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CD8 T Cells Are Essential for Recovery from a Respiratory Vaccinia Virus Infection

John Goulding,*,† Rebecka Bogue,* Vikas Tahiliani,*,† Michael Croft,* and Shahram Salek-Ardakani*,†

The precise immune components required for protection against a respiratory Orthopoxvirus infection, such as human smallpox or monkeypox, remain to be fully identified. In this study, we used the virulent Western Reserve strain of vaccinia virus (VACV-WR) to model a primary respiratory Orthopoxvirus infection. Naive mice infected with VACV-WR mounted an early CD8 T cell response directed against dominant and subdominant VACV-WR Ags, followed by a CD4 T cell and Ig response. In contrast to other VACV-WR infection models that highlight the critical requirement for CD4 T cells and Ig, we found that only mice deficient in CD8 T cells presented with severe cachexia, pulmonary inflammation, viral dissemination, and 100% mortality. Depletion of CD8 T cells at specified times throughout infection highlighted that they perform their critical function between days 4 and 6 postinfection and that their protective requirement is critically dictated by initial viral load and virulence. Finally, the ability of adoptively transferred naive CD8 T cells to protect RAG$^{-/-}$ mice against a lethal VACV-WR infection demonstrated that they are both necessary and sufficient in protecting against a primary VACV-WR infection of the respiratory tract. *The Journal of Immunology*, 2012, 189: 2432–2440.

O rthopoxvirus genus members, including variola virus (VARV), ectromelia virus (ECTV), monkeypox, and vaccinia virus (VACV), are large dsDNA viruses that encode transcription and replication machinery that facilitates their life cycle within the cytoplasm of the host cell (1). Despite the global eradication of VARV, the etiological agent of human smallpox, the continuing threat of intentional or accidental release of VARV and a growing number of human respiratory monkeypox and natural VACV infections has propelled a renewed interest in Orthopoxvirus research (2–6). Historically, in vivo models have used both VACV and ECTV to simulate a primary infection and vaccination regimens to understand the essential immune requirements needed for protection. Collectively, these studies emphasized the necessity for CD4 T cells and B cell-derived Ig in protecting against a primary and secondary Orthopoxvirus infection (7–11). However, the majority of these studies used infection models that do not represent the natural route of a primary Orthopoxvirus infection in humans. To address, this a number of respiratory Orthopoxvirus infection models were developed (12–17).

In many respects, intranasal (i.n.) infection with the highly virulent mouse-adapted Western Reserve strain of VACV (VACV-WR) simulates the spread of smallpox virus throughout the respiratory tract, resulting in severe disease that is associated with pulmonary inflammation, intra-alveolar edema, hemorrhage, peribronchial and perivascular inflammation, and subsequent death (16, 18, 19). Initial VACV-WR replication is thought to occur in the respiratory epithelium and alveolar macrophages before the development of a transient viremia that spreads virus to peripheral reticuloendothelial cells throughout the host (12, 20). Over the past several years, a number of studies established an important role for innate immune cells, such as NK cells (21, 22), monocytes (23, 24), and dendritic cells (25), during primary VACV infections; however, the relative contribution of adaptive immune cells, in particular CD8 T cells, remains unclear.

In this study, we show that a respiratory VACV-WR infection elicits a robust Ag-specific CD8 T cell response that is followed shortly thereafter by the development of a VACV-specific humoral response that is completely dependent on CD4 T cell help. Unexpectedly we found that CD8, but not CD4, T cell-deficient or -depleted mice failed to recover from infection and presented with elevated viral titers and 100% mortality by day 9 postinfection (p.i.). Selective depletion of CD8 T cells at specified times throughout infection highlighted a critical role for CD8 T cells between days 4 and 6 p.i. Finally, as revealed by the ability of adoptively transferred naive CD8 T cells to protect RAG$^{-/-}$ mice against a lethal VACV-WR infection, we show that CD8 T cells are both necessary and sufficient to protect against a primary VACV-WR infection of the respiratory tract.

Materials and Methods
Mice

The experiments performed conformed to the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research. All experiments were completed in compliance with regulations of the La Jolla Institute Animal Care Committee and in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. Eight- to twelve-week-old female

*Division of Immune Regulation, La Jolla Institute for Allergy and Immunology, San Diego, CA 92037; and †Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL 32610-0275

Address correspondence and reprint requests to Dr. Shahram Salek-Ardakani, Department of Pathology, Immunology, and Laboratory Medicine, University of Florida, P.O. Box 100275, Gainesville, FL 32610-0275. E-mail address: ssalek@ufl.edu

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Abbreviations used in this article: ECTV, ectromelia virus; GC, germinal center; i.n., intranasal(ly); LN, lymph node; NYCBOH, New York City Board of Health; p.i., postinfection; VACV, vaccinia virus; VACV-WR, Western Reserve strain of vaccinia virus; VARV, variola virus; WT, wild-type.

C57BL/6J (wild-type [WT]), RAG$^{-/-}$, CD8$^{-/-}$, MHC I$^{-/-}$, MHC II$^{-/-}$, and μMT mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Vaccinia virus and stock preparation

VACV-WR, New York City Board of Health (NYCBOH), and Lister strains were purchased from the American Type Culture Collection, grown in HeLa cells, and subsequently titered on VeroE6 cells, as described previously (26).

Respiratory VACV-WR infection model

Naive mice were anesthetized by isoflurane inhalation and infected i.n. with 1.25×10^3 to 2×10^6 PFU of the indicated VACV strain, with daily measurements of body weight, lung pathology, and viral titers, as described previously (27). No animal was 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 as a result of severe disease (weight loss >25%).

VACV titer assay

After i.n. infection, specified 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.

Flow cytometric analysis

Spleens, lungs, and lymph nodes (LNs) 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. In addition, before mechanical disruption, the lung tissue was treated for 1 h at 37°C with 250 μg Collagenase D (Roche), followed by treatment for 10 min at 4°C with 100 mM 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 50 μM 2-ME (Sigma-Aldrich) and enumerated using a BD automated Vi-CELL counter.

T cell, NK cell, NKT cell, and germinal cell staining. Cells were washed with FACS buffer (PBS and 2% FCS) and stained with anti-Fc II/III receptor mAb 2.4G2 for 15 min at 4°C. After an additional wash in FACS buffer, the following Abs were incubated with the cells for 30 min at 4°C; CD4 (RM4-5; BD Pharmingen), CD8 (53-6.7; BD Pharmingen), CD3 (145-2C11; eBioscience), NK1-1 (PK136; eBioscience) DX5 (CD49b) (DX5, eBioscience) were used to determine T cell, NK, and NKT cell subsets, whereas anti-mouse IgD (11-26; Southern Biotech), CD138 (281-2, RDI) and PNA (FITC; Vector Laboratories), FAS (Jo2; BD Pharmingen) delineated plasma cell and germinal center (GC) B cells, respectively (28). All samples were acquired on a FACSCalibur flow cytometer or Canto II (BD Bioscience) and analyzed using FlowJo software (Tree Star).

