Memory CD8+ T Cells Specific for a Single Immunodominant or Subdominant Determinant Induced by Peptide-Dendritic Cell Immunization Protect from an Acute Lethal Viral Disease

Sanda Remakus,a,b Daniel Rubio,a,c Xueying Ma,a Alessandro Sette,d and Luis J. Sigal,a

Fox Chase Cancer Center, Immune Cell Development and Host Defense Program, Philadelphia, Pennsylvania, USA; Jefferson Medical College of Thomas Jefferson University, Department of Microbiology and Immunology, Philadelphia, Pennsylvania, USA; Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, Campus de Cantoblanco, Madrid, Spain; and La Jolla Institute for Allergy and Immunology, La Jolla, California, USA

The antigens recognized by individual CD8+ T cells are small peptides bound to major histocompatibility complex (MHC) class I molecules. The CD8+ T cell response to a virus is restricted to several peptides, and the magnitudes of the effector as well as memory phases of the response to the individual peptides are generally hierarchical. The peptide eliciting a stronger response is called immunodominant (ID), and those with smaller-magnitude responses are termed subdominant (SD). The relative importance of ID and SD determinants in protective immunity remains to be fully elucidated. We previously showed that multispecific memory CD8+ T cells can protect susceptible mice from mousepox, an acute lethal viral disease. It remained unknown, however, whether CD8+ T cells specific for single ID or SD peptides could be protective. Here, we demonstrate that immunization with dendritic cells pulsed with ID and some but not all SD peptides induces memory CD8+ T cells that are fully capable of protecting susceptible mice from mousepox. Additionally, while natural killer (NK) cells are essential for the natural resistance of nonimmune C57BL/6 (B6) to mousepox, we show that memory CD8+ T cells of single specificity also protect B6 mice depleted of NK cells. This suggests it is feasible to produce effective antiviral CD8+ T cell vaccines using single CD8+ T cell determinants and that NK cells are no longer essential when memory CD8+ T cells are present.

During viral infections, viral proteins are degraded by the proteolytic machinery of the cell into small peptides. Peptides with the appropriate motif and that are 8 to 10 amino acids long bind to major histocompatibility class I (MHC-I) molecules in the endoplasmic reticulum and are transported to the cell surface for presentation to CD8+ T cells, which use clonotopic T cell receptors (TCR) encoded by somatically recombined genes to recognize specific MHC-I-bound peptides, also known as determinants (1). The magnitude of the CD8+ T cell response to the various MHC-I determinants of a virus is generally hierarchical, a phenomenon called T cell immunodominance (59, 60). The determinant that elicits the highest number of CD8+ T cells is termed immunodominant (ID), and those that induce smaller but detectable responses are known as subdominant (SD). Some peptides may bind to MHC-I molecules but be ignored by the CD8+ T cell response. Immunodominance is the result of many interacting factors affecting antigen-presenting cells (APC), such as antigen processing and presentation, and T cells, such as differences in precursor frequency, T cell receptor affinity, competition for activating stimuli, etc. (59, 60).

At the peak of an antiviral response, the frequency of virus-specific CD8+ T cells can be as high as 60 to 80% of the total CD8+ T cells (13, 38). These cells produce effector molecules, such as gamma interferon (IFN-γ), which has antiviral and immunomodulatory effects, and perforin (Prf) and granzyme B (GzB), which kill infected cells through granule exocytosis. If the virus is controlled, ~90% of the antiviral CD8+ T cells die, but some remain as memory CD8+ T cells (38). In the resting state, memory CD8+ T cells do not express effector molecules. However, upon antigen encounter, they rapidly become effectors and proliferate. In this way, they help to quickly control secondary infection by the same or similar viruses. It is thought that memory CD8+ T cells play an important role in vaccine protection, and there is a strong impetus in designing new vaccines that induce protective antiviral CD8+ T cell memory. Therefore, it is of interest to determine the level of protection that can be conferred by memory CD8+ T cells specific for ID or SD determinants during a lethal viral infection.

It has been reported that peptide-dendritic cell (DC) vaccination with a Listeria monocytogenes ID determinant reduced bacterial burden (3). It has also been shown that immunization with recombinant vaccinia virus (VACV) expressing various lymphocytic choriomeningitis virus (LCMV) determinants protected mice from lethal intracranial LCMV challenge infection (28, 29, 56). In addition, the same VACV recombinants (41) and DNA vaccines expressing the LCMV nucleoprotein (NP) containing an ID determinant (34) protected mice from chronic LCMV clone 13 infection administered intravenously (i.v.). Presently, it remains unknown whether memory CD8+ T cells specific for single ID or SD determinants can protect from a lethal acute systemic viral infection that spreads via the lympho-hematogenous route in its natural host.

