Memory CD8$^+$ T cells are gatekeepers of the lymph node draining the site of viral infection

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It is uncertain how immunity protects against systemic viral diseases. Here, we demonstrate that in the absence of persistent virus, not only antibodies but also recall responses by long-lived memory CD8$^+$ T cells prevent mousepox, a disease caused by ectromelia virus, a close relative of the virus of human smallpox. Moreover, we show that to protect, recall CD8$^+$ T cells directly kill targets in the lymph node draining the primary site of infection thus curbing systemic viral spread. Therefore, our work provides the basis for a model where lymph nodes are not just organs where lymphocytes become activated and proliferate but also the sites where a major fight against virus spread takes place.

immunity | poxvirus | T cell memory | vaccines

It is well established that circulating antiviral antibodies can protect against many viral diseases. However, whether and how virus-specific memory CD8$^+$ T cells can protect from systemic viral disease remains a matter of heated debate (1–5). The current model proposes that upon activation in secondary lymphoid organs, CD8$^+$ T migrate to sites of viral replication to kill infected cells (6–8). It has been argued, though, that this mechanism is too slow to prevent acute viral diseases caused by viruses that spread systemically from the primary site of infection (5).

Viruses can be subdivided into those that cause localized infections at the site of entry such as influenza (respiratory tract) and human papillomavirus (skin) and those that cause systemic disease. Many viruses that cause systemic infections spread in a stepwise manner: (*i*) replication at the site of entry (e.g., skin, respiratory epithelium, gut), (*ii*) spread via the afferent lymphatic vessels to and replication in the local (draining) lymph node (D-LN) or other secondary lymphoid organs such as Peyer patches or tonsils, (*iii*) spread via the efferent lymphatic vessels and the thoracic duct to the blood causing a primary viremia, and (*iv*) seeding and replication in target organs. This type of viral spread is used by orthopoxviruses (OPV) such as those that cause smallpox in humans [variola virus (VARV)] and mousepox in mice [ectromelia virus (ECTV)] as well as many other human and animal pathogens in other genera such as Enterovirus (polio, cocksackie), Aphthovirus (foot-and-mouth disease), Rubivirus (rubella), Flavivirus (Yellow Fever, Dengue, West Nile), Rubulavirus (mumps), Morbillivirus (measles), Varicellovirus (chickenpox), and others (9).

Mousepox is an excellent model to study protection against acute systemic viral infections in general and smallpox in particular (10). As with all OPV, ECTV is genetically and antigenically very similar to VARV and to the virus of the smallpox vaccine, vaccinia virus (VACV) (11). Moreover, similar to smallpox, mousepox can be prevented by immunization with VACV. The natural route of ECTV entry is via skin abrasions in the footpad (10) followed by systemic spread through the D-LN as described above. In mousepox-susceptible strains such as BALB/c, the virus reaches very high titers in liver and other organs (12), and most mice die from acute liver necrosis 7–15 days p.i (10). The few surviving mice experience pronounced weight loss and, after a secondary viremia and spread of the virus to the skin, develop the characteristic rash of OPV infections from which they generally recover (10, 13).