VACV-WR–specific IFN-γ cytokine production. CD8 and CD4 T lymphocyte VACV-WR–specific cytokine production was assessed as previously described (26, 28). Briefly, after lysing RBCs, splenocytes and lung cells from infected mice were plated in round-bottom 96-well microtiter plates in 200 μl RPMI 1640 medium (Invitrogen) supplemented with 10% FCS (Omega Scientific), 1% L-glutamine (Invitrogen), 100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μM 2-ME (Sigma-Aldrich). Then, 10 μg/ml of the indicated MHC class I- or class II-restricted VACV peptide was added and incubated for 1 h at 37°C. The VACV peptide epitopes used in this study to identify virus-specific T cells were predicted and synthesized, as described previously (29–31). GolgiPlug (BD Biosciences) was added to the cultures, according to the manufacturer's instructions, and the incubation was continued for 9 h. Cells were then stained with anti-CD4 (RM4-5) or anti-CD8 (53-6.7) and anti-CD62L (MEL-14; all from 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) for 30 min at 4°C. Samples were analyzed for their proportion of cytoplasmic cytokines after gating on CD4 or CD8 CD62L^{low} T cells using a FACSCalibur flow cytometer and CellQuest and FlowJo software.

In vivo CD8 and CD4 T cell depletion

Hybridomas were cultured in protein-free hybridoma medium II (Invitrogen), and mAbs were isolated by dialysis of supernatant. Groups of VACV-WR–infected mice were depleted of CD8 and/or CD4 T cells with

anti-CD8 (clone 2.43; 200 μg/mouse) and anti-CD4 (GK1.5; 200 μg/mouse) given in one i.v. injection 3 d prior, as well as i.p. injections on days −1 and every 3 d thereafter until the termination of the experiment. T cell depletion was confirmed by flow cytometry of peripheral blood and lung tissue.

In vivo naive CD8 T cell transfer

Naive CD8 T cells (CD3$^+$CD8$^+$CD44^{low}) were isolated from naive WT C57BL/6J mice. Briefly, naive spleens were homogenized to a single-cell suspension, as described above, and anti-CD8 MicroBeads (Miltenyi Biotec) were 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 FACSAria flow cytometer. Subsequently, 5×10^6 naive CD8 T cells/mouse were transferred into age-matched RAG$^{-/-}$ mice via the retro-orbital plexus.

Measurement of serum VACV-WR–specific IgG isotype titers

Serum was obtained after centrifugation of blood samples collected with a heparinized capillary pipette from the retro-orbital plexus. All samples were stored at −20°C until analyzed for Ab titer. The level of specific Abs against VACV-WR in serum was quantitated by ELISA, as previously described (32–34).

In vivo passive VACV-WR immunization

VACV-WR immune or control serum was prepared from naive (WT) mice that were infected i.n. with VACV-WR (1×10^4 PFU/mouse). Age-matched naive C57BL/6J mice were injected i.p. with 250 μl serum from the indicated mice. The following day, mice were anesthetized by inhalation of isoflurane and infected i.n. with 1×10^4 PFU VACV-WR. Mice were weighed daily for 2 wk following infection and were euthanized at a predetermined time point or if they lost >25% of their initial body mass.

Statistical analysis

Tests were performed using Prism 4.0 software (GraphPad, San Diego, CA). Statistical analysis was performed using the two-tailed, unpaired Student t test with 95% confidence intervals, unless indicated otherwise. Two-way ANOVA was used to determine differences in weight loss profiles, and the Mantel–Cox test was used for survival analysis. Unless indicated otherwise, data represent the mean ± 1 SEM, with $p < 0.05$ considered statistically significant.

Results

Respiratory VACV-WR infection results in severe lung inflammation

To study the role of CD8 T cells in the susceptibility to respiratory infection with VACV, naive C57BL/6 WT controls were infected via the i.n. route with a low (1×10^4 PFU), medium (5×10^4 PFU), or high (1×10^6 PFU) dose of VACV-WR, with daily measurements of body weight, lung pathology, and viral titers (Fig. 1A). Mice infected with ≥5×10^4 PFU of VACV-WR had lost >25% of their initial weight by day 7 p.i. (Fig. 1A), when they were euthanized for humane reasons. These mice were hunched, had significantly labored breathing and ruffled fur, and were minimally responsive to manipulation. H&E-stained sections of lungs were evaluated for the presence of inflammation, hemorrhage, edema, and necrosis. Alterations were not observed in any lung samples obtained at 1 and 2 d p.i. (data not shown). However by day 6 (Fig. 1B, *middle panels*), the majority of virus-infected mice had moderate to severe, multifocal, mixed (predominantly mononuclear) inflammation that tended to be focused around the bronchioles and blood vessels. The degree of bronchiolar epithelial hyperplasia and necrosis was moderate to severe, with several bronchioles being affected. Generally, there was mild to moderate, multifocal, vascular necrosis and occasionally mild to moderate hemorrhage and edema. Thus, the lesion characteristics were consistent with a diagnosis of moderate to severe viral bronchopneumonia characterized by mononuclear inflammation, bronchiolar hyperplasia, and necrosis.
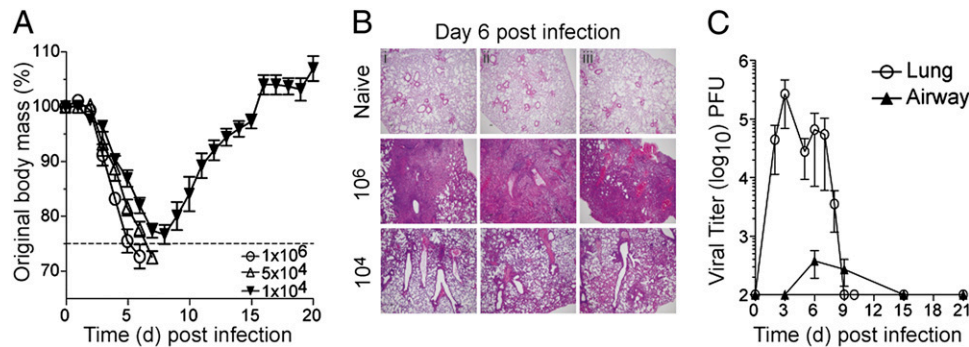
FIGURE 1. Respiratory VACV-WR infection results in severe lung inflammation. WT C57BL/6J mice were infected i.n. with increasing PFU of VACV-WR. Body mass was monitored daily (**A**) and lung inflammation was determined by staining with H&E (original magnification ×10) (**B**). (**i–iii**) Three representative micrographs are shown for two separate PFU doses. (**C**) VACV-WR tissue-specific viral titers were measured at specified times following infection with 1×10^4 PFU. Body mass and viral titers are presented as mean ± 1 SEM of three separate experiments containing 5–12 mice/group.

