Perforin-dependent CD4$^+$ T-cell cytotoxicity contributes to control a murine poxvirus infection

Min Fang[a], Nicholas A. Siciliano[b], Adam R. Hersperger[b], Felicia Roscoe[a], Angela Hu[a], Xueying Ma[a], Ahamed R. Shamsedeen[a], Laurence C. Eisenlohr[b], and Luis J. Sigal[a,1]

[a]Immune Cell Development and Host Defense Program, Fox Chase Cancer Center, Philadelphia, PA 19111; and [b]Department of Microbiology and Immunology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107

Edited by Bernard Moss, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, and approved May 16, 2012 (received for review February 6, 2012)

CD4$^+$ T cells are generally regarded as helpers and regulators of the immune response. Although cytolytic CD4$^+$ T cells have been described, whether those generated during the course of a viral infection play a role in virus control remains unknown. Here we show that during acute infection with ectromelia virus, the mouse homolog of the human virus of smallpox, large numbers of CD4$^+$ T cells in the draining lymph node and liver of resistant mice have a cytotoxic phenotype. We also show that these cells kill targets in vivo in a perforin-dependent manner and that mice with specific deficiency of perforin in CD4$^+$ T cells have impaired virus control. Thus, perforin-dependent CD4$^+$ T-cell killing of infected cells is an important mechanism of antiviral defense.

CD4$^+$ T lymphocytes | cytotoxic T lymphocytes | viral immunology | viral pathogenesis

T-cell–mediated immunity is a major mechanism providing specific protection against viruses. CD8$^+$ T cells recognize peptides associated with MHC class I (MHC I) proteins and CD4$^+$ T cells recognize peptides associated with MHC class II (MHC II) molecules. Traditionally, CD4$^+$ T lymphocytes have been described as helpers and regulators of the immune response, and cytotoxic T lymphocyte (CTL) effector functions have been attributed mostly to CD8$^+$ T cells. However, the existence of MHC II-restricted CD4$^+$ CTL has been recognized since the late 1970s (1). CD4$^+$ CTL clones have been generated in humans and mice (2, 3). The presence of CD4$^+$ CTL during primary infection were initially documented in mice that lacked the normal complement of CD8$^+$ T cells (4, 5) or during chronic or persistent viral infections (6, 7), supporting the notion that CD4$^+$ CTL can be generated by chronic exposure to antigen or repeated in vitro stimulation. However, CD4$^+$ CTL capable of killing target cells in vivo have been described more recently in immunocompetent mice infected with the Armstrong strain of lymphocytic choriomeningitis virus (LCMV) (8) and with West Nile Virus (9), indicating that CD4$^+$ CTL can also be generated during acute viral infections.

CD8$^+$ CTL mainly kill targets by releasing perforin (Prf) and granzymes, such as granzyme B (GzB) through granule exocytosis (GE) (10, 11). Although human and mouse CD4$^+$ T cells have been shown to lyse cells through Fas-Fas ligand interactions (12–14), GE-mediated CD4$^+$ T-cell cytotoxicity has also been shown in human infections with HIV, human cytomegalovirus, Epstein-Barr virus, herpes simplex virus, influenza virus, and vaccinia virus (2, 15–20).

Regarding a possible role for CD4$^+$ CTL in protective immunity, previous work by others showed that adoptive transfer of in vitro primed CD4$^+$ CTL protected from lethal influenza infection in a Prf-dependent manner (17). However, whether CD4$^+$ CTL generated during the course of a viral infection play any role in virus control remains to be demonstrated (3), most likely because it has been difficult to distinguish from the protective effects of CD8$^+$ CTL.

The Orthopoxvirus (OPV) Ectromelia virus (ECTV), the agent of mousepox, has host specificity for the mouse. This virus is very similar to variola virus, the agent of human smallpox, the zoonotic monkeypox virus, and the smallpox vaccine species vaccinia virus (VACV) (21–25). Following infection in the footpad, its natural route of entry, ECTV infects the popliteal draining lymph node (D-LN) and spreads through the lympho-hematogenous (LH) route to seed visceral organs, mainly the liver and the spleen. Of interest, ECTV LH spread is usually used as the textbook example for the many human and animal natural viruses that, with some variations, spread through the LH route (26, 27). Although ECTV infects all mouse strains, the outcome of primary infection following footpad inoculation varies. Susceptible strains, such as BALB/c, develop mousepox, which is characterized by uncontrolled replication of the virus in the liver and spleen. Most susceptible mice die with extensive liver damage within the first 14 d postinfection (dpi), and those that survive develop the typical skin rash of poxvirus infections. In resistant strains, such as C57BL/6 (B6), the virus also infects the liver and spleen but the mice do not show overt symptoms of disease because the replication of the virus is controlled by the combined action of innate and adaptive immune mechanisms (21, 23, 25, 28–33).

Our previous studies and those of others have shown that CD4$^+$ T-cell help-independent CD8$^+$ T-cell responses are essential for the early—and that CD4$^+$ T-cell help-dependent Ab responses are essential for the late but not the early—control of primary ECTV infections (29, 34–36). Previous work suggested that, in addition to providing help to B cells, CD4$^+$ T cells may also have a direct effector function to control ECTV but the specific mechanism was not identified (31). Here we report that the CD4$^+$ T-cell responses to ECTV are very large, particularly in the LN, draining the primary site of infection, and in the liver. Moreover, we show that most responding CD4$^+$ T cells have characteristics of CTL because they are GzB$^+$. We also show that the responding CD4$^+$ T cells have in vivo Prf-dependent, MHC II-restricted CTL activity and that CD4$^+$ directly contributes to controlling the virus in a Prf-dependent manner. Thus, our work is unique in showing that CD4$^+$ CTL have an important role in controlling a viral infection.