Results

Memory CD8$^+$ T Cells Protect from Mousepox. As in humans with smallpox, passive immunization with anti-OPV antiserum can protect naïve susceptible mice from mousepox (10). To determine whether memory CD8$^+$ T cells are also protective, BALB/c mice were immunized with VACV and boosted with ECTV or VACV. One month to 1 year after the booster immunization, mice were killed and their CD8$^+$ T cells were magnetically purified. For simplicity, we refer to these as memory CD8$^+$ T cells albeit only ≈5% were truly virus-specific (not shown). Memory CD8$^+$ T cells (≈5×10^6) or control CD8$^+$ T cells from naïve mice were inoculated into naïve (nonirradiated) BALB/c mice. One day posttransfer, mice were infected in the footpad with the Moscow strain of ECTV (14). As shown in Table 1 (experiments 1–5) and Fig. 1*A*, all memory CD8$^+$ T cell recipient mice were protected from death, visible signs of mousepox, and weight loss even when the memory CD8$^+$ T cells were obtained from donors that were immunized as long as 1 year before. Protection was due to memory CD8$^+$ T cells and not other contaminating cells such as memory CD4$^+$ T or memory B cells because the CD8-depleted lymphocytes from immunized mice did not prevent death (Table 1, experiment 5), strongly suggesting that memory CD4 or B cells do not protect. As expected, most control mice that received naïve CD8$^+$ T cells died (Table 1, experiments 1–5), and the few that survived experienced generalized skin rash and profound weight loss (Fig. 1*A*). Histological analysis showed that 7 days postinfection (p.i.), the spleens of naïve CD8$^+$ T cell recipients were necrotic and almost completely depopulated of lymphocytes (Fig. 1*B*), and their livers were severely damaged with extensive hepatocellular necrosis without lymphocytic infiltration (Fig. 1*B*). In contrast, the spleens of memory CD8$^+$ T cell recipients were not necrotic and had enlarged germinal centers indicating an ongoing immune response (Fig. 1*B*). Also, although their livers had necrotic areas, the damage was less extensive than in naïve CD8$^+$ T cell recipients, i.e., the necrotic foci were fewer and each contained many infiltrating lymphocytes (Fig. 1*B*). Thus, absence of clinical symptoms of mousepox was accompanied by major reduction in tissue damage. As expected (13), control mice that were passively immunized with antibodies in the form of ECTV antiserum (Table 1, experiment 1, and Fig. 1*A*) or that had been actively immunized with VACV 1 month earlier (Table 1, experiment 1, and Fig. 1*A*) were also protected from disease and death. Therefore, similar to active VACV immunization or passive immunization with Abs, long-lived memory CD8$^+$ T cells protect

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Abbreviations: D-LN, draining lymph node; ECTV, ectromelia virus; GzB, granzyme B; ND-LN, nondraining lymph node; OPV, orthopoxvirus(es); p.i., postinfection; VACV, vaccinia virus.

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Table 1. Anti-OPV memory CD8$^+$ T cells protect mice from mousepox and death after footpad ECTV challenge

Experiment	Recipient inoculation	Donor immunization	Donor boost	Time boost to transfer	Time transfer or immunization to challenge	Mousepox/total	Dead/total
1	Memory CD8$^+$ T cells	VACV	ECTV	1 month	1 day	0/5	0/5
	Naïve CD8$^+$ T cells	—	—	—	1 day	5/5	3/5
	Antiserum	VACV	ECTV	>2 months	1 day	0/5	0/5
	VACV	NA	NA	NA	1 month	0/5	0/5
2	Memory CD8$^+$ T cells	VACV	ECTV	4 months	1 day	0/5	0/5
	Naïve CD8$^+$ T cells	—	—	—	1 day	5/5	3/5
3	Memory CD8$^+$ T cells	VACV	VACV	>2 months	1 day	0/5	0/5
	Naïve CD8$^+$ T cells	—	—	—	1 day	5/5	5/5
4	Memory CD8$^+$ T cells	VACV	VACV	1 year	1 day	0/5	0/5
	—	NA	NA	NA	NA	5/5	5/5
5	Memory CD8$^+$ T cells	VACV	VACV	>2 months	1 day	0/5	0/5
	Memory CD8$^-$ cells	VACV	VACV	>2 months	1 day	5/5	5/5
6	Memory CD8$^+$ T cells	VACV	VACV	>2 months	1 month	0/5	0/5
	Naïve CD8$^+$ T cells	—	—	—	1 month	5/5	5/5
7	Memory CD8$^+$ T cells	VACV	VACV	>2 months	4 months	0/5	0/5
	Naïve CD8$^+$ T cells	—	—	—	4 months	5/5	5/5

Naïve BALB/c mice were inoculated with purified CD8$^+$ ($\approx 5 \times 10^6$ per mouse) or CD8$^-$ ($\approx 10^7$ per mouse) LN and spleen cells from donor mice that had been infected and boosted (1 month apart) with the indicated viruses. One day, 1 month, or 4 months later, recipient mice were challenged with ECTV in the footpad, and mousepox (assessed by clinical signs) and death were determined. All experiments are representative of at least three repeats except for experiment 6, which was performed once. In experiments 5 and 6, anti-VACV antibody was assayed in sera just before challenge. No anti-VACV antibody was detected at dilutions of the sera ranging from 10^{-1} to 10^{-8}. The time of death for all nonsurviving mice was 9 ± 2 days. NA, not applicable.

from acute viral disease and death. Our data also show that anti-VACV and anti-ECTV memory CD8$^+$ T cells are equally effective at protecting challenged mice from mousepox demonstrating the high degree of conservation of the protective CD8$^+$ T cell determinants in these two OPV.