Mice infected i.n. with 1×10^4 PFU VACV-WR produced a less severe disease, reduced weight loss (Fig. 1A), and reduced lung inflammation and pathology (Fig. 1B, *bottom panels*) at day 6 p.i. All mice began to recover from disease starting at day 7, returning to their original mass by day 14 p.i. (Fig. 1A). The titer of infectious virus in lung tissue and airways of VACV-WR–infected mice followed the pattern observed with regard to the weight loss and histological assessment (Fig. 1C). Although a large amount of infectious virus was recovered from VACV-WR–infected lungs at days 4 and 6 p.i., little or no virus could be detected by day 10 p.i. Thus, infection with 1×10^4 PFU VACV-WR promoted a robust immune response that led to virus clearance from the lung within 10 d and protected all animals from death; therefore, 1×10^4 PFU was used to further characterize the protective components of a primary respiratory infection with VACV-WR.

Mice lacking adaptive immunity fail to control a respiratory VACV-WR infection

To determine the importance of T and B cells, we studied infection in $RAG^{-/-}$ mice deficient in mature T and B cells as the result of an inability to perform V(D)J recombination (35). Following infection with 1×10^4 PFU VACV-WR, the initial weight loss and illness score were comparable to WT mice; however, progressive weight loss and illness in the $RAG^{-/-}$ mice resulted in 100% mortality by day 12 p.i., suggesting an important contribution of B and/or T cells in controlling VACV-WR infection (Fig. 2A). Histopathological analysis of the lung tissue on days 7 and 10 p.i. highlighted extensive lung pathology, cellular infiltrate, alveoli destruction, and pulmonary edema in $RAG^{-/-}$ mice compared with WT controls (Fig. 2B). $RAG^{-/-}$ mice also failed to contain initial viral titers in the lung and displayed signs of viral dissemination, as determined by the significant levels of virus in their ovaries (Fig. 2C).

Robust mucosal and systemic T cell responses to a respiratory VACV-WR infection

To determine whether recovery from a primary respiratory VACV-WR infection in WT mice correlated with the presence of T cells at the site of infection, groups of age-matched naive WT mice were infected i.n. with VACV-WR, and the kinetics of lymphocyte recruitment to the lung and spleen were determined. The number of cells recovered from the lung increased between days 3 and 7, peaked between days 7 and 10, and decreased by day 15 (Fig. 3A). The majority of the lymphocytes infiltrating the lung and spleen were CD8 T cells (Fig. 3A, 3B). Between days 7 and 10 p.i., the number of CD8 T cells in the lung was ≥10–20-fold greater than the number of CD4 T cells. To determine the specificity and functionality of the T cell response at the peak of the primary

response, total lung and spleen cells were isolated on day 10 p.i. and stimulated ex vivo with different VACV peptides. Because the extent of CD8 and CD4 T cell VACV responses is large, with a total of 49 CD8 and 14 CD4 Ags recognized (29–31), we used 8 immune-dominant Ags to explore the diversity of VACV-specific T cell response. IFN-γ–producing CD8 T cells specific for B8R, B16R, J3R, and A8R viral Ags dominated both the lung and splenic T cell response, whereas VACV-specific CD4 T cells targeted multiple epitopes with similar magnitude (Fig. 3C, 3D). The emergence of VACV-specific T cells in the lung tissue and their secretion of IFN-γ in these sites paralleled the clearance of virus from the lungs of infected mice and the cessation of weight loss, suggesting that effector CD4 and/or CD8 T cells play critical roles in recovery from infection.

B cell and Ig responses to a respiratory VACV-WR infection

IgG and IgM class-specific end point ELISA was used to investigate the kinetics of VACV-specific Ig in the serum of mice infected i.n. with VACV-WR. Postinfection, VACV-specific serum IgG was still
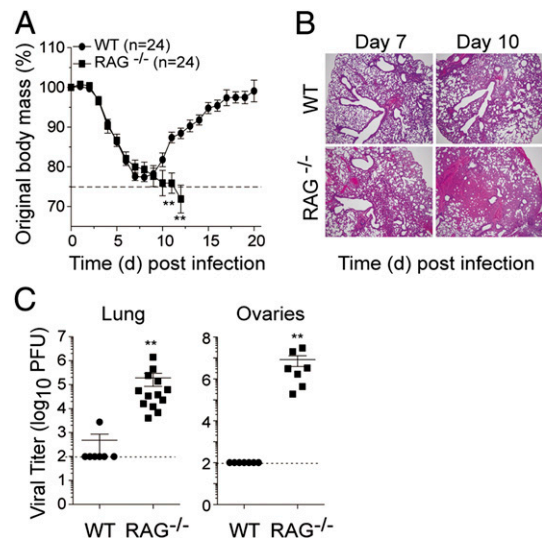

FIGURE 2. Adaptive immunity is necessary to control a respiratory VACV-WR infection. (**A**) WT C57BL/6J and $RAG^{-/-}$ mice were infected i.n. with 1×10^4 PFU VACV-WR and weighed daily. Lung inflammation was assessed by H&E staining (original magnification ×10) on days 7 and 10 p.i. (**B**), and viral titers were also measured in the lung and ovaries on day 10 p.i. (**C**). Body mass and viral titers are presented as the mean ± 1 SEM of four independent experiments with four to eight mice/group. The Student t test with the Bonferroni correction was used to determine statistical significance. *$p < 0.05$, **$p < 0.01$.
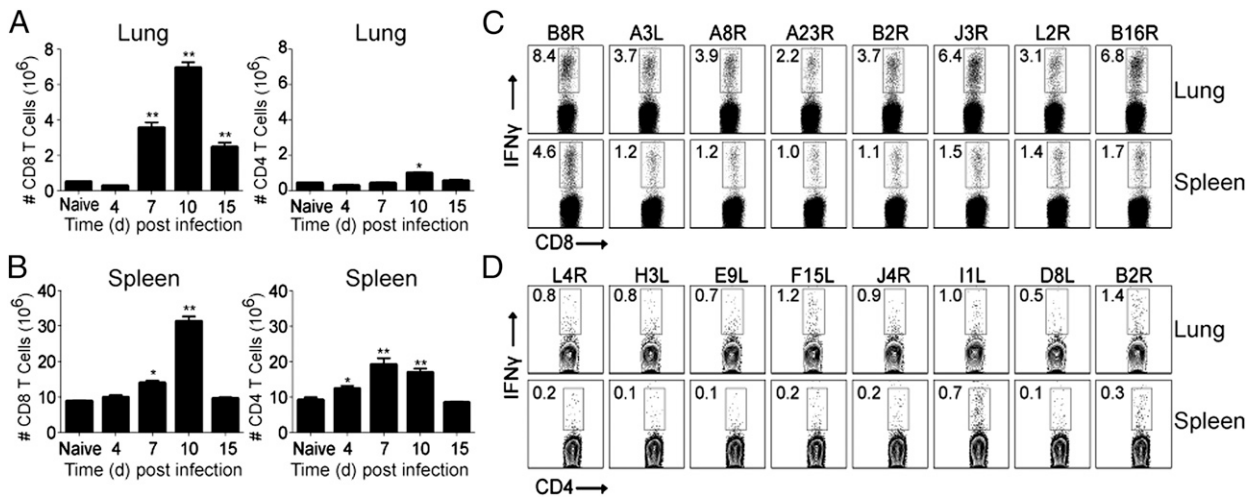
FIGURE 3. Lung and splenic T cell responses to a respiratory VACV-WR infection. WT C57BL/6J mice were infected i.n. with 1×10^4 PFU of VACV-WR. Total lung (**A**) and spleen (**B**) CD8 and CD4 T cell numbers were measured at various time points p.i. The percentage of VACV-WR–specific CD8 (**C**) and CD4 (**D**) IFN-γ–producing T cells in the lung and spleen were also enumerated on day 10 p.i. T cell numbers are presented as the mean ± 1 SEM of three separate experiments containing five to eight mice/group or as a representative FACS plot from the represented tissue. The Student t test with the Bonferroni correction was used to determine statistical significance *$p < 0.05$, **$p < 0.01$.