Natural killer (NK) cells are cells of the innate immune system that are essential for resistance to several primary viral infections (9, 10, 25, 26, 43). Similar to CD8+ T cells, their main effector...
mechanism are the production of IFN-γ and killing of infected cells by granule exocytosis (5, 7, 18, 22, 32). Different from CD8+ T cells, however, NK cells recognize infected cells using germ line-encoded activating receptors rather than antigen-specific receptors (30). Because NK cells do not need to expand clonotypically, they can contribute to virus control during the first few days of infection, when the adaptive response is still incipient. Because their effector functions overlap, it remains possible that NK cells are no longer required when antiviral memory CD8+ T cells are present at relatively high frequencies; however, this possibility has not been thoroughly explored.

Orthopoxviruses (OPV) are a genus of highly conserved DNA viruses that includes, among others, variola virus, the causative agent of smallpox in humans, VACV, the virus used as the smallpox vaccine, and ectromelia virus (ECTV), the causative agent of mousepox in mice (16). Different from VACV, which is often used as the prototypic OPV, ECTV naturally infects the mouse. When inoculated with as little as 1 PFU in the footpad, its natural route of infection, ECTV causes disease and death in susceptible mouse strains, including BALB/c (H-2d) (54) and B6.D2-(D6Mit149-D6Mit15)/LusJ, a congenic strain of C57BL/6 (B6) that carries the distal portion of chromosome 6 of the susceptible DBA/2 strain, and referred to here as B6.D2-D6 (H-2d) (11, 15). On the other hand, B6 mice are naturally resistant to mousepox but become susceptible if depleted of NK cells before or soon after infection (9, 10, 43).

We have previously shown that memory CD8+ T cells can protect susceptible mice from lethal mousepox (58). Therefore, ECTV infection of susceptible mice serves as a model to understand the mechanisms of CD8+ T cell protective immunity. Work by others has shown that the sequence of the ID H-2 Kbeta-restricted determinant TSYKFESV (amino acids 20 to 27 of the B8R protein) of VACV is fully conserved in ECTV (48). We found that several SD determinants of VACV are also fully conserved and serve as SD determinants in ECTV. Armed with this knowledge, we immunized susceptible B6.D2-D6 mice with DCs pulsed with the ID or SD peptides. We found that this method of immunization resulted in the induction of a high frequency of memory CD8+ T cells to some but not all the peptides. B6.D2-D6 mice immunized with those peptides that successfully induced high frequencies of memory CD8+ T cells were protected from mousepox, regardless of their immunodominance hierarchy during infection. Additionally, we found that B6 mice immunized with TSYKFESV-pulsed DCs remained resistant to mousepox after NK cell depletion (10, 25, 43), suggesting that when memory CD8+ T cells are present, NK cells may no longer be required for resistance to viral diseases. Our findings are important for a thorough understanding of the mechanisms of protective T cell immunity and for the rational development of CD8+ T cell vaccines.

MATERIALS AND METHODS

Ethics statement. All experiments were performed following guidelines of the National Institutes of Health. The Fox Chase Cancer Center Institutional Animal Care and Use Committee approved the experimental protocols involving animals.

Viruses. Initial stocks of the wild-type (WT) ECTV Moscow (6, 15) were obtained from ATCC (VR-1374). New stocks of ECTV WT were expanded in B5-C1 cells infected with 0.1 PFU/cell as described previously (57). Briefly, B5-C1 cells in T150 flasks were infected with 0.1 PFU/cell. After 3 or 4 days cells were collected, resuspended in phosphate-buffered saline (PBS), frozen and thawed three times, and stored in aliquots at −80°C as virus stock. Virus titers in ECTV stocks were determined by plaque assays on confluent B5-C-1 cells by using 10-fold serial dilutions of the stocks in 0.5 ml Dulbecco's modifed Eagle's medium (DMEM)−2.5% fetal bovine serum (FBS) in 6-well plates (2 wells/dilution) for 1 h. Two milliliters of fresh DMEM−2.5% FBS was added, and the cells were incubated at 37°C for 5 days. Next, the medium was aspirated and the cells were fixed for 1 h with 3.7% paraformaldehyde, washed with water, and stained with 0.1% crystal violet in 20% ethanol. The fix/stain solution was subsequently aspirated, the cells air dried, the plaques counted, and PFU/ml values in stocks were calculated accordingly.