Results

ECTV Induced Large Numbers of Potentially Cytolytic CD4$^+$ T Cells. CD11a and CD49d coexpression marks activated CD4$^+$ T cells induced by antigen exposure (37, 38). When we determined the

Author contributions: M.F. and L.J.S. designed research; M.F., N.A.S., A.R.H., F.R., A.H., X.M., and A.R.S. performed research; L.C.E. contributed new reagents/analytic tools; M.F., N.A.S., A.R.H., L.C.E., and L.J.S. analyzed data; and M.F. and L.J.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: luis.sigal@fccc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202143109/-/DCSupplemental.

CD4$^+$ T-cell response to ECTV in C57BL/6 (B6) mice using these markers, we found that at 5 dpi ~60% of the CD4$^+$ T cells in the D-LN were CD11a$^+$ CD49d$^+$, indicating strong virus-specific CD4$^+$ T-cell responses. Interestingly, ~60% of the CD11a$^+$ CD49d$^+$ CD4$^+$ T cells were also GzB$^+$ (Fig. 1*A*). In the reverse analysis, ~95% of the GzB$^+$ CD4$^+$ T cells were CD11a$^+$ CD49d$^+$, indicating that GzB expression occurs only in antigen experienced cells (Fig. 1*B*). At 7 dpi, ~70% of the CD4$^+$ T cells in the D-LN and liver were CD11a$^+$ CD49d$^+$ and ~60% of these were GzB$^+$. In the spleen ~40% were CD11a$^+$ CD49d$^+$ and 40% of these were GzB$^+$ but the reverse analysis showed that the majority of the GzB$^+$ but not of the GzB$^-$ CD4$^+$ T cells in all organs were CD11a$^+$CD49d$^+$ (Fig. S1*A*). When the total CD4$^+$ T cells were considered, ~40% in the D-LN but low background frequencies in the liver or spleen were GzB$^+$ at 5 dpi, and 40% in the D-LN and liver and 15% in the spleen were GzB$^+$ at 7 dpi; however, the absolute number of GzB$^+$ cells was higher in the spleen than in the other organs (Fig. 1*C*).

To further address the extent of the CD4$^+$ T-cell response to ECTV, we inoculated B6 mice at different stages of infection with BrdU intraperitoneally. Three hours later, proliferation by BrdU incorporation was determined by flow cytometry in various organs. BrdU incorporation by CD4$^+$ T cells peaked at 5 dpi and remained high at 7 dpi in the D-LN. Incorporation of BrdU in spleen and liver was low at 5 dpi but increased significantly at 7 dpi. Of interest, the proliferative response was higher in the D-LN and liver than in the spleen. More important, most of the CD4$^+$ T cells that incorporated BrdU$^+$ expressed GzB (Fig. S1*B*). We also found that CD4$^+$ T cells from mice at 7 dpi and exposed to infected cells ex vivo expressed surface CD107a, a sign of degranulation (Fig. S1*C*). Thus, the CD4$^+$ T-cell responses to ECTV are large, particularly in the D-LN and liver and, consistent with a CTL phenotype, most of the responding CD4$^+$ T cells are GzB$^+$.

VACV MHC II-Restricted CD4$^+$ T-Cell Determinant I1L$_{7–21}$ Is also an ECTV Determinant. To identify ECTV CD4$^+$ T-cell determinants, we screened B6 mice for responses to the previously described VACV determinants H3L$_{272–286}$, I1L$_{7–21}$, and B5$_{46–60}$ (39, 40), which are highly conserved in ECTV. Compared with uninfected mice, a significantly larger proportion of the splenic CD4$^+$ T cells from infected mice stained with I-Ab-I1L$_{7–21}$ (QLVFNSISAR-ALKAY in VACV and the tetramer; QLIFNSISARALKAY in ECTV, bold underlined letters indicate a different amino acid between VACV and ECTV) but not with tetramers loaded with H3L$_{272–286}$, B5$_{46–60}$ or control CLIP peptide (Fig. S2*A*). In addition, a small but clear population of splenic CD4$^+$ T cells from infected mice produced IFN-γ when stimulated in vitro with the ECTV variant of I1L$_{7–21}$ (Fig. S2*B*). Moreover, more CD4$^+$ T cells stained with I-A^b-I1L$_{7–21}$ at 5 dpi in the D-LN and at 7 dpi in the liver in infected compared with uninfected mice (Fig. 2*A*). Furthermore, there was a significantly higher number of I1L$_{7–21}$ but not H3L$_{272–286}$ specific CD4$^+$ T cells in the spleens and livers at 5 and 7 dpi compared with uninfected mice in ELISPOT assays (Fig. 2*B* and Fig. S2*C*). Hence, the VACV CD4$^+$ T-cell determinant I1L$_{7–21,}$ (39) is also an ECTV CD4$^+$ T-cell determinant.

MHC II$^+$ Cells Are Targets of Virus-Specific Cytolytic CD4$^+$ T Cells in ECTV-Infected Mice. Most cells infected with ECTV in the D-LN are MHC II$^+$, as revealed by infection with a recombinant ECTV that expresses enhanced green fluorescence protein (ECTV-OVA197-386-IRES-EGFP) (Fig. 3*A*). We next tested whether ECTV induces CD4$^+$ CTL capable of killing MHC II$^+$ targets pulsed with I1L$_{7–21}$ in vivo. Splenocytes from B6.CD45.1 (CD45.1$^+$) mice stained with 0.8 μM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE^{low}) and pulsed with I1L$_{7–21}$ peptide, or stained with 4 μM CFSE (CFSE^{high}) and mock-pulsed, were cotransferred 1:1 into ECTV-infected B6 (CD45.2$^+$) mice at different days postinfection. Sixteen hours after transfer, the specific killing of MHC II$^+$ and MHC II$^-$ CFSE^{low} targets was determined in different organs (Fig. 3*B*). At 5 dpi, MHC II-restricted in vivo killing of target cells pulsed with the I1L$_{7–21}$ peptide was observed in the D-LN but not in the liver or spleen (Fig. 3*C*). However, strong MHC II-restricted specific killing was observed in the liver and to a lesser extent in the spleen and nondraining lymph node (ND-LN) at 7 dpi. Thus, the in vivo killing of MHC