undetectable on day 10, when the animals were already recovering from disease (Fig. 1A, 1C). Low levels of VACV-specific IgG were measured on day 15; however, they increased thereafter, reaching maximal levels by day 148. Previously, we and other investigators showed that almost all of the anti-VACV IgG response after i.p. infection is CD4 T cell help dependent (7, 33, 36). Therefore, we used MHC class II-deficient mice, which lack CD4 T cells, to evaluate the T cell help requirement for production of anti-VACV IgG after i.n. infection. Similar to B cell-deficient mice, no anti-VACV IgG was found in MHC II$^{-/-}$ mice (Fig. 4B). Analysis of the relative contribution of virus-specific IgG isotypes indicated that the levels of IgG1 (data not shown) and IgG2c also peaked between days 30 and 148 p.i. and that IgG2c was the most abundantly produced isotype (Fig. 4C). IgM was also produced in response to respiratory VACV-WR infection; however, it had more rapid kinetics than did IgG and peaked in titer between days 7 and 10 p.i., before diminishing by day 30 p.i. (Fig. 4D).

Mediastinal LNs drain the lung and are a site where mucosal immune responses are initiated against Ags reaching the lung. Indeed, we found high frequencies of GC B cells (PNA$^+$FAS$^+$) within these LNs (Fig. 4E, *upper panels*), consistent with the presence of IgG Ab in the serum (Fig. 4A). Frequencies of GC B cells declined over time, reaching basal levels by day 30 (data not shown). The development of mediastinal LN B cells positive for the plasma cell differentiation marker CD138 followed similar kinetics to those of GC B cells (Fig. 4E, *lower panels*). Together, these data highlighted that the development of GC and Ig-secreting plasma cells following a respiratory VACV infection occurred after the peak of the T cell response but was maintained over the course of several months.

CD4 T cells and Ig are not required for protection against a respiratory VACV-WR infection

To assess the contribution of CD4 effector T lymphocytes in virus clearance and recovery, we infected MHC II$^{-/-}$ mice, which are devoid of CD4 T cells, with VACV-WR and monitored their weight loss over time. MHC II$^{-/-}$ mice displayed comparable weight loss and recovery profiles to those observed in WT mice (Fig. 5A), even though Ig production was inhibited (Fig. 4B). Also, in vivo depletion of CD4 T cells in WT mice did not substantially modify either survival (Fig. 5A) or virus clearance from

the respiratory tract (data not shown). Consistent with this, lung pathology and lung infiltrate were similar between CD4 T cell-deficient and WT isotype-treated (Ig) control mice (Fig. 5B).

To address the role of Ig during a primary respiratory infection with VACV-WR more directly, serum was prepared from mice that were infected with VACV-WR 4, 7, 10, 15, or 148 d prior to the start of the experiment and transferred i.p. into naive WT mice. Serum from uninfected (naive) mice was used as control. The following day, all mice were challenged with a sublethal i.n. inoculum of VACV-WR, and weight loss was monitored for 15 d (Fig. 5C). All mice that received serum from day 4 and day 7 VACV-infected mice (anti-VACV IgG$^-$, anti-VACV IgM^{low}) displayed comparable weight loss and recovery to mice that received control serum (Fig. 5D). In contrast, mice that received hyperimmune serum isolated after day 10 of a respiratory VACV infection (anti-VACV IgG$^+$) exhibited a significant reduction in weight loss (Fig. 5E) and accelerated recovery. These data, together with the kinetics of GC B cell development and viral clearance, suggest that humoral responses develop too late to be of significant value in the initial containment of VACV replication in the lung.

An early CD8 T cell response is necessary for protection during a respiratory VACV-WR infection

One likely explanation for recovery of CD4-depleted or MHC II$^{-/-}$ mice after a respiratory VACV-WR infection is the ability of CD8 T cells to compensate for the lack of CD4 and Ab responses. To evaluate this, MHC II$^{-/-}$ mice were infected with VACV-WR, and primary ex vivo CD8 T cell responses were measured on day 10, the peak time for anti-VACV responses (Fig. 6A). In agreement with our previous data in the i.p. infection model (27, 36), we found that CD4 T cell help is not required for the generation of VACV-specific effector CD8 T cells after i.n. infection. Indeed, the frequency of IFN-γ–producing virus-specific CD8 T cells was slightly elevated in the lung and spleens of VACV-infected MHC II$^{-/-}$ mice compared with WT controls.