For the determination of virus titers in spleens, the spleens were removed from experimental mice on the indicated days after footpad infection, made into a single-cell suspension between two frosted slides, and resuspended in 10 ml complete RPMI medium. One-milliliter aliquots of the cell suspensions were frozen and thawed three times, and titers were determined in 10-fold serial dilutions of the cell lysates as described above. Virus titers were calculated as PFU/spleen. To determine the virus titers in liver, a portion of the liver was weighed and homogenized in medium by using a tissue lyser (Qiagen). The virus titers were calculated as PFU/gram of liver.

Mice and infections. The Fox Chase Cancer Center Institutional Animal Care and Use Committee approved the experimental protocols involving animals. C57BL/6 mice were purchased from Taconic when they were 8 to 10 weeks of age and were rested at least a week before use in experiments. The B6.D2-(D6Mit149-D6Mit15)/LusJ (B6.D2-D6) mice were initially purchased from Jackson Laboratory and bred in the Fox Chase Cancer Center Laboratory Animal Facility. Unless indicated, mice were infected with ECTV in the left footpad with 25 μl PBS containing 3 × 106 PFU. Following infections, mice were observed daily for signs of disease (lethargy, ruffled hair, weight loss, skin rash, eye secretions) and imminent death (unresponsiveness to touch, lack of voluntary movements).

In vivo cytotoxicity assays. In vivo cytotoxicity assays were performed as described elsewhere (14). Briefly, red blood cell-depleted spleenocytes from naive B6 mice were split into two populations. One population was labeled with a high concentration of carboxyfluorescein succinimidyl ester (CFSE) at 4 μM (CFSEhigh) and pulsed with SIINFEKL or a VACV/ECTV determinant, TSYKFESV (88R290-27), SIFRFLN1 (J3R189-209), KSYNYML (A3L20-237), ITYRFYL1 (A8R189-196), or STLNFNLN (E7R130-137) (GenScript) at a final concentration of 1 μg/ml. For in vivo cytotoxicity assays in DC-vaccinated memory mice, the second population of lymphocytes was labeled with a low concentration of CFSE (0.8 μM; CFSElow) and was pulsed with the SIINFEKL peptide at a final concentration of 1 μg/ml. The two cell populations were mixed together in a 1:1 ratio, and 2 × 106 cells were injected i.v. into naïve or ECTV-infected B6 mice or naïve or DC-vaccinated memory mice. For naïve and ECTV-infected B6 recipient and naïve and DC-vaccinated memory mice, at 4 and 18 h, respectively, after target cell inoculation, the recipient mice were sacrificed and the presence of CFSElow and CFSEhigh cells was determined by flow cytometry in cell suspensions of lymph nodes and spleens from individual mice. To calculate the percent specific lysis, the following formula was used: \( [1 - \text{(ratio for unprimed/ratio for primed)}] \times 100 \), where the ratio for unprimed and primed were calculated as the percent CFSElow/percent CFSEhigh (21).

Histopathology. Livers were aseptically collected, and 0.5- to 1.0-g liver sections were fixed in formalin and embedded in paraffin blocks. Serial sections were stained with hematoxilin and cosin (H&E) or immunostained with EVM135.