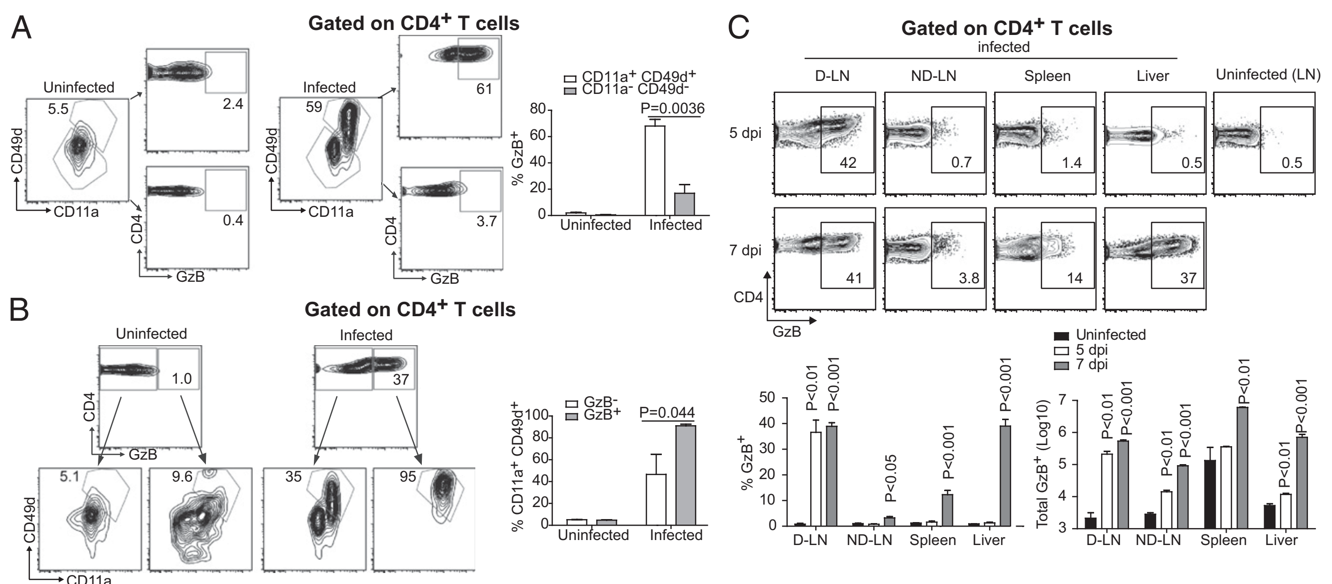

Fig. 1. ECTV infection induces a large number of potentially cytolytic CD4$^+$ T cells. B6 mice were infected with 3×10^3 pfu ECTV or left uninfected. (*A*) Representative flow cytometry plots with gating strategy and summary graphs showing the frequency of GzB expression in CD11a$^+$CD49d$^+$ and CD11a$^-$CD49d$^-$ CD4$^+$ T cells at 5 dpi. (*B*) As in *A* but showing the frequency of CD11a and CD49d coexpression in GzB$^+$ and GzB$^-$ CD4$^+$ T cells. (*C*) Representative flow cytometry plots and column graphs showing the frequency and absolute number of GzB expression in CD4$^+$ T cells at 5 and 7 dpi. Shown are the significant *P* values between the corresponding column vs. the same organ in the uninfected controls. All of the data are representative of two to four similar experiments.

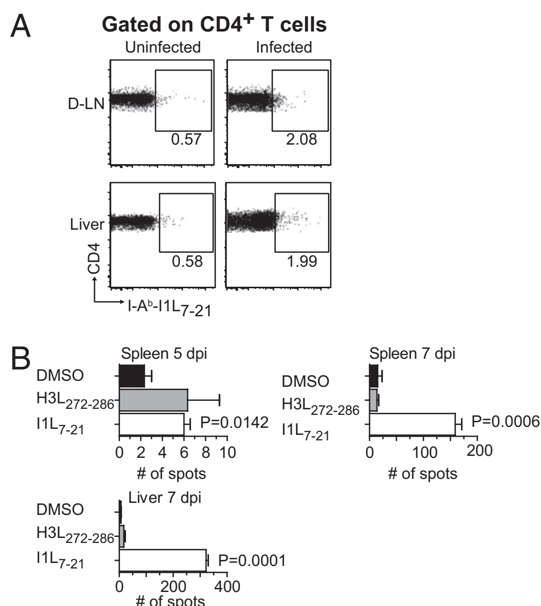

Fig. 2. The VACV MHC II-restricted CD4+ T-cell determinant $I1L_{7–21}$ is also an ECTV determinant. B6 mice were infected with 3×10^3 pfu ECTV or left uninfected. (*A*) The D-LN and liver cells stained with I-A^b-$I1L_{7–21}$ tetramer at 7 dpi. (*B*) ELISPOT assay for CD4+ T cells from indicated organs at 5 or 7 dpi cultured with DMSO (negative control), ECTV $H3L_{272–286}$, ECTV $I1L_{7–21}$ and ConA (positive control). *P* values that were significant when comparing with DMSO control are shown. Data are representative of two or three similar experiments. A picture of the ELISPOT plate is shown in Fig. S2.