To test whether CD8 T cells can protect in the absence of CD4 T cells, we depleted both subsets of T cells simultaneously, starting before infection and continuing during the observation period. Strikingly, depletion of CD8 T cells in CD4-depleted mice resulted in 100% mortality by day 9 p.i. (Fig. 6B, *left panel*), implying that the CD8 subset was indeed required for the survival of the CD4-
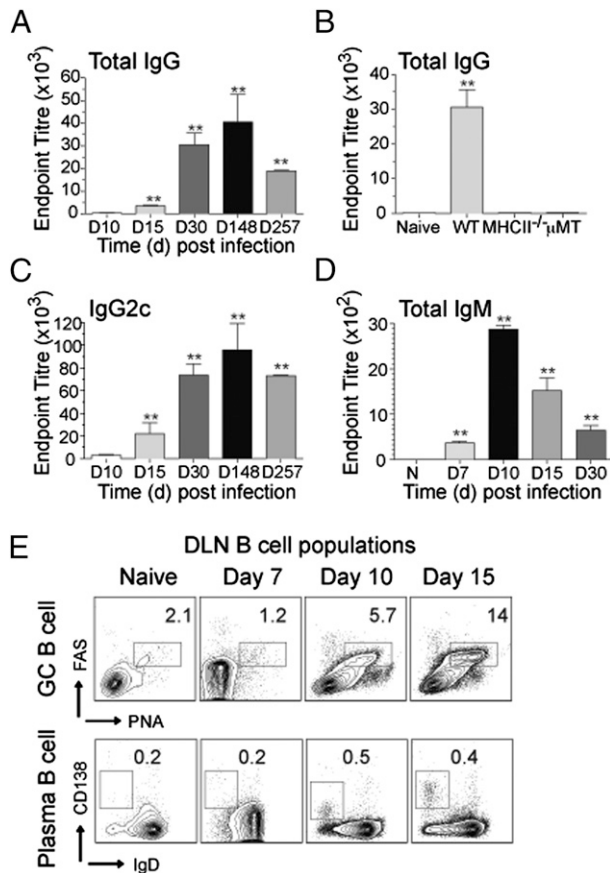
FIGURE 4. B cell and Ig responses following a respiratory VACV-WR infection. WT C57BL/6J mice were infected i.n. with 1×10^4 PFU of VACV-WR. Absolute serum concentrations of VACV-WR–specific IgG (**A**), IgG2c (**C**), and IgM (**D**) were determined in WT mice by ELISA at specific days p.i. (**B**) Total IgG levels were also measured on day 30 p.i. in MHC II$^{-/-}$ (CD4 T cell-deficient) and μMT (B cell-deficient) mice. End point Ig titers equate to the dilution needed to produce an absorbance value of 0.2. (**E**) Lung-draining LN GC (B220$^+$FAS$^+$PNA$^+$) and plasma cell (B220$^+$CD138$^+$IgD$^-$) development was also measured at specified times following infection. All Ig levels are presented as the mean ± 1 SEM of three independent experiments with five to eight mice/group or as a representative FACS plot. The Student t test with the Bonferroni correction was used to determine statistical significance. **$p < 0.01$.

deficient mice. This result was phenocopied in MHC I$^{-/-}$, CD8$^{-/-}$, and WT mice depleted of CD8 T cells throughout the course of a VACV-WR infection (Fig. 6B, *right panel*). All mice deficient in CD8 T cells presented with profoundly altered lung architecture, with multiple foci of perivascular and peribronchial inflammation consisting of polymorphonuclear and some mononuclear cells (Fig. 6C). Consistent with this, VACV clearance was markedly impaired in CD8 T cell-depleted mice, which showed 1,000- and 10,000-fold increased viral load at day 7 p.i. compared with WT controls (Fig. 6D). Virus titers in the ovaries were also highly elevated (1,000–10,000-fold) p.i. with VACV-WR, similar to titers found in RAG$^{-/-}$-infected mice (Fig. 2C). A concern with systemically administered depleting Abs is their unexpected effect on other cell types important for protection. To verify this, we measured the proportion and total number of lung and splenic NK and NKT cells and found no reduction in mice transiently depleted of CD8α$^+$ cells (Supplemental Fig 1).

To determine the time at which CD8 T cells provide their protective function, we depleted CD8 T cells in WT mice starting on days −1, 3, or 6 after a primary infection. Mice depleted of

CD8 T cells throughout the course of infection or on day 3 onward failed to control the primary VACV-WR infection, which resulted in 100% mortality by day 8 p.i. (Fig. 6E). Both groups of mice presented with similar lung pathology, systemic inflammation and viral dissemination to that observed in MHC I$^{-/-}$ and CD8$^{-/-}$ mice (data not shown). Mice depleted of CD8 T cells after day 6 post–VACV-WR infection demonstrated comparable weight loss and survival to the isotype-treated (Ig) control mice (Fig. 6E). This time-dependent importance for CD8 T cells was further supported by the observation that VACV-specific CD8 T cells, which are capable of producing IFN-γ, began entering the lung tissue on day 4.5 d p.i., before increasing significantly by day 5.5 p.i. (Fig. 6F, 6G). Thus, our results demonstrate that an early CD8 T cell response to respiratory VACV infection is crucial for host defense, whereas CD4 T cells do not appear to be required for virus clearance or for the induction of this protective response.

Initial viral load and virulence critically determine the requirement for CD8 T cells in protection against a respiratory VACV infection

Next, we hypothesized that the protective requirement for CD8 T cells during a primary anti-VACV immune response in the lung may vary with the infective inoculum and the virulence of the strain used for infection. First we assessed the role of CD8 T cells during a respiratory VACV-WR infection with a reduced inoculum bolus. WT mice infected with 1.25×10^3 PFU of VACV-WR, almost a log less than our determined sublethal dose, experienced moderate weight loss (10–15%) but quickly recovered and went on to maintain normal weight after day 10 (Fig. 7A). Strikingly, mice depleted of CD8 T cells throughout this low-dose VACV infection presented weight loss and survival that were comparable to the control-treated mice (Fig. 7A). To investigate the requirement for CD8 T cell immunity during a less virulent VACV infection, we used two live-attenuated VACV variants that differ in their expression of several virulence factors that determine their replicative capacity and virulence in mice (37, 38). This reduced virulence is reflected by the need to use a significantly higher infectious dose (2×10^6 PFU/mouse) to provoke a measurable immune response, as well as the lack of significant weight loss or outward signs of illness throughout infection. Similar to a low-dose VACV-WR infection, the depletion of CD8 T cells throughout infection with Lister or NYCBOH did not affect weight loss, illness, or recovery (Fig. 7B, 7C). Therefore, CD8 T cells are necessary for protection against a respiratory VACV-WR infection but are not required when the infectious inoculum is reduced or a less virulent VACV strain is used.