In the experiments above, we did not find VACV by plaque assays in donor mice at the time of transfer (not shown). This indicated that virus persistence was not required to maintain the ability of memory CD8$^+$ T cells to protect from viral disease. However, the manipulation of the CD8$^+$ T cells during the purification process could have resulted in nonspecific activation. To ensure that the transferred memory CD8$^+$ T cells were resting at the time of ECTV challenge, experiments were performed where memory CD8$^+$ T cells were parked in the recipient mice for 1 or 4 months before ECTV. Of note, the recipient mice did not seroconvert during the parking period indicating that VACV had not been transferred with the CD8$^+$ T cells (data not shown). Also in this case, all memory CD8$^+$ T cell recipient mice survived the infection without visible signs of disease or weight loss (Table 1, experiments 6 and 7, and Fig. 1*A*). Therefore, we conclude that memory CD8$^+$ T cells that had been rested for at least 4 months in the absence of Ag can protect from an acute and lethal systemic viral disease such as mousepox.

Only Immunocompetent Mice Can Be Protected by Memory CD8$^+$ T Cells or Antiserum. The previous experiments demonstrated that either memory CD8$^+$ T cells or circulating antibodies can protect mice from mousepox in the context of a fully competent but naïve immune system. We have recently shown that resistance and survival of genetically resistant B6 mice to primary mousepox requires CD8$^+$ T cells at the early stages of infection and Abs at a later phase (15). However, it was possible that this requirement for both components of the immune system to resist mousepox applied to genetically resistant naïve mice but not to genetically susceptible mice when protected by either memory T cells or Abs. To determine whether memory CD8$^+$ T cells can also protect in the absence of a functional B cell compartment, we transferred memory CD8$^+$ T cells into B cell-deficient BALB/c (B cell KO) mice (16). This resulted in a very marginal increase in the survival time of the mice (Fig. 2*A*, compare yellow and blue lines). Moreover, a combination of strong CD4$^+$ and CD8$^+$ T cell response was not sufficient for protection in the absence of Ab because B cell KO mice immunized with VACV [which by itself did not cause disease but induced potent CD4 and CD8 responses (data not shown)] also succumbed to mousepox, albeit with a delay of 1–2 weeks, as compared with naïve B cell KO mice (Fig. 2*A*, compare yellow and green lines). This indicates that when *de novo* production of Ab is not possible, memory T cells (CD8$^+$ and CD4$^+$ together) control ECTV only transiently. To determine whether antibodies can protect in the absence of a functional CD8$^+$ T cell compartment, BALB/c mice were antibody-depleted of CD8 T cells and challenged with ECTV. Notably, all CD8$^+$ T cell-depleted mice that received antiserum still succumbed to ECTV infection (Fig. 2*B*, red line), whereas, as before, all nondepleted mice that received antiserum were protected from death (Fig. 2*B*, black line). Therefore, in the BALB/c susceptible background, circulating antibodies are protective but only in the presence of CD8$^+$ T cells. Together, these data show that functional T cell and B cell compartments are required to control ECTV even in the case of preexisting humoral or cellular immunity, respectively. These results support a model in which preexisting antibodies or memory CD8$^+$ T cells alone cannot provide sterilizing immunity but can prevent disease by delaying spread or replication of the virus long enough to give other arms of the immune system the necessary time to respond. These results are disappointing given the current interest in designing vaccines to protect immunodeficient individuals.

Memory CD8$^+$ T Cells Curb Virus Spread from the D-LN to Vital Organs. We next determined the kinetics of ECTV replication and spread in mice that received memory or naive CD8$^+$ T cells. As shown in Fig. 3*A*, during the first week p.i., ECTV reached high titers in the footpads of both naïve and memory CD8$^+$ T cell recipients, indicating that memory CD8$^+$ T cells do not protect from clinical illness by decreasing replication at the primary site of infection. In addition, memory CD8$^+$ T cells did not significantly decrease virus loads in the D-LN during the first 5 days p.i. However, memory CD8$^+$ T cell recipient mice had a highly significant reduction of virus loads on days 3–4 p.i. in the spleen