Bone marrow-derived dendritic cells and vaccination. We generated bone marrow-derived CD11c+ DCs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) as described previously (23). Lipopolysaccharide (100 ng/ml; Sigma) was added on the last day to induce maturation. After 5 to 7 days in culture, the cells were collected, incubated for 1 h with 1 μg peptide, washed exten-
amino acids of 37 VACV SD determinants (36) were fully conserved in ECTV. At least several of these peptides were ECTV has been shown to be fully conserved and a major determinant in h after inoculation into ECTV-infected B6 mice, splenocytes were cultured at 37°C in 96-well plates. For each sample, 2 × 10⁶ cells were incubated with no peptide or 0.1 μg/mL TSYKFESV, SIFRFLNI, or SIIN FEKL for restimulation in the presence of brefeldin A (BFA; Sigma) and monensin (Golgi plug; Becton, Dickinson [BD]). After 2 h, 0.4 μg of CD107a antibody (Ab; Biolegend) was added to measure degranulation. After a total of 5 h of restimulation, supernatant of Ab 2.4G2 (anti-Fcy II/III receptor; ATCC) was added to block nonspecific binding of labeled Ab to Fc receptors. The cells were then stained for cell surface molecules, fixed, permeabilized, and stained for intracellular molecules by using the Cytofix/Cytoperm kit (BD) according to the manufacturer’s instructions. The following Abs were used: anti-CD3 (145-2C11; Biolegend), anti-CD4 ( GK1.5; Biolegend), anti-CD8a (53-6.7; Biolegend), anti-IFN-γ (clone XMG11; Biolegend), anti-CD14 (Sa14-2; Biolegend), anti-CD16 (93; Biolegend), anti-CD19 (6D5; Biolegend), anti-CD107a (1D4B; Biolegend), and phycoerythrin-Cy5.5-labeled anti-human GzB (Caltag), which cross-reacts with mouse GzB (57). For VACV/ECTV- and SIINFEKL-specific CD8⁺ T cells, H-2 K⁺Ig recombinant fusion protein (Dimer-X; BD) was incubated with synthetic TSYKFESV, ITYRFYLI, SIFRFLNI, KS YNYMILL, and SIINFEKL peptides and used as recommended by the manufacturer. On some occasions, instead of K⁺-peptide dimers, we used K⁺-TSYKFESV, -SIFRFLNI, -KSYNYMILL, and -STLNFNNL tetramers prepared by the NIAID Tetramer Facility or K⁺-TSYKFESV or -SIINFEKL tetramers prepared in our laboratory according to published methods (47). Stained cells were analyzed by flow cytometry at the Fox Chase Cell Sorting Facility using an LSR II system (BD). At least 100,000 cells were analyzed.

Data display and statistical analysis. Unless indicated, all displayed data correspond to one representative experiment of at least two similar experiments with groups of three to six mice. Spleens and livers were analyzed individually, and results are representative of at least two independent experiments. Statistical analysis was performed using GraphPad Prism 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 t test or the Mann-Whitney test as necessary. When applicable, data are displayed with means ± standard error of the means (SEM). P values were determined between SIINFEKL-immunized or TSYKFESV-immunized mice and all other groups. Data were analyzed to P levels of 0.05, 0.01, and 0.001, as shown in the figures and described in the figure legends below (when not marked in the figures, the differences were not statistically significant).

RESULTS
Identification of H-2 K⁺-restricted ECTV determinants. By using intracellular staining for IFN-γ, we and others previously identified 49 determinants that account for the majority (94.8%) of the CD8⁺ T cell response to VACV Western Reserve (WR) in B6 mice (36, 48, 61). Of interest, the ID determinant TSYKFESV has been shown to be fully conserved and a major determinant in ECTV (36, 61). When we compared sequences, we found that the amino acids of 37 VACV SD determinants (36) were fully conserved in ECTV. At least several of these peptides were ECTV determinants, because a significant proportion of CD8⁺ T lymphocytes from ECTV-infected B6 mice were stained with K⁺ multimers loaded with the conserved VACV SD peptides SIFRFLNI, KSYNYMILL, ITYRFYLI, and STLNFNNL, albeit at a lower frequency than when loaded with TSYKFESV (Fig. 1A and B). Also, 4 h after inoculation into ECTV-infected B6 mice, splenocytes pulsed with any of the SD peptides were killed significantly less than when pulsed with TSYKFESV but significantly more than when pulsed with the control SIINFEKL (Fig. 1C and D). Thus, while less pronounced, probably due to the kinetics of the assay, the in vivo cytotoxicity results were consistent with those of K⁺ multimer staining.