CD8 T cells are sufficient for protection during a respiratory VACV-WR infection

We further addressed the ability of CD8 T cells to mediate protective immunity by adoptive transfer into naive RAG$^{-/-}$ mice. Splenic naive CD8 T cells (CD3$^+$CD8$^+$CD44^{low}) were enriched by magnetic bead purification and then sorted by FACS to a purity of 99–100%. 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 1×10^4 PFU VACV-WR and monitored for signs of weight loss and illness (Fig. 8A). As demonstrated previously, RAG$^{-/-}$ mice fail to survive infection and succumb by day 10 p.i. (Fig. 8B, 8C). In marked contrast, RAG$^{-/-}$ mice that received naive WT CD8 T cells were protected and exhibited survival rates comparable to WT controls (Fig. 8B, 8C). These results suggest that CD8 T cells can act independently of a humoral immune response to confer resistance to a respiratory VACV-WR infection.
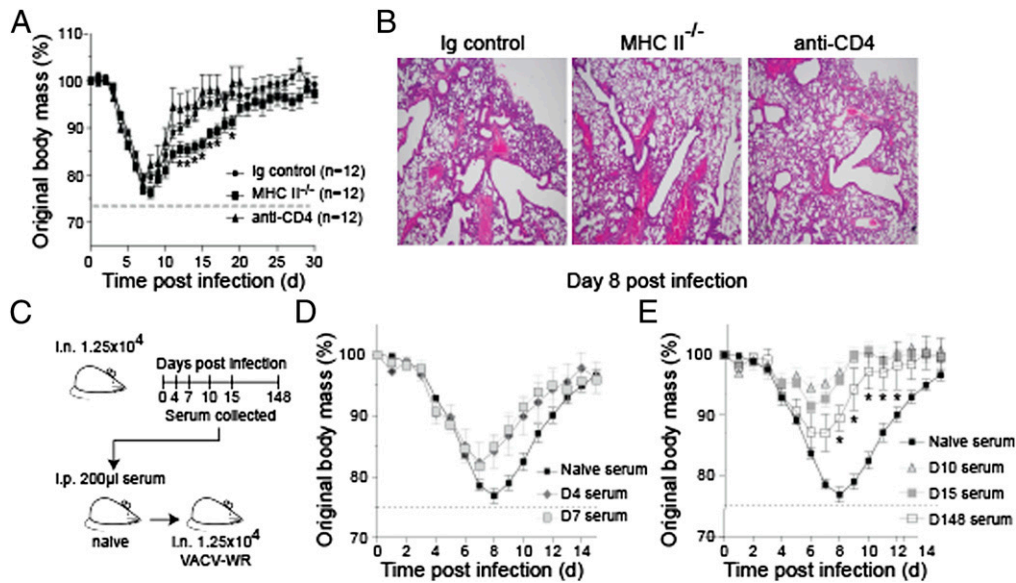
FIGURE 5. CD4 T cells and Ig are not required for protection against a respiratory VACV-WR infection. WT C57BL/6 and CD4 T cell-deficient mice (CD4 depleted and MHC II$^{-/-}$) were infected i.n. with 1×10^4 PFU VACV-WR. Body mass (**A**) and lung inflammation (**B**) were assessed on specified days p.i. (**C**) In a separate experiment, 200 μl of control (naive) and hyperimmune serum, isolated from WT mice on days 4, 7, 10, 15, and 148 post-i.n. infection with 1×10^4 PFU VACV-WR, was injected i.p. into naive WT mice. (**D** and **E**) Twenty-four hours later, the passively immunized mice were infected i.n. with 1×10^4 PFU VACV-WR, and their body mass was monitored daily. Data represent mean ± 1 SEM of three independent experiments with four to eight mice/group. The Student t test with the Bonferroni correction was used to determine statistical significance. *$p < 0.05$.

Discussion

To our knowledge, the current study is the first systematic evaluation of the host adaptive immune response to a respiratory VACV infection. We demonstrate that VACV infection results in a cell-mediated immune response with the induction of virus-specific CD8 CTLs and CD4 T cells, as well as a humoral response with the production of VACV-specific Ig. In contrast to alternative routes of VACV infection, we found that VACV-specific CD8 T cells, rather than CD4 T cells and Ig, are necessary and sufficient for clearing virus from the respiratory tract and protection against VACV-induced lung pathology and death. Most significantly, the protective requirement for CD8 T cells was critically dictated by the initial infection load and VACV strain virulence. These experiments help to define the precise immune mechanisms that govern the efficient generation of protective immunity against acute respiratory poxvirus infections.

For many years, the antiviral function of CD8 T cells in resistance against a primary infection with VACV has remained controversial. An early study by Spriggs et al. (8) showed that β2m$^{-/-}$ mice, which are devoid of CD8 T cells, were able to survive a s.c. infection with VACV-WR, even at doses exceeding 10^8 PFU, and they experienced little, if any, outward signs of viremia or illness. These results demonstrated directly that CD8 T cells are not required for the clearance of a VACV infection as long as humoral immunity is intact. Similarly, Xu et al. (7) demonstrated that, despite a robust cytotoxic CD8 T cell response in C57BL/6 mice following an i.p. VACV-WR infection, depletion of CD8 T cells by mAb or deficiency in CD8 cells did not alter virus clearance or survival in comparison with control-treated mice. On the contrary, CD4 T cells were shown to be essential for clearing an i.p. VACV infection. Accordingly, CD4-depleted and MHC class II-deficient mice were compromised in their ability to clear virus at day 14 p.i., and they harbored 100–1000-fold greater titers than did WT mice at day 20 p.i. B cell-deficient mice showed a similar inability to clear VACV as did CD4-depleted mice. This failure of CD4 T cell-depleted, MHC class II$^{-/-}$ and Igh$^{-/-}$ mice to clear a pri-

mary i.p. VACV infection was attributed to their inability to mount an effective Ig response. Thus, following an i.p. infection with VACV, CD4-dependent virus-specific Ig responses appear to be the most important effector mechanism required for clearing the virus from infected tissues.

Our data now extend these observations by demonstrating that, in the context of a respiratory VACV-WR infection, CD8 T cells are both necessary and sufficient for recovery from disease. Interestingly, despite a robust CD4 T cell and IgG response, we found that both CD4-depleted WT and MHC class II$^{-/-}$ mice, which are unable to elicit effective CD4 T cell or Ig responses, cleared the virus from their lungs at similar rates as did WT mice. A detailed kinetic analysis indicated that VACV replication in the lung is under control before serum IgG can be detected. Our data indicated that i.n. VACV-infected mice did not develop circulating anti-VACV IgG until day 15, with a strong IgG response present by day 30. This is significantly later than the time point at which the presence of VACV in the lung can be detected. Consistent with this, the passive transfer of serum, collected from days 4- and 7-infected mice, into naive recipients failed to protect them from disease caused by an i.n. VACV challenge. In contrast, mice that received hyperimmune serum isolated after day 10 exhibited a significant reduction in weight loss and accelerated recovery following an i.n. VACV challenge. These data provide further evidence to suggest that the development of a virus-specific Ig response occurs too slowly to limit VACV replication in the respiratory tract following a primary infection with VACV-WR. Rather, they suggest that the dominant role of a VACV-specific IgG response is to protect against secondary infections. However, the small delay in weight gain between days 10 and 18 p.i. observed in MHC II$^{-/-}$ mice suggests a possible role for CD4 T cells and/or Ig during the recovery period.