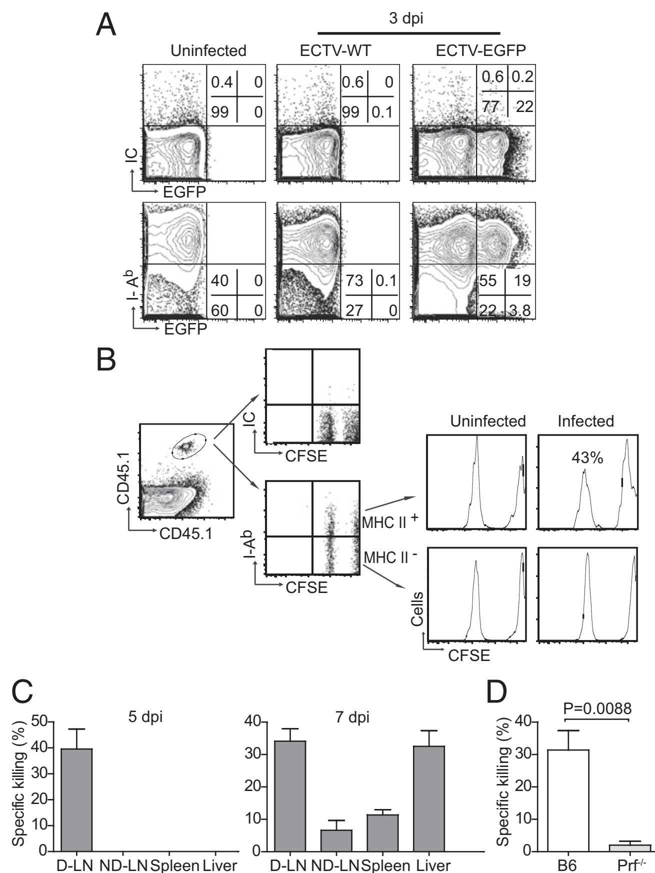

Fig. 3. MHC II+ cells are targets of virus-specific cytolytic CD4+ T cells in ECTV-infected mice. (*A*) B6 mice were infected with 3×10^3 pfu ECTV expressing GFP or left uninfected. At 3 dpi, cells from the D-LN were stained with isotype control or anti-MHC II mAb. Data are representative of three similar experiments. (*B*) Strategy for the determination of in vivo MHC II-restricted killing. At the indicated days postinfection, the mice were cotransferred with a 1:1 mixture of 10^7 naive splenocytes from B6-CD45.1 mice labeled with 4 μM CFSE (CFSE^{high}) or labeled with 0.8 μM CFSE (CFSE^{low}) and pulsed with $I1L_{7–21}$. After 16 h the mice were killed and the cells double-stained for CD45.1 to facilitate identification of the transferred cells and for MHC II or isotype control (IC). The ratio of CFSE^{high} and CFSE^{low} cells in the CD45.1+ MHC II+ and CD45.1+ MHC II− cells was determined by flow cytometry and specific killing calculated as detailed in *Methods*. (*C*) B6 mice. Specific killing at 5 and 7 dpi in the D-LN, ND-LN, spleen, and liver as indicated. (*D*) Specific killing in the liver of the indicated mice at 7 dpi. The mice had been treated with the anti-OPV drug cidofovir to prevent death of the Prf$^{-/-}$ mice.

II-restricted $I1L_{7–21}$ pulsed targets was consistent with the frequencies of GzB+ CD4+ T cells in the different organs and with the kinetics of their response.

In addition to GzB, killing by GE requires Prf. Although GzB-deficient mice are not readily accessible, Prf-deficient (Prf$^{-/-}$) mice can be obtained commercially. Thus, we compared MHC II-restricted killing of targets pulsed with $I1L_{7–21}$ in the livers of B6 and Prf$^{-/-}$ mice infected with ECTV. Because Prf$^{-/-}$ mice are highly susceptible to mousepox, all groups were treated with the poxvirus inhibitor Cyodofovir at 4 dpi. We found that killing was absent in the Prf$^{-/-}$ mice, indicating that during ECTV infection, MHC II-restricted killing in vivo depends on the GE pathway (Fig. 3*D*).

CD4+ T Cells Have a Direct Role in Virus Control. Optimal primary CD8+ T-cell responses to ECTV infection are CD4+ T-cell–independent, as demonstrated in CD4-depleted and CD4-deficient mice (29, 35), and as we also show here in MHC II-deficient (MHC II$^{-/-}$) mice (Fig. 4*A*). Furthermore, although production of Abs to ECTV requires CD4+ T-cell help and anti-ECTV Abs are required for long-term survival to mousepox, the control of ECTV at 7 dpi is completely independent of Abs (34, 36). To determine whether the effector function of CD4+ T cells is required for efficient virus control, we measured virus titers at 7 dpi in B6 mice depleted of CD4+ T cells, CD8+ T cells, CD4+ and CD8+ T cells, and in MHC II$^{-/-}$ mice. The virus titers in the liver and spleen of the CD4+ T-cell–depleted or MHC II$^{-/-}$ mice were 10- to 100-fold higher than in intact B6 mice, but lower than in B6 mice depleted of CD8+ T cells. Depletion of CD4+ and CD8+ T cells resulted in significantly higher virus titers in the spleen compared with any single depletion (Fig. 4*B*). Thus, although CD8+ T cells appear to be more efficient, the effector functions of CD4+ significantly contribute to control ECTV loads. We also compared virus titers in CD4+ T-cell–depleted, as well as CD4$^{-/-}$ and CD8$^{-/-}$, mice to those of CD40$^{-/-}$ and B-cell–deficient mice, which are unable to make Ab responses but mount normal CD4+ and CD8+ T-cell responses (34). Confirming the preponderance of effector CD8+ T cells but also the significant contribution of effector CD4+ T cells to ECTV control, we found no differences in the virus titers between CD40$^{-/-}$, B-cell–deficient, and wild-type B6 mice. However, there were 10- to 100-fold higher virus titers (measured only in spleens) (Fig. 4*C*) of CD4+ T-cell–depleted or CD4$^{-/-}$ mice compared with wild-type B6 mice, and even higher virus loads in CD8$^{-/-}$ mice. When tested for survival, CD8$^{-/-}$ mice succumbed ∼7 dpi because of lack of early virus control by CD8+ T cells; B-cell–deficient mice and CD40$^{-/-}$ mice succumbed more than 40 dpi. On the other hand, MHC II$^{-/-}$ mice succumbed ∼20 dpi, indicating that in addition to their ability to provide help to B cells, CD4+ T cells have a direct role in controlling ECTV (Fig. 4*D*).