Variable responses to immunization with DCs pulsed with ECTV/VACV CD8⁺ T cell determinant peptides. Mousepox-susceptible B6 congenic B6.D2-D6 mice were immunized and boosted (1 week apart) with DCs pulsed with various ECTV/ VACV peptides or the control, SIINFEKL. Four weeks after boost, CD8⁺ T cells specific for the different peptides were identified by staining with K⁺ multimers loaded with the relevant peptides (Fig. 2A). Immunization with DCs pulsed with ID TSYKFESV as well as SD KSYNYMILL and SIFRFLNI, or control SIINFEKL, resulted in similarly high numbers of K⁺-peptide-specific memory CD8⁺ T cells. On the other hand, immunization with DCs pulsed with SD ITYRFYLI or STLNFNNL did not result in a significant increase in the frequency of cells that stained with the specific K⁺-peptide complexes (Fig. 2B). When we performed in vivo cytotoxicity assays (14) in draining lymph nodes (D-LN) and spleen, there was some significant killing in mice immunized with ITYRFYLI-DC (which did not induce a significant proportion of specific memory CD8⁺ T cells as detected by K⁺-multimer staining), but this was significantly much lower than in mice immunized with TSYKFESV-DC or SIFRFLNI-DC (Fig. 2C to E). Therefore, the number of CD8⁺ T cells induced by peptide-pulsed DC immunization varies with the immunizing peptide, and the killing efficiency in vivo is affected by the frequency of memory CD8⁺ T cells that each peptide induces and by other unknown factors.

Memory CD8⁺ T cells elicited by immunization with peptide-pulsed DCs respond to ECTV infection. B6.D2-D6 mice were immunized and boosted with DCs pulsed with TSYKFESV, SIFRFLNI, or SIINFEKL as a control. SD SIFRFLNI was chosen for comparison with ID TSYKFESV, because it induced comparable frequencies of K⁺-peptide-specific memory CD8⁺ T cells (Fig. 2A and B). Six to 8 weeks after boosting, the mice were infected with ECTV, and 7 days postinfection (dpi) the CD8⁺ T cell responses were examined in the livers (the main target organ of ECTV) and spleens (Fig. 3). Consistent with our previous finding that naive B6.D2-D6 mice do not mount CD8⁺ T cell responses when challenged with WT ECTV (11), none of the SIINFEKL-immunized mice mounted a TSYKFESV or SIFRFLNI CD8⁺ T cell response in either the spleen or the liver. On the other hand, mice immunized with DC-TSYKFESV had high frequencies and absolute numbers of CD8⁺ T cells that stained with K⁺-TSYKFESV (Fig. 3B and E), but only background numbers of cells that stained with K⁺-SIFRFLNI (Fig. 3C and F). In mice immunized with DC-SIFRFLNI, K⁺-SIFRFLNI (Fig. 3C and F) stained a high proportion and high absolute numbers of CD8⁺ T cells, but a relatively high number of cells also stained with K⁺-TSYKFESV (Fig. 3B and E). This was not due to cross-reactivity, because each cell stained with only one tetramer (Fig. 3A and D). Thus, in the presence of memory cells to SD SIFRFLNI, a primary response to the ID TSYKFESV was rescued, but not vice versa. Rescue of a primary response by memory CD8⁺ T cells after DC immunization is consistent with our finding that adoptively transferred memory CD8⁺ T cells can rescue a primary response in otherwise-unresponsive B6.D2-D6 mice. This rescue of a primary response is likely due to the ability of the memory cells to lower virus loads.
thereby preventing the death of naïve lymphocytes (S. Remakus et al., submitted for publication).

We also analyzed the effector CD8$^+$ T cell responses after in vitro restimulation with peptide. TSFKFESV restimulation resulted in a significant increase in the frequency of CD8$^+$ T cells expressing IFN-γ in liver mononuclear cells (Fig. 4A and C) and splenocytes (Fig. 4B and D) from DC-TSYKFESV-immunized mice. Similarly, SIFRFLNI restimulation of liver mononuclear cells (Fig. 4A and C) and splenocytes (Fig. 4B and D) from DC-SIFRFLNI-immunized mice resulted in a significantly increased frequency of IFN-γ$^+$ CD8$^+$ cells. CD8$^+$ T cells from the livers of DC-SIINFEKL-immunized mice significantly upregulated IFN-γ expression following restimulation with SIINFEKL, albeit to low levels, likely because they did not expand (Fig. 4A and C). On the other hand, SIINFEKL restimulation of splenocytes from DC-SII

![Graph A](image1.png)