The current study provides several lines of evidence that suggest that trafficking of CD8 T cells into a VACV-infected mouse lung is critical for viral clearance, survival, and recovery. First, the emergence of IFN-γ–producing VACV-specific CD8 T cells in the
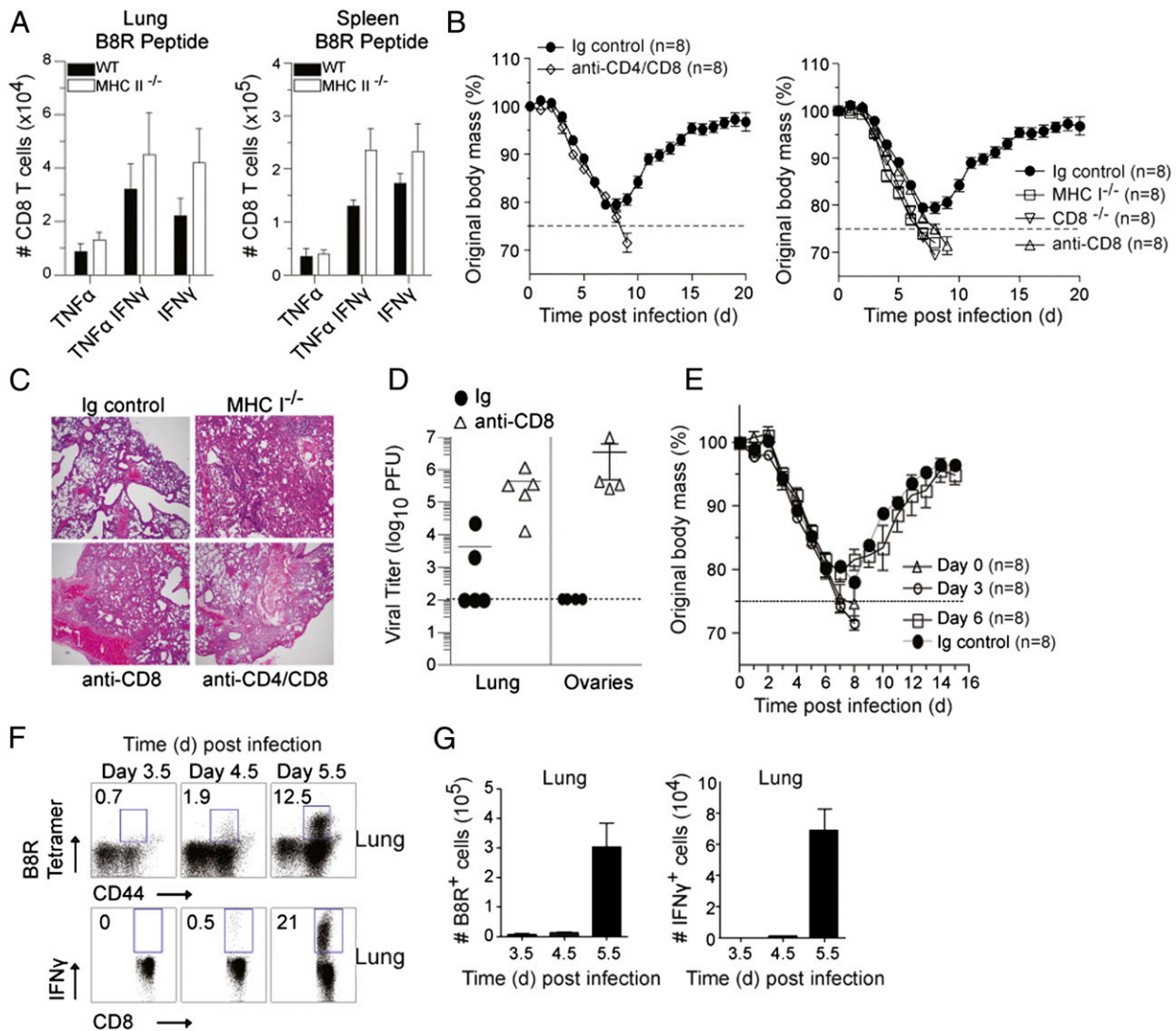
FIGURE 6. An early CD8 T cell response is necessary for protection during a respiratory VACV-WR infection. (**A**) Total lung and splenic IFN-γ– and TNF-α–producing CD8 T cells in WT C57BL/6 and MHC II$^{-/-}$ mice 8 d following i.n. infection with 1×10^4 PFU VACV-WR were assessed after ex vivo stimulation with B8R peptide. Subsequently, WT, CD4 and CD8 (**B**, *left panel*) and CD8-alone (B, *right panel*) T cell-deficient mice were infected i.n. with 1×10^4 PFU VACV-WR and monitored for weight loss. Lung inflammation (**C**) and tissue viral titers (**D**) in mice deficient in CD8 T cells were assessed at day 8 p.i. (**E**) Weight loss and survival were measured in WT mice that were depleted or not of CD8 T cells at the same time as or 3 or 6 d after i.n. infection with 1×10^4 PFU VACV-WR. In addition, the percentage (**F**) and total number (**G**) of lung B8R tetramer$^+$ and IFN-γ–producing CD8 T cells were enumerated on days 3.5, 4.5, and 5.5 p.i. Data are mean ± 1 SEM of three independent experiments with four to eight mice/group.

lung tissue paralleled the clearance of virus from the lungs of infected mice and the cessation of weight loss. Second, by depleting CD8 T cells with mAb treatment throughout the course of infection, we found that, in the absence of Ig, CD8 T cells are able to provide protection against an i.n. VACV infection in CD4-depleted mice. Third, the absence of CD8 T cells in MHC class I$^{-/-}$, CD8$^{-/-}$ mice or mice transiently depleted of this subset failed to clear VACV, which resulted in severe lung immunopathology and 100% mortality by day 9 p.i. Fourth, our kinetic studies indicated that CD8 T cells perform their protective role between days 4 and 6 post-i.n. VACV infection. Interestingly, this was prior to the peak (days 7–10) of the CD8 T cell response, suggesting that relatively small numbers of CD8 T cells are capable of controlling VACV infection in the lung. Lastly, we observed that the adoptive transfer of large numbers of naive polyclonal CD8 T cells, presumably containing a very low frequency of VACV-reactive CD8 T cell clones, into RAG 1$^{-/-}$ re-

cipient mice resulted in a significantly reduced viral load in the lungs and fully protected mice from death following challenge with a lethal dose of VACV-WR. Collectively, these results demonstrate that CD8 T cells are a critical component of the adaptive immune response that act independently of CD4 T cell and humoral immune responses to control an acute respiratory VACV infection.