Prf Deficiency Exclusively in CD4+ T Cells Results in Deficient ECTV Control. To investigate whether CD4+ CTL are required to efficiently control ECTV, we depleted B6 mice of CD4+ T cells,

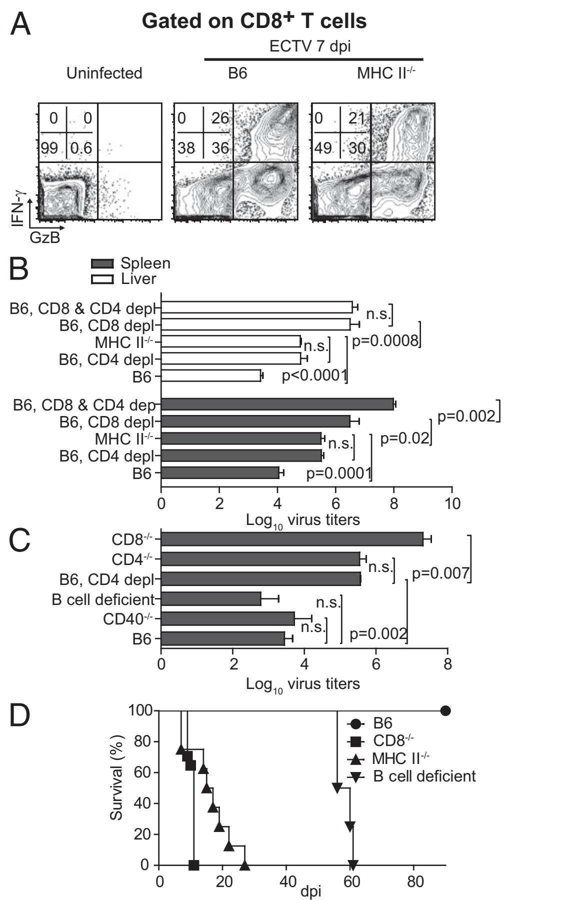

Fig. 4. CD4+ T cells have a direct role in virus control. (*A*) B6 and MHC $II^{-/-}$ mice were infected with 3×10^3 pfu ECTV. CD8+ T-cell responses were determined in the spleen at 7 dpi. Data correspond to a pool from three mice and is representative of three similar experiments. An uninfected B6 mouse is shown as control. (*B*) Virus titers in the indicated organs of the indicated mice at 7 dpi. mAb 2.43 and GK1.5 were used to deplete CD8+ and CD4+ T cells, respectively. (*C*) As in *B* but with the indicated mice. (*D*) Survival of the indicated mice following ECTV infection. Data correspond to three mice per group and is representative of three similar experiments.

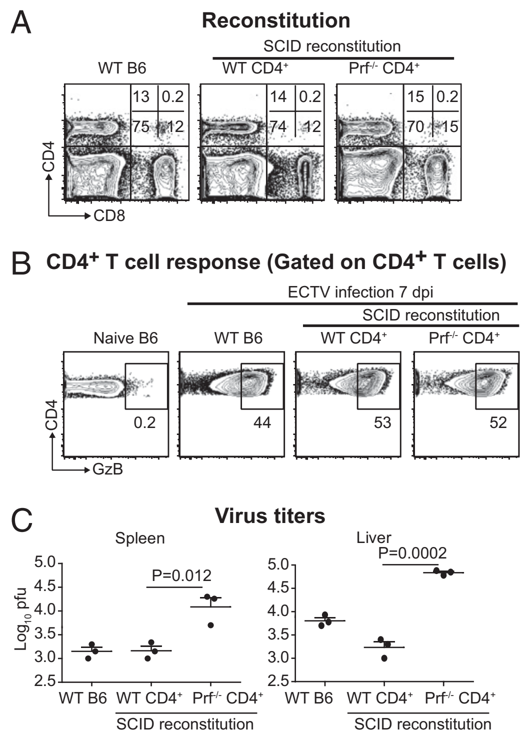

Fig. 5. Prf deficiency exclusively in CD4+ T cells results in deficient ECTV control. SCID mice were reconstituted with 10^8 of a 84/16 mixture of splenocytes from CD4-depleted B6 mice and magnetically purified CD4+ T cells from B6 or $Pfr^{-/-}$ mice. (*A*) CD4+ and CD8+ T cells in peripheral blood 2 wk after reconstitution. Data correspond to one representative mouse per group. A B6 control mouse is also shown. (*B*) Three weeks after reconstitution, SCID mice and B6 controls were infected with ECTV. Plots show expression of GzB and CD4 in gated CD4+ T-cell from the livers at 7 dpi. Data correspond to one representative mouse/group. (*C*) Virus titers at 7 dpi in spleen and liver of reconstituted SCID and control B6 mice. Data are representative of two experiments with three mice per group each.

harvested their splenocytes, mixed them with magnetically purified $CD4^+$ T cells from B6 or $Prf^{-/-}$ mice in an 84:16 ratio, and transferred them into SCID B6 mice. Two weeks after reconstitution, the mice had normal proportions of $CD4^+$ and $CD8^+$ T cells in the blood (Fig. 5*A*). Three weeks after reconstitution, the mice were challenged with ECTV. At 7 dpi, a similarly large proportion of $CD4^+$ T cells in the livers of the reconstituted SCID and control B6 mice expressed GzB (it should be noted that there are no good commercial Abs to identify Prf^+ cells by flow cytometry) (Fig. 5*B*). However, the virus titers in the liver and spleen of SCID mice reconstituted with $Prf^{-/-}$ $CD4^+$ T cells were significantly higher than in mice reconstituted with wild-type $CD4^+$ T cells (Fig. 5*C*). Thus, in mice that had a specific deficiency of Prf in $CD4^+$ T cells, the control of ECTV was defective. These data are unique in demonstrating that $CD4^+$ CTL generated during the course of a viral infection play a significant role in controlling the virus.