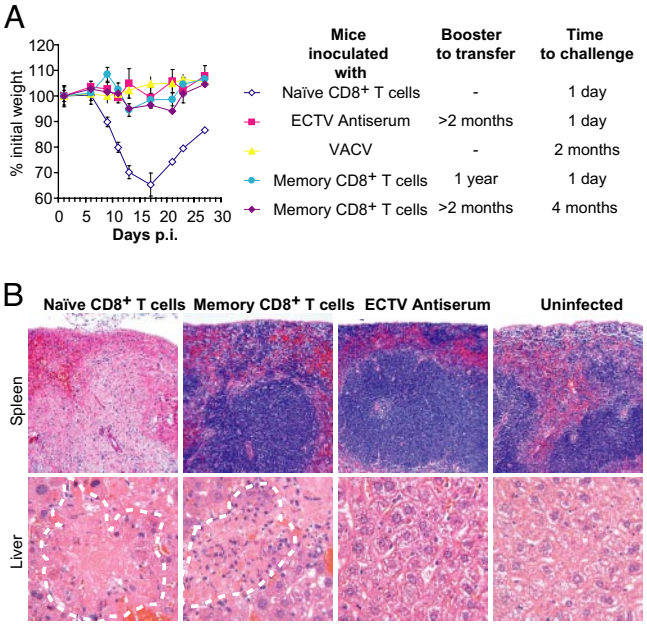

Fig. 1. Long-lived memory CD8$^+$ T cells prevent mousepox. (*A*) Weight loss. BALB/c mice were treated as indicated and at different times posttreatment were infected in the footpad with ECTV. The body weight was determined over the following 4 weeks. Data are expressed as percentage average of initial weight ±SEM. All groups consisted of five mice except the VACV-treated group, which consisted of eight mice. In the group of mice treated with naïve CD8$^+$ T cells, only one mouse survived past day 13 p.i. Experiments were repeated three times, except the challenge after 4 months post memory CD8$^+$ T cell inoculation, which was performed once. (*B*) Histopathology. Mice were treated as indicated and 1 day later were infected with ECTV in the footpad. Seven days p.i., mice were killed, and organs were collected and processed for histopathology. Microphotographs representative of three mice. (Magnification of hematoxylin/eosin stains: spleen, ×10; liver, ×24.) Stippled lines demarcate the sites of liver necrosis.

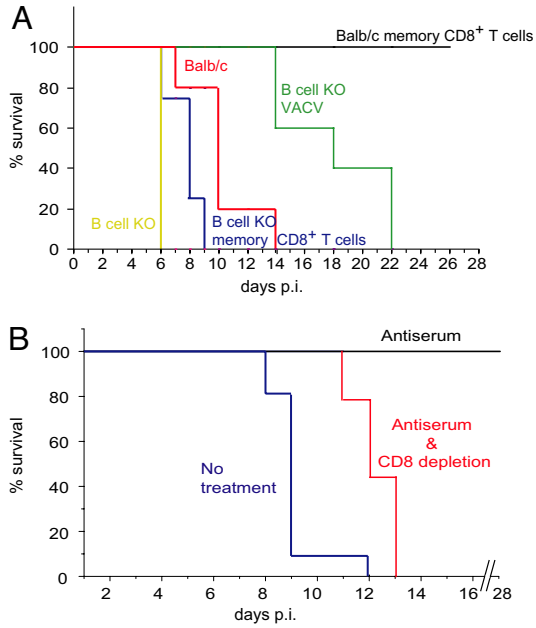

Fig. 2. Ab and memory CD8$^+$ T cells protect from mousepox and death but only in immunocompetent mice. (*A*) Memory CD8$^+$ T cells do not protect BALB/c mice unable to generate antibodies. Groups of four or five BALB/c or B cell KO mice were treated as indicated and challenged with ECTV in the footpad. Survival was monitored for 4 weeks. The experiment is representative of two. Data for B cell KO mice treated with memory CD8$^+$ T cells correspond to a separate experiment where untreated B cell KO mice also died on day 6, and all BALB/c that received memory CD8$^+$ T cells survived. (*B*) Antibodies do not protect mice unable to generate CD8$^+$ T cell responses. Groups of five BALB/c mice were treated as indicated and infected with ECTV in the footpad. Survival was monitored for 4 weeks. The experiment shown is representative of three.