As described above, much of the existing literature investigating the protective components of primary VACV infections use i.p., intradermal, or s.c. infectious routes to simulate human vaccination and understand Ig development. This critical difference may explain the conflicting evidence for the requirement for CD8 T cells in protection against primary VACV infections. It is plausible that the inoculation of different tissues affects both the propensity of viral replication and dictates the kinetics and, thus, the relative importance, of an ensuing cellular or humoral immune response. We highlight that, despite infecting with 100–1000 times less
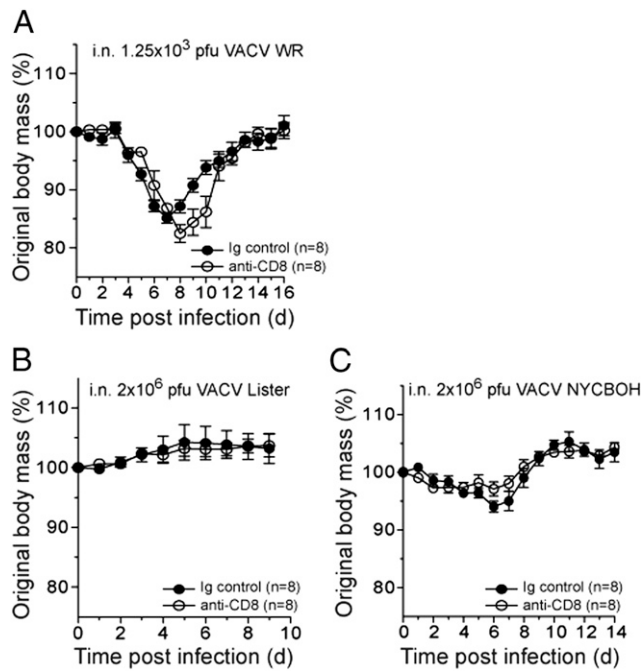
FIGURE 7. Initial viral load and virulence critically determine the requirement for CD8 T cells in WT protection against a respiratory VACV-WR infection. WT C57BL/6 mice were depleted of CD8 T cells and infected i.n. with 1.25×10^3 PFU VACV-WR (low dose) (**A**), 2×10^6 PFU VACV Lister (**B**), or 2×10^6 PFU VACV NYCBOH (**C**) and monitored for weight loss. Data are mean $\pm$ 1 SEM of three independent experiments with four to eight mice/group.

VACV than that used during an i.p. or s.c. infection, CD8 T cells are necessary for protection following a respiratory VACV-WR infection. This suggests that the route of infection dictates either the virulence of VACV or its capacity to replicate rapidly and, thus, the requirement for a protective CD8 T cell response. Essentially, the greater the virulence or capacity for viral replication, the greater the requirement for CD8 T cells for protection. This concept is supported by evidence generated using the mouse-adapted and highly virulent ECTV. Analogous to a respiratory VACV infection, ECTV infection requires a rapid CD8 T cell response to contain initial viral titers before virus-specific Ig is generated and assists in eliminating virus from infected tissues (39, 40). We also show that a reduction in the primary infectious dose, a parameter of replicative capacity, or decreased VACV virulence abrogated the requirement for CD8 T cells for protec-

tion. Clearly, an important component of replicative capacity is the amount of time available for viral replication before a protective immune response is elicited. We demonstrate that CD8 T cell–mediated immunity in the lung occurs between days 4 and 6 p.i. This suggests the existence of a time-dependent titer threshold. One can consider that if the titer of VACV in the lung or infected tissue does not reach a specific threshold, CD8 T cells are not required for protection. However, if this threshold is surpassed (by a greater inoculum dose or increased rate of viral replication), CD8 T cells are required and are essential for protection. In addition to viral titer, the time taken to reach this threshold or for alternative mechanisms of immunity to develop might explain the differential requirement for CD8 T cells during other primary VACV infection models. We demonstrate that, despite the generation of a long-lived virus-specific IgG response following a respiratory VACV-WR infection, significant GC B cell number or Ig titers were not detected until day 10 p.i. In contrast, following an i.p. VACV-WR infection, GC and plasma B cells are readily observed in the spleen as early as day 5, implying that the speed of Ag mobilization, presentation, and Ig production might negate the requirement for CD8 T cells when infected via this route (33, 34). The ability of Ig to gain access to the site of infection might also impact the effectiveness of alternative protective immune mechanisms. For instance, it was shown in mice and in humans that a neutralizing IgM response following VACV immunization is present within the first 5 d p.i. and likely contributes to virus control before the development of IgG (33). However, our serum-transfer data suggest that the presence of IgM on day 7 following an i.n. VACV infection provides little, if any, protection, again highlighting the requirement for an early CD8 T cell response. Although pentameric IgM isotypes contain a functional J chain that allows *trans*-epithelial secretion, its protective role during a respiratory VACV infection remains to be determined.

In summary, this study adds to the current literature by providing evidence that CD8 T cells are both necessary and sufficient for protecting against a primary VACV-WR infection of the respiratory tract. These results highlight the plasticity of the immune system in combating VACV infections administered via different routes. Moreover, they have important implications in extending our understanding of host–pathogen interactions, as well as in the development of novel vaccines and therapeutics for human respiratory Orthopoxvirus infections.

Disclosures
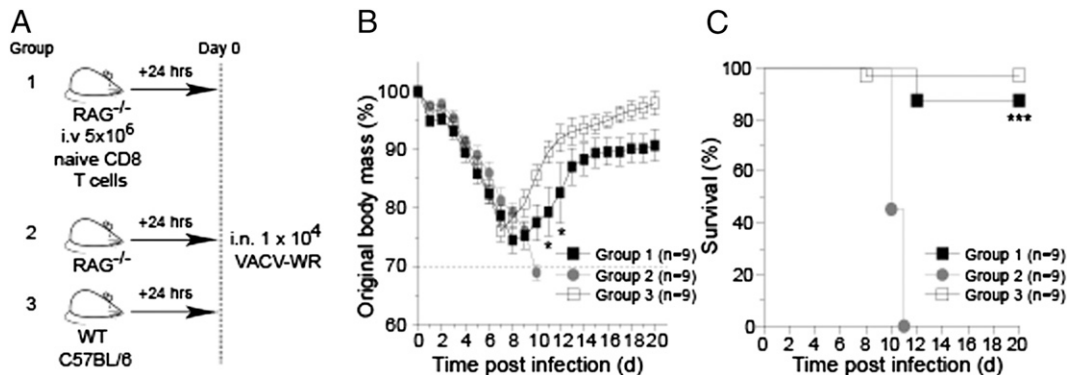
The authors have no financial conflicts of interest.

FIGURE 8. CD8 T cells are sufficient for protection during a respiratory VACV-WR infection. (**A**) A total of 5×10^6 naive (CD3^{+}CD8^{+}CD44^{low}) WT CD8 T cells were transferred i.v. into RAG^{−/−} mice that were infected i.n. 24 h later with 1×10^4 PFU VACV-WR. Body mass (**B**) and illness and survival (**C**) were followed throughout the experiment. Data are mean $\pm$ 1 SEM of three independent experiments with four or five mice/group. Two-way ANOVA and Mantel–Cox tests were used to determine statistical significance. *$p < 0.05$, ***$p < 0.001$.

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