Discussion

Previous studies in mousepox-resistant B6 mice suggested that in addition to their helper functions, $CD4^+$ T cells may have an effector role in controlling ECTV infection (31). At the time, the authors did not investigate the mechanism but proposed it did not involve cytotoxicity. Here we found that the $CD4^+$ T-cell responses to ECTV are very strong and dominated by cells that express GzB, in particular in the D-LN and liver, where at the peak of the response they reached ~40% of the total $CD4^+$ T cells. To our knowledge, such extensive expression and tissue compartmentalization of a cytolytic molecule in $CD4^+$ T cells during a viral infection has not been documented.

The large proportion of GzB^+ $CD4^+$ T cells that we detected in the D-LN and liver also suggested that $CD4^+$ T-cell responses might be more extensive than generally thought. Our analysis of the kinetics of the $CD4^+$ T-cell response by BrdU incorporation confirmed that the $CD4^+$ T-cell responses are massive in the D-LN and liver but smaller in the spleen. Our results also indicated that the majority of the responding $CD4^+$ T cells ($CD11a^+$ $CD49d^+$) are GzB^+ in the D-LN and liver and, to a smaller extent, also in the spleen. Although it is commonly thought that the antiviral $CD4^+$ T-cell responses are quantitatively much smaller than those of $CD8^+$ T cells, our data are consistent with a recent report suggesting that $CD4^+$ T-cell responses are much larger than previously appreciated and showing that the anti-LCMV $CD4^+$ T-cell responses detected in peripheral blood lymphocytes comprised 50% of the $CD4^+$ T cells (38). Nevertheless, the $CD4^+$ T-cell responses to $I1L_{7–21}$, the most dominant determinant in our experiments and which is also the most dominant determinant yet indentified for VACV (39), consisted of no more than 1.5% of the total $CD4^+$ T cells. This finding contrasts with the $CD8^+$ T-cell response where 5–8% of the total $CD8^+$ T cells

are directed toward the dominant determinant $B8R_{20–27}$ (35, 41). One possibility is that for $CD4^+$ T cells, the total response spreads among a much broader set of determinants. Alternatively, the dominant determinant may remain to be discovered.

The existence of $CD4^+$ CTL in humans and mice is well documented (2, 3). Furthermore, it has been shown that ex vivo generated $CD4^+$ CTL can protect mice from influenza virus infection (17). However, whether the cytolytic $CD4^+$ T cells that develop during the course of a viral infection can play a significant protective role remained elusive (3), most likely because of their overlapping function with NK cells and $CD8^+$ T cells. Our finding that most of the antiviral $CD4^+$ T cells in the D-LN and liver expressed GzB, a molecule that is required for GE cytotoxicity, and that many ECTV-infected cells were MHC II^+, offered the possibility of addressing this important issue. Our analysis demonstrated that the antiviral $CD4^+$ CTL kill targets through GE in an MHC II-restricted manner, confirmed that $CD4^+$ T cells have a direct effector function in controlling ECTV infection, and demonstrated that the enhanced control of ECTV by $CD4^+$ T cells requires expression of Prf. To our knowledge, this demonstration that the $CD4^+$ CTL that develop during the course of a viral infection have a significant role in controlling a virus is unique. Many viruses that naturally infect humans and animals spread through the LH route and infect (MHC II^+) dendritic cells and macrophages in the D-LN (26). Thus, it is very possible that the cytolytic activity of the $CD4^+$ T cells also plays a role in their control. Thus, our results have important implications for our understanding of the functions of the immune system. Moreover, because $CD4^+$ CTL are so prevalent, the ECTV model offers a tractable system to identify the molecular mechanisms that result in their induction in vivo and this may be important for the development of novel vaccines.

Methods

Mice. The Fox Chase Cancer Center Institutional Animal Care and Use Committee approved the experimental protocols involving animals. Homozygous B6.129S-H2d$^{lAb1\text{-}Ea}$ (MHC $II^{-/-}$),B6.129S2-Igh-6$^{tm1Cgn/J}$ (B-cell–deficient), B6.129S2-Cd4$^{tm1Mak/J}$ ($CD4^{-/-}$), B6.129S2-Cd8a$^{tm1Mak/J}$ ($CD8^{-/-}$), C57BL/6-Prf1$^{tm1Sdz/J}$ ($Prf^{-/-}$), and B6.129P2-Cd40$^{tm1Kik/J}$ ($CD40^{-/-}$) were originally purchased from the Jackson Laboratories and bred at the Fox Chase Cancer Center Laboratory Animal Facility in specific pathogen-free rooms. Genotyping was according to the vendor's protocols. B6 mice were purchased from Taconic Farms or the Jackson Laboratories. CB6.CB17-*Prkdc^{scid}*/SzJ (SCID mice lacking T and B cells in C57BL/6J background) mice were purchased from the Jackson Laboratory. B6-LY5.2/Cr (B6-CD45.1) were purchased from the Frederick National Laboratory for Cancer Research. For reconstitution, splenocytes from B6 mice depleted of $CD4^+$ T cells 3 d before were mixed in an 84/16 ratio with magnetically purified $CD4^+$ cells (Miltenyi Biotech; Automacs and $CD4^+$ beads following the manufacturer's instructions) from wild-type B6 or $Prf^{-/-}$ mice and 10^8 transferred intravenously into SCID mice.

Infections. For infections, sex-matched 8- to 12-wk-old animals were used. Mice were infected in the left footpad with 25 μL PBS containing 3×10^3 pfu unless otherwise indicated. When indicated, 400 μg cidofovir (Vistide) was injected 4 dpi intraperitoneally.

Cell Culture, ECTV Moscow, Virus Production, and Determination of Virus Titers. Cell culture, ECTV Moscow, virus production, and determination of virus titers were done as described previously (42). ECTV-OVA197-386-IRES-EGFP was produced by homologous recombination as described previously (43) by introducing a C-terminal fragment of chicken ovalbumin coding for amino acids 197–386, followed by the internal ribosomal entry site from Encephalomyocarditis virus in front of EGFP.