(day 3, 69-fold, $P = 0.007$; day 4, 100-fold difference, $P = 0.00067$) and on day 4 in the liver (23-fold, $P = 0.001$) as compared with naïve CD8$^+$ T cell recipients. These results demonstrate that memory CD8$^+$ T cells protect from acute disease and death by decreasing virus loads in vital organs either by limiting the spread of ECTV from the D-LN to vital organs or by rapidly (within 3 or 4 days) migrating to vital organs to kill infected cells. Of interest, active immunization of control animals with VACV did not significantly reduce virus titers in the footpad either, clearly demonstrating that even active immunization that targets cellular as well as humoral immunity fails to protect the primary site of infection. This is consistent with reports that anti-OPV immunity is not sterilizing (13, 17, 18).

Next, we determined the kinetics of memory CD8$^+$ T cells recall responses in D-LN, a nondraining LN (ND-LN), and the spleen. Memory and naïve CD8$^+$ T cells from BALB/c mice were labeled with the fluorochrome CFSE (19, 20) and transferred to naïve Thy1KO BALB/c mice (21). (In experiments that are not shown, we found that Thy1 KO mice were susceptible to mousepox and inoculation of memory CD8$^+$ T cells from wild-type BALB/c mice protected them. Moreover, their memory CD8$^+$ T cells protected BALB/c mice.) One day after transfer, recipient mice were challenged with ECTV in the footpad, and the responses of donor (Thy1$^+$) CD8$^+$ T cells were determined by flow cytometry at different times p.i. On day 3 p.i., the D-LN of memory CD8$^+$ T cell recipients infected with ECTV had a detectable memory CD8$^+$ T cell response as evidenced by the presence of ≈18% Thy1$^+$ cells that had already divided (percentage Thy1$^+$ cells above background that showed decreased CFSE fluorescence). Most of these cells expressed the molecules granzyme B (GzB) (Fig. 3*B*) and IFN-γ (data not shown), indicating that they were armed effectors. This proliferation of the donor cell population, however, did not result in a net increase in the proportion of donor-derived Thy1$^+$ cells (data not shown). The response in the D-LN was clearly driven by the infection with ECTV because no response was detected in memory CD8$^+$ recipient mice that remained uninfected (Fig. 3*B*). Of interest, no memory CD8$^+$ T cell responses were yet detected in the spleens (data not shown) despite the fact that at this time memory CD8$^+$ T cell recipients already had a significant decrease in splenic virus loads (Fig. 3*A*). By day 4 p.i., there was still no change in the proportion of donor (Thy1$^+$) lymphocytes in any of the secondary lymphoid organs (Fig. 3*C*, contour plots). However, the proportion of divided cells in the D-LN that expressed IFN-γ (22%) and/or GzB (37%) was now larger (Fig. 3 *B* and *C*), whereas the memory CD8$^+$ T cell responses in the spleen still remained undetectable. On days 5 and 7 p.i., the proportion of donor (Thy1$^+$) cells in the D-LN increased to 20% and 68%, respectively, and the vast majority had divided eight or more times and expressed IFN-γ and/or GzB. Strong responses were now also apparent in the spleen, indicating that the D-LN response precedes the splenic response by ≈2 days. On day 7 p.i., most donor cells in the ND-LN were CFSE negative, indicating that they had divided at least eight times. Because we did not find cells from intermediate generations in ND-LN and the overall proportion of Thy1$^+$ cells did not increase, these data suggest that the effector cells were virus-specific migrants from the D-LN or the spleen. Most likely, this indicates that the migration of effector T cells from the D-LN and spleen to other organs occurred not earlier than day 5 p.i. even though the liver was