**FIG 1** Identification of H-2 K$^b$-restricted ECTV determinants. (A) B6 mice were infected with 3,000 PFU of ECTV in the footpad, and splenocytes were analyzed 7 dpi. Representative flow cytometry plots show the frequencies of CD8$^+$ cells positive for H-2 K$^b$-TSFKFESV, -SIFRFLNI, -KSYNYMLL, -ITYRFYLI, and -STLNFNNL. The dot plots represent the frequency of CD8$^+$ cells that were stained with the indicated K$^b$-peptide dimer in 3 independent experiments for all peptides except for ITYRFYLI, for which data are from 2 independent experiments. (B) Graphs showing summary data of flow cytometry plots from panel A. Open symbols represent naïve mice, and closed symbols represent ECTV-infected mice. Each data point corresponds to pooled splenocytes from 2 to 3 mice in one experiment. (C) Representative in vivo cytotoxicity data. Naïve and VACV-immune mice were inoculated i.v. with a 1:1 mixture of B6 splenocytes labeled with 0.8 μM CFSE (CFSELow) or splenocytes labeled with 4 μM CFSE and pulsed with the TSFKFESV (CFSEhigh). Mice were killed 4 h after target inoculation, and the proportions of CFSElow and CFSEhigh cells were determined by flow cytometry in spleens of individual mice. Histograms are gated on CFSE-positive cells of naïve and ECTV-infected B6 mice, as indicated. (D) Summary of in vivo cytotoxicity assays using the indicated ECTV/VACV peptide-pulsed targets. Numbers indicate percentages of specific killing of CFSEhigh cells, calculated as detailed in Materials and Methods. Data correspond to means ± SEM for 3 or more independent experiments, where n was 15 for TSFKFESV, n was 12 for SIFRFLNI, n was 12 for KSYNYMLL, n was 10 for ITYRFYLI, n was 3 for STLNFNNL, and n was 11 for SIINFEKL. P values (*, P < 0.05; ***, P < 0.001) shown were determined for comparisons TSFKFESV and all other groups. The P value determined for SIINFEKL versus all other groups was <0.001 (data not shown).
nized with DCs pulsed with ID TSYKFESV or SD SIFRFLNI, but not with irrelevant SIINFEKL, mount strong recall CD8⁺ T cell responses to ECTV. The results also showed that mice immunized with ID TSYKFESV do not mount a primary response to SD SIFRFLNI (Fig. 3 and 4), while mice immunized with SD SIFRFLNI mount a non-cross-reactive primary response to the ID TSYKFESV (Fig. 3).

Productive peptide-DC immunization results in protective immunity against lethal mousepox. B6.D2-D6 mice primed and boosted 6 to 8 weeks earlier with DCs pulsed with the ID TSYKFESV, SD SIFRFLNI, or ITYRFYLI (all of which induced a high frequency of memory cells, as detected by Kᵦ-peptide staining), with SD KSYNYMLL or STLNFNFLN (which did not induce a significant number of memory CD8⁺ T cells) or control SIINFEKL (which induced a high frequency of memory CD8⁺ T cells upon immunization but is not an ECTV determinant) were challenged with ECTV. All the mice immunized with DCs pulsed with ID TSYKFESV and SD SIFRFLNI survived the infection and lost <2% of their weight (Fig. 5A and B). Mice immunized with KSYNYMLL-pulsed DCs were also highly protected, because 80% survived the infection and lost <10% of their weight. On the other hand, all mice immunized with DCs pulsed with ITYRFYLI or STLNFNFLN (which did not generate a significant response), or with SIINFEKL, succumbed to the infection and lost ≥10% of their weight. Still, ITYRFYLI-DC immunization was somewhat protective, because death was delayed by 4 days compared with SIINFEKL immunized mice (Fig. 5A and B).