Flow Cytometry. BrdU incorporation, isolation of liver lymphocytes, and intracellular staining was done as described previously (34, 42, 43). For MHC class II tetramer staining, APC labeled I-A^b -CLIP (PVSKMRMATPLLMQA), -$H3L_{272–286}$, -$I1L_{7–21}$, and -$B5_{46–60}$ tetramers were prepared by the National Institute of Allergy and Infectious Diseases tetramer facility and used according to their standard protocol. Peptide restimulation was performed for 6 h with bone marrow-derived primary dendritic cells obtained as described previously (44), and pulsed with 2 μg/mL peptide.

In Vivo Killing Assays. For in vivo killing assays, single-cell suspensions of lymphocytes from naive B6.Ly5.2 (CD45.1) mice (National Cancer Institute) were split into two. One set was labeled with 4 μM CFSE (CFSE^{high}) and the second set was labeled with 0.8 μM CFSE (CFSE^{low}) and pulsed with 1 μg/mL $I1L_{7–21}$ peptide). The two populations were mixed in a 1:1 ratio and 2×10^7 total cells were injected intravenously into recipient mice. Lymphocytes were isolated 16 h later from lymph nodes, livers, and spleens. The presence of CFSE^{low}, $CD45.1^+$, CFSE^{high}, MHC II^+ and MHC II^- cells was determined by flow cytometry. Specific lysis was calculated as (percentage CFSE^{high}/percentage CFSE^{low}). Percentage specific lysis = [1 − (ratio unprimed/ratio primed) × 100]. At least 100,000 cells were analyzed by flow cytometry using a LSR II system (BD Biosciences).

IFN-γ ELISPOT Assay. Groups of three B6 mice were infected in the footpad with 3×10^3 pfu ECTV. Cells isolated from the livers and spleens from each group were pooled ($n = 3$) and 5×10^5/well incubated at 37 °C and 5% CO_2 for 18 h in 96 well ELISpot plates (Millipore) together with 2×10^5 splenocytes from naive mice that had been pulsed with 10 μg/mL of the indicated peptide. IFN-γ^+ T-cell responses were assayed using IFN-γ ELISPOT (BD) as directed by the manufacturer. Spots were counted using ImmunoSpot software (Cellular Technology).

In Vivo Depletions. Depletion of $CD4^+$ and $CD8^+$ T cells was performed by intraperitoneal inoculation of 200 μg anti-CD4 mAb GK1.5 or 200 μg anti-CD8 mAb 2.43 1 d before ECTV infection. The efficiency of depletion was >95%, as determined by flow cytometric analysis of white blood cells one day after Ab inoculation.

Data Displayed and Statistical Analysis. Unless indicated, all displayed data correspond to one representative experiment of at least three similar experiments with groups of three to five mice. When indicated, the LNs, livers, and spleens from the mice in a group were pooled. Statistical analysis was performed using Prism (GraphPad Software) software. For survival studies, *P* values were obtained using the Log-rank (Mantel-Cox) test. All other statistical analyses were performed using an unpaired two-tailed Student *t* test or the Mann–Whitney test as applicable. When applicable, data are displayed with mean ± SEM.

ACKNOWLEDGMENTS. We thank Fox Chase Cancer Center Laboratory Animal, Flow Cytometry, and Tissue Culture Facilities for their services; Ms. Lucy Aburto for technical assistance; Ms. Holly Gillin for assistance in the preparation of the manuscript; and the National Institute of Allergy and Infectious Diseases (NIAID) Tetramer Facility for MHC II tetramers. This work was supported by NIAID Grants R01AI048849, R01AI065544, and 5U19AI083008 (to L.J.S.) and NCI Grant P30CA006927 (to the Fox Chase Cancer Center).

1. Billings P, Burakoff S, Dorf ME, Benacerraf B (1977) Cytotoxic T lymphocytes specific for I region determinants do not require interactions with H-2K or D gene products. *J Exp Med* 145:1387–1392.
2. Brown DM (2010) Cytolytic CD4 cells: Direct mediators in infectious disease and malignancy. *Cell Immunol* 262:89–95.
3. Marshall NB, Swain SL (2011) Cytotoxic CD4 T cells in antiviral immunity. *J Biomed Biotechnol* 2011:954602.
4. Muller D, et al. (1992) LCMV-specific, class II-restricted cytotoxic T cells in beta 2-microglobulin-deficient mice. *Science* 255:1576–1578.
5. Zajac AJ, Quinn DG, Cohen PL, Frelinger JA (1996) Fas-dependent CD4+ cytotoxic T-cell-mediated pathogenesis during virus infection. *Proc Natl Acad Sci USA* 93: 14730–14735.
6. Krensky AM, Ault KA, Reiss CS, Strominger JL, Burakoff SJ (1982) Generation of long-term human cytolytic cell lines with persistent natural killer activity. *J Immunol* 129:1748–1751.
7. Suni MA, et al. (2001) CD4(+)CD8(dim) T lymphocytes exhibit enhanced cytokine expression, proliferation and cytotoxic activity in response to HCMV and HIV-1 antigens. *Eur J Immunol* 31:2512–2520.
8. Jellison ER, Kim SK, Welsh RM (2005) Cutting edge: MHC class II-restricted killing in vivo during viral infection. *J Immunol* 174:614–618.
9. Brien JD, Uhrlaub JL, Nikolich-Zugich J (2008) West Nile virus-specific CD4 T cells exhibit direct antiviral cytokine secretion and cytotoxicity and are sufficient for antiviral protection. *J Immunol* 181:8568–8575.
10. Trapani JA, Smyth MJ (2002) Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2:735–747.

