experiments containing three to five mice per group; weight loss data were analyzed using a two-way ANOVA to determine statistical significance. Survival data use combined survival data across combined experiments using the Mantel-Cox test. **p < 0.01.
is administered via the i.p. route (45). Therefore, whether CD8 T cells that develop during the course of VACV-WR infection use direct cytolytic pathways to kill virus-infected cells remained elusive. To address this important issue, we focused our studies on a respiratory model of VACV-WR infection. This model provided us with a unique opportunity to examine, for the first time to our knowledge, the effector mechanism/s used by CD8 T cells to protect against a more natural mucosal infection in which CD4 T cells and Ab responses play little or no role. Our data reveal strikingly different outcomes when VACV-WR enters the host via the respiratory tract.

The most significant finding in the current study is that virus-specific CD8 T cells need to produce IFN-γ to protect against the lethal effects of a highly virulent respiratory VACV-WR infection. This conclusion is based on several independent lines of evidence. First, mice that lack IFN-γ or IFN-γR were unable to clear VACV-WR, and all succumbed to infection by day 8. Most notably, the extent of lung pathology, virus dissemination from the lung to visceral tissues, and the time of death closely resembled what we had shown previously in mice deficient in CD8 T cells (12). Second, peak mRNA and protein levels of lung IFN-γ coincided with the peak of the VACV-specific CD8 T cell response. Third, kinetic depletion studies indicated that IFN-γ primarily performed its protective role between days 3 and 6 p.i., which again mirrored what we had found earlier for CD8 T cells (12). Fourth, besides the inability to produce IFN-γ, VACV-reactive CD8 T cells isolated from the lung and lymph nodes of IFN-γ or IFN-γR−/− mice displayed the same level of activation, expansion, TNF secretion, and the ability to locate within infected lung tissue as did WT cells, thus indicating that T cell–intrinsic effects of IFN-γ are minimal in comparison with its effects on other host cell types. The lack of intrinsic effects on CD8 T cells has also been reported in the context of murine CMV (46), Sendai virus (47), influenza virus (48), and also in i.v. (49) and i.p. (50) VACV-WR infection models. Most significantly, IFN-γ did not impact the ability of CD8 T cells to acquire cytolytic activity. In the absence of IFN-γ, we found that lung-infiltrating VACV-specific CD8 T cells displayed normal or, even in some cases, elevated levels of granzyme, perforin, TRAIL, and FASL and readily expanded, TNF secretion, and the ability to locate within infected lung tissue as did WT cells, thus indicating that T cell–intrinsic effects of IFN-γ are minimal in comparison with its effects on other host cell types. The lack of intrinsic effects on CD8 T cells did not affect the ability of mice to recover (data not shown), in accordance, far fewer lung-infiltrating VACV-specific CD8 T cells were transferred into RAG−/- recipients as compared with IFN-γ−/- recipients, the infection was cleared with the same kinetics as those seen in WT (RAG−/+). In striking contrast, RAG−/- mice that received IFN-γ−/-/CD8 T cells failed to control the virus, developed clinical symptoms similar to those observed in nontransferred IFN-γ−/- and IFN-γR−/- mice, and all succumbed to the infection. Similarly, the transfer of naive polyclonal WT CD8 T cells into IFN-γR−/- mice also failed to provide protection against a respiratory VACV-WR infection. Interestingly, when naive polyclonal WT CD8 T cells, presumably containing a very low frequency of VACV-reactive CD8 T cell clones, were transferred into naive IFN-γ−/- hosts, all mice were fully protected from death, but had a noticeably delayed recovery phase when compared with WT (IFN-γ−/-) mice. One interpretation of these data could be that, in addition to CD8 T cells, IFN-γ derived from other cell types in the host is needed for optimal viral clearance and recovery. We considered other potential lymphocyte populations that may be involved in the production of IFN-γ including B cells and virus-specific CD4 T cells, both of which are elevated in the lung after respiratory VACV infection (12). However, in our earlier study (12), we had shown that the absence of CD4 and B cells had little or no effect on the ability of mice to recover from a respiratory VACV-WR infection. We also found that NK cells are recruited to the lung and readily produce IFN-γ in response to VACV-WR infection (data not shown). In this regard, prior reports have indicated that NK cells play a significant role in limiting VACV-WR viral replication following intradermal (54), i.p. (55), and i.v. (56) infection. Consequently, we anticipated these cells to be crucial for resistance to a respiratory VACV-WR infection. Unexpectedly, however, depletion of NK cells at the time of VACV-WR infection did not affect the ability of mice to recover (data not shown), suggesting that these cells were not the critical source of IFN-γ in the lung. In striking contrast, absence of CD8 T cells showed a dramatic effect. Mice deficient in or depleted of CD8 T cells at the time of VACV-WR infection were highly susceptible to a respiratory VACV-WR infection (12). Therefore, the interpretation that we currently favor is that the observed delay in recovery is likely due to the low precursor frequency of virus-specific CD8 T cells transferred into IFN-γ−/- mice. In accordance, far fewer adoptively transferred VACV-specific WT CD8 T cells were recovered from the lungs of IFN-γ−/- recipients as compared with endogenous virus-specific CD8 T cells in the same host or WT
controls that did not receive any cells. Regardless, the fact that such small numbers of IFN-γ-producing CD8 T cells are able to protect against death is, in our view, a strong illustration of the potency of this effector mechanism in combating a highly virulent respiratory poxvirus infection.

The antiviral properties of IFN-γ have been established in several virus infection models, including HSV (57), CMV (58), lymphocytic choriomeningitis virus (59, 60), and ectromelia virus (61, 62). However, in most cases, it was not determined whether these are due to CD8 T cell autonomous expression of IFN-γ. Anti–IFN-γ treatment of mice infected in the footpad with ectromelia virus resulted in death within 7 d, ~5 d earlier than those that lack CD8 T cells (61–63). Similarly, the early defense against VACV-WR administered via the i.v. route was severely defective in lack CD8 T cells (61–63). Interestingly, CD8 T cells protect against the lethal effects of influenza infection by employing multiple effector mechanisms many of which are redundant (72). Whether differences in virus virulence, cell tropism, viremia, viral immune modulatory strategies, or the speed at which alternative modes of immunity develop influences the extent of redundancy in antiviral effector mechanisms employed by CD8 T cells are some areas that clearly need further investigation. Answering these questions will be important for establishing novel vaccines and therapeutics for existing and emerging human respiratory viral infections.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


Wild type (WT) C57BL/6J and IFN-γR−/− mice were intranasally (i.n.) infected with 1.25 x 10^4 plaque forming units (PFU) of VACV-WR. On day 8 post infection, WT and IFN-γR−/− lung cells were stained with CD3ε, CD8α, CD44, B8R tetramer, or stimulated with B8R peptide and subsequently stained for intracellular IFN-γ and TNF. Total numbers (a) of CD3ε+CD8α, CD3ε+CD8α+CD44high, B8R tetramer-positive T cells, relative frequency of CD3ε+CD8α+CD44high B8R tetramer (b; left panel) and representative plots for cytokine staining (b; right panel), gated on CD8α+CD62Llow cells, are indicated. Total numbers of day 8 lung CD8α+TNF producing CD8 T cells (c) are also quantified. In situ CD8 T cell location (peribronchial and parenchymal) in IFN-γR−/− mice was also determined in day 8 infected OCT embedded lung sections fluorescently stained for actin (green) and CD8α (magenta) (d). Data are presented as the mean ± one SEM of two independent experiments with 8 mice per group; or as a representative FACS plot or micrograph from the represented mouse strain.