Next, we compared virus loads and pathology in protected versus control unprotected mice. At 7 dpi, TSYKFESV- and SIFRFLNI-immunized mice had significantly lower virus loads in the spleen and liver than did SIINFEKL-immunized mice (Fig. 5C and D). Moreover, 7 dpi the livers of TSYKFESV- and SIFRFLNI-immunized mice had significantly fewer necrotic foci than SIINFEKL-immunized mice. Different from the foci in SIINFEKL-im-
B6.D2-D6 mice were immunized with peptide-pulsed DCs and 2 months later infected with 3,000 PFU of ECTV in the footpad. At 7 dpi, livers and spleens were analyzed in individual mice. (A) Representative flow cytometry plots showing frequencies of CD8^+ cells in the livers that were Kb-TSYKFESV^+ or Kb-SIFRFLNI^+. (B) Column graphs correspond to summary data presented in the flow cytometry plots of panel A. Frequencies and absolute numbers of positive CD8^+ cells are shown for mice immunized with Kb-TSYKFESV as indicated. Data correspond to five mice per group ± SEM and are representative of two independent experiments. (C) Summary data as described for panel B, but showing the frequencies and absolute numbers of CD8^+ cells that were Kb-SIFRFLNI^+. (D) Representative flow cytometry plots showing frequencies of CD8^+ cells that were Kb-TSYKFESV^+ or Kb-SIFRFLNI^+ in the spleen. (E) Column graphs corresponding to summary data for the frequencies of CD8^+ cells that were Kb-TSYKFESV^+. Data correspond to results for five mice per group ± SEM and are representative of two independent experiments. (F) Summary data as described for panel B, but for Kb-SIFRFLNI^+ cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
FIG 4 Memory CD8⁺ T cells elicited by immunization with peptide-pulsed DCs become activated in the liver and spleen in response to ECTV infection. B6.D2-D6 mice were immunized with DCs pulsed with the indicated peptides and 2 months later infected with 3,000 PFU of ECTV in the footpad. (A and B) At 7 dpi, livers and spleens were analyzed in individual mice. Representative flow cytometry dot plots are shown for IFN-γ and GzB expression in liver mononuclear cells (A) and splenocytes (B). The top row shows control unimmunized, uninfected mice. All graphs are gated on CD8⁺ CD4⁻ cells. (C to F) Graphs show the summary data for the frequencies of CD8⁺ T cells expressing IFN-γ (C and D) or GzB (E and F) in the livers and spleens. Data correspond to five mice per group ± the SEM and are representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
FIG 5 Productive peptide-DC immunization results in protective immunity against lethal mousepox infection. B6.D2-D6 mice were immunized and boosted (1 week apart) with the indicated peptide-DC dimer and challenged more than 1 month later with 3,000 PFU of ECTV in the footpad. (A) Survival curve of B6.D2-D6 mice. The experiment is representative of three, with n of 5 per group. (B) Body weights over the course of infection. Data are expressed as the percent initial weight ± the SEM. (C and D) Virus titers in spleen and liver, respectively. Data correspond to 5 mice per group ± SEM and are representative of three independent experiments. (E) Liver histopathology (H&E stain) and immunohistochemistry (anti-EVM135 stain). Original magnifications are indicated. Data correspond to 5 mice per group ± the SEM and are representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
CD8+ T cells induced by peptide-DC immunization protect mice against lethal mousepox infection in the absence of NK cells. B6 mice were immunized with peptide-pulsed DCs. (A) Representative data for the percentage of Kb-TSYKFESV-positive CD8+ in PBMCs from naive B6 mice or B6 mice vaccinated with TSYKFESV-DC. (B) Column graphs correspond to summary data presented in the panel A flow cytometry plots for frequencies of CD8+ cells that were Kb-TSYKFESV+. Data correspond to 3 independent experiments (± SEM; n = 7 for naive mice and n = 15 for TSYKFESV-vaccinated mice). (C) Survival of naive and TSYKFESV-DC-immunized B6 mice that were depleted of NK cells with the PK136 monoclonal antibody i.p. and challenged with ECTV. Data correspond to 2 independent experiments ± SEM for naive (n = 8) and TSYKFESV-immunized (n = 10) mice. ***, P < 0.001.

NK cells are no longer required for resistance to mousepox following effective peptide-DC immunization. Because the effecter mechanisms of CD8+ T cells and NK cells overlap, we next tested whether NK cells are dispensable for protection when anti-ECTV memory CD8+ T cells are present. B6 mice were immunized with TSYKFESV-DC, which resulted in a significant increase in the frequency of CD8+ T cells that stained with Kb-TSYKFESV, as measured 4 weeks after booster immunization (Fig. 6A and B). At 6 to 8 weeks postboost, TSYKFESV-DC-immunized and control unimmunized B6 mice were depleted of NK cells and were challenged with ECTV 1 day later. Eighty percent of TSYKFESV-DC-immunized mice survived, while all controls died (Fig. 6C). Thus, memory CD8+ T cells of single specificity significantly protected mice from lethal mousepox in the absence of NK cells.

DISCUSSION

In this study, we have demonstrated that memory CD8+ T cells of single specificity induced by immunization with DCs pulsed with viral peptides protect from an acute lethal viral disease. Furthermore, we showed that CD8+ T cells directed to the ID as well as to those SD determinants that were effective at inducing a significant CD8+ T cell response upon DC-peptide immunization were highly protective. Moreover, we showed that protection can be achieved even in the absence of NK cells, which are essential for resistance to primary ECTV infection.