11. Trapani JA, et al. (1998) Efficient nuclear targeting of granzyme B and the nuclear consequences of apoptosis induced by granzyme B and perforin are caspase-dependent, but cell death is caspase-independent. *J Biol Chem* 273: 27934–27938.
12. Janssens W, et al. (2003) CD4+CD25+ T cells lyse antigen-presenting B cells by Fas-Fas ligand interaction in an epitope-specific manner. *J Immunol* 171:4604–4612.
13. Stalder T, Hahn S, Erb P (1994) Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. *J Immunol* 152:1127–1133.
14. Nikiforow S, Bottomly K, Miller G, Münz C (2003) Cytolytic CD4(+)-T-cell clones reactive to EBNA1 inhibit Epstein-Barr virus-induced B-cell proliferation. *J Virol* 77: 12088–12104.
15. Casazza JP, et al. (2006) Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *J Exp Med* 203:2865–2877.
16. Appay V, et al. (2002) Characterization of CD4(+) CTLs ex vivo. *J Immunol* 168: 5954–5958.
17. Brown DM, Dilzer AM, Meents DL, Swain SL (2006) CD4 T cell-mediated protection from lethal influenza: Perforin and antibody-mediated mechanisms give a one-two punch. *J Immunol* 177:2888–2898.
18. Khanolkar A, Yagita H, Cannon MJ (2001) Preferential utilization of the perforin/granzyme pathway for lysis of Epstein-Barr virus-transformed lymphoblastoid cells by virus-specific CD4+ T cells. *Virology* 287:79–88.
19. Niiya H, et al. (2005) Differential regulation of perforin expression in human CD4+ and CD8+ cytotoxic T lymphocytes. *Exp Hematol* 33:811–818.
20. Mitra-Kaushik S, Cruz J, Stern LJ, Ennis FA, Terajima M (2007) Human cytotoxic CD4+ T cells recognize HLA-DR1-restricted epitopes on vaccinia virus proteins A24R and D1R conserved among poxviruses. *J Immunol* 179:1303–1312.
21. Esteban DJ, Buller RM (2005) Ectromelia virus: The causative agent of mousepox. *J Gen Virol* 86:2645–2659.
22. Fenner F (1981) Mousepox (infectious ectromelia): Past, present, and future. *Lab Anim Sci* 31:553–559.
23. Fenner F (1994) Mousepox (ectromelia). *Virus Infections of Rodents and Lagomorphs, Virus Infections of Vertebrates*, ed Osterhaus A (Elsevier Science, Amsterdam, New York), Vol 5, p 412.
24. Fenner F (1949) Mouse-pox; infectious ectromelia of mice; a review. *J Immunol* 63: 341–373.
25. Fenner F, et al. (1988) *Smallpox and its Eradication* (World Health Organization, Geneva), p 1460.
26. Flint SJ, Enquist LW, Racaniello VR, Skalka AM (2009) *Principles of Virology* (ASM Press, Washington, DC), 3rd Ed.
27. Virgin HW (2007) Pathogenesis of viral infection. *Fields' Virology*, eds Fields BN, Knipe DM, Howley PM (Wolters kluwer/Lippincott Williams & Wilkins, Philadelphia), 5th Ed Vol 1, pp 335–336.
28. Wallace GD, Buller RM (1985) Kinetics of ectromelia virus (mousepox) transmission and clinical response in C57BL/6j, BALB/cByj and AKR/J inbred mice. *Lab Anim Sci* 35: 41–46.
29. Buller RM, Holmes KL, Hügin A, Frederickson TN, Morse HC, 3rd (1987) Induction of cytotoxic T-cell responses in vivo in the absence of CD4 helper cells. *Nature* 328:77–79.
30. Buller RM, Palumbo GJ (1991) Poxvirus pathogenesis. *Microbiol Rev* 55:80–122.
31. Karupiah G, Buller RM, Van Rooijen N, Duarte CJ, Chen J (1996) Different roles for CD4+ and CD8+ T lymphocytes and macrophage subsets in the control of a generalized virus infection. *J Virol* 70:8301–8309.
32. Karupiah G, Fredrickson TN, Holmes KL, Khairallah LH, Buller RM (1993) Importance of interferons in recovery from mousepox. *J Virol* 67:4214–4226.
33. Parker AK, Parker S, Yokoyama WM, Corbett JA, Buller RM (2007) Induction of natural killer cell responses by ectromelia virus controls infection. *J Virol* 81:4070–4079.
34. Fang M, Sigal LJ (2005) Antibodies and CD8+ T cells are complementary and essential for natural resistance to a highly lethal cytopathic virus. *J Immunol* 175:6829–6836.
35. Fang M, Sigal LJ (2006) Direct CD28 costimulation is required for CD8+ T cell-mediated resistance to an acute viral disease in a natural host. *J Immunol* 177:8027–8036.
36. Chaudhri G, Panchanathan V, Bluethmann H, Karupiah G (2006) Obligatory requirement for antibody in recovery from a primary poxvirus infection. *J Virol* 80: 6339–6344.
37. Butler NS, et al. (2012) Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection. *Nat Immunol* 13:188–195.
38. McDermott DS, Varga SM (2011) Quantifying antigen-specific CD4 T cells during a viral infection: CD4 T cell responses are larger than we think. *J Immunol* 187: 5568–5576.
39. Moutaftsi M, et al. (2007) Vaccinia virus-specific CD4+ T cell responses target a set of antigens largely distinct from those targeted by CD8+ T cell responses. *J Immunol* 178: 6814–6820.
40. Sette A, et al. (2008) Selective CD4+ T cell help for antibody responses to a large viral pathogen: Deterministic linkage of specificities. *Immunity* 28:847–858.
41. Tscharke DC, et al. (2005) Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines. *J Exp Med* 201:95–104.
42. Fang M, Cheng H, Dai Z, Bu Z, Sigal LJ (2006) Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial protection from a lethal orthopoxvirus infection in a natural host. *Virology* 345:231–243.
43. Fang M, Lanier LL, Sigal LJ (2008) A role for NKG2D in NK cell-mediated resistance to poxvirus disease. *PLoS Pathog* 4:e30.
44. Siciliano NA, Skinner JA, Yuk MH (2006) *Bordetella bronchiseptica* modulates macrophage phenotype leading to the inhibition of CD4+ T cell proliferation and the initiation of a Th17 immune response. *J Immunol* 177:7131–7138.

PNAS PNAS PNAS