Other laboratories have previously studied the differential protective abilities of memory CD8+ T cells specific for single ID or SD determinants during LCMV infection (20, 28, 29, 34, 41, 44, 49–51, 53, 56). However, the pathogenesis of LCMV is very different from that of ECTV. Natural LCMV infection in the mouse occurs in utero and results in a chronic infection rather than an acute disease (4). Depending on the dose, route, and clone, experimental intraperitoneal (i.p.) or i.v. infection results in transient acute or chronic infection without major symptoms and causes fatal meningitis only after intracerebral inoculation.

Somewhat analogous studies have also been performed following infection with respiratory viruses. For example, Fu et al. generated a DNA construct encoding full-length NP with two mutations (NPmut) that eliminated the ID determinant NP147–155 from influenza virus A/PR/8/34. This allowed for the detection of the immunorecessive determinant NP218–226 (19). NP218–226 behaves as a typical immunorecessive determinant in that specific CD8+ T cell response, which can be detected only when the ID determinant is absent during priming (39, 40, 42). BALB/c mice were immunized intramuscularly with NPmut DNA and were protected against cross-strain challenge with A/HK/68 (H3N2). Also, Cole et al. demonstrated that the hierarchy of CD8+ T cell determinants recognized in Sendai virus can be selectively altered by immunization against an SD determinant, with the resulting CD8+ T cell response following virus challenge directed predominantly to the subdominant determinant (8). In addition, Kast et al. showed that peptide immunization with the ID peptide of Sendai virus protected mice from a lethal challenge (27). In these experiments, protection conferred by memory CD8+ T cells specific for an SD determinant was not assessed. These studies differed from ours because, different from ECTV infection, influenza and Sendai viruses produce disease by replicating at the primary site of infection rather than by spreading systemically. Moreover, we examined protection by CD8+ T cells against subdominant rather than immunorecessive determinants, and we found that the response to the ID determinant was not abrogated in the presence of memory cells to the SD determinant.

Regarding infection with the related OPV VACV, studies of DNA vaccines containing ID or SD determinants from simian or human immunodeficiency virus showed a reduction in virus titers in ovaries of mice infected i.p. with recombinant VACV expressing the relevant determinants (24, 33). Snyder et al. showed protection against lethal secondary intranasal (i.n.) VACV challenge in HLA-A2 transgenic mice by vaccination with an MHC-I-restricted T cell determinant. However, mice with a memory CD8+ T cell response to a single determinant did not have complete protection, as some mice lost weight and some mice died. This did not occur in mice previously immunized with the whole virus (46). Cornberg et al. showed that VACV-E7R-specific memory
CD8$^+$ T cell responses to the influenza virus A/PR8/34 NP immunodominant epitope ASNENMEM by DC immunization, even though they have high affinity for MHC-I. In support, we have been unable to induce responses to the influenza virus A/PR8/34 NP immunodominant epitope ASNEMMEM by DC immunization, even though it has an 8 nM affinity for Dd (45). DCs have an endopeptidase activity at their plasma membrane that has been shown to degrade the K$^d$-restricted tyrosinase epitope YMDGTMQV, precluding its recognition by CD8$^+$ T cells (2). Thus, it is possible that, similar to YMDGTMQV, peptides such as ITYRYFLI, STLNFNNL, and ASNENMETM, but not the immunogenic peptides, are unsuitable for DC immunization because they are preferentially degraded at the surface of DCs. Another possibility is that, despite their high affinity for MHC-I, the half-lives of the peptide–MHC-I complexes at the surface of DCs varies significantly. As an example, the half-life of Db-ASNENMETM at the surface of DCs is about 45 min, while the half-lives of the peptide–MHC-I complexes at the surface of DCs are about 20 min. This suggests that peptide degradation is more rapid at the surface of DCs than in the endosomal compartments.

In summary, our study provides us with a better understanding of the mechanisms of acquired protection to highly infectious OPV. In addition, because ECTV spreads through the lymphohematogenous route, our findings may be relevant for the many unrelated viruses that spread via this route (17, 52). Moreover, our work contributes to the efforts of rational vaccine development by providing information about mechanisms of acquired protection that may be applicable to other pathogenic viruses that cause acute or chronic viral diseases.

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