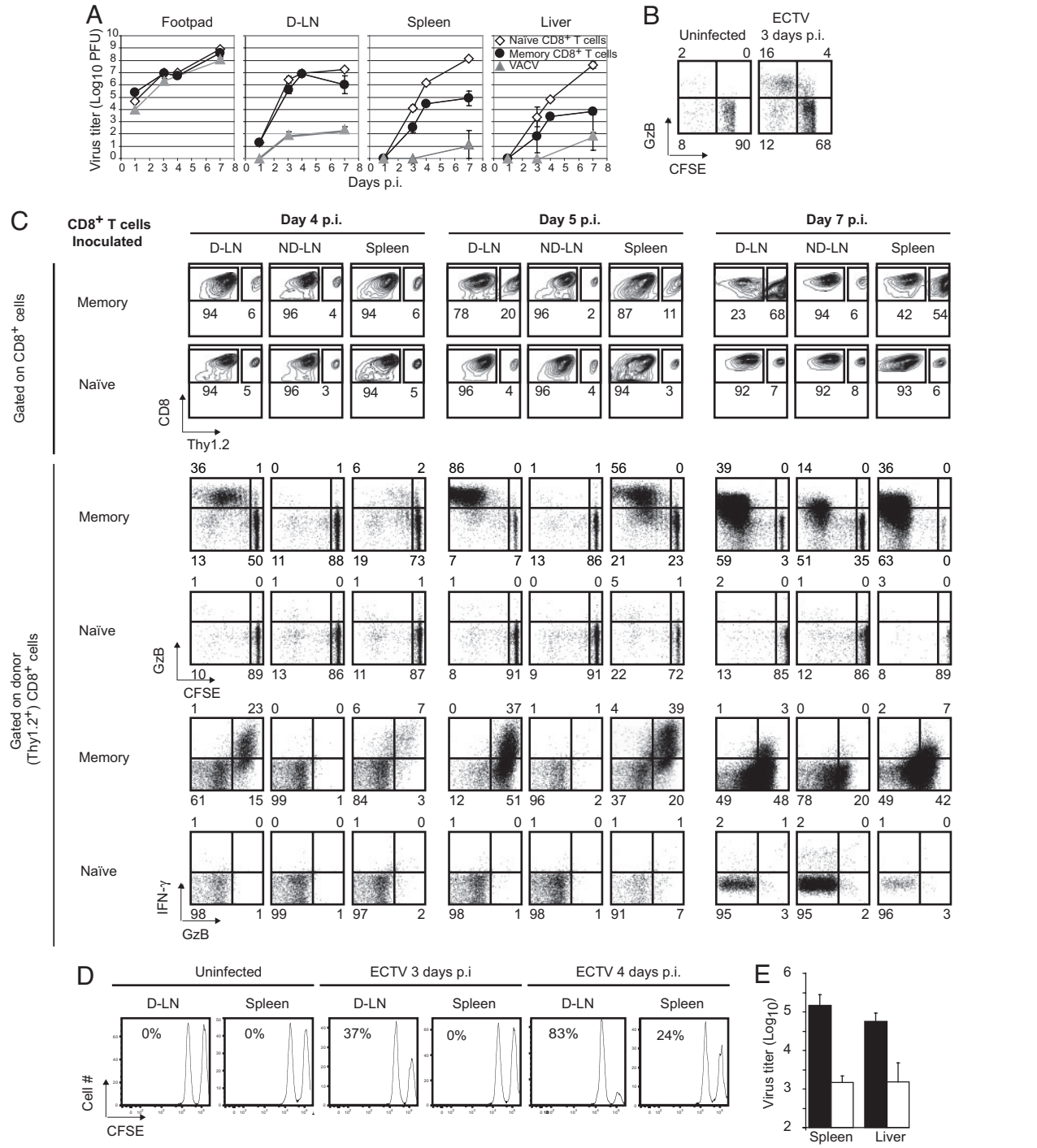

Fig. 3. Memory CD8$^+$ T cells reduce virus spread to vital organs. (*A*) Virus titers. BALB/c mice were transferred with memory or naïve CD8$^+$ T cells or immunized with VACV. One day after adoptive cell transfer or 2 months after VACV immunization, mice were infected in the footpad with ECTV. One, 3, 4, and 7 days p.i., mice were killed and virus titers were determined in the indicated organs. Data corresponds to four or five mice per group ±SEM, and most of the time points were repeated at least twice. DL, detection limit. (*B* and *C*) Memory CD8$^+$ T cells respond rapidly in the D-LN. Thy1 KO mice were adoptively transferred with CFSE-labeled CD8$^+$ T cells from naïve or memory (VACV-immune) BALB/c mice. Animals were challenged or not with ECTV in the footpad and donor (Thy1$^+$) CD8$^+$ T cell responses were determined in D-LN, ND-LN, and spleen by intracellular IFN-γ and GzB staining at different times p.i. CFSE dilution into daughter cells was used to determine proliferation of donor-derived CD8$^+$ T cells. Stains are as indicated. Data correspond to pools of LN or spleens from two to five mice. In *B*, both dot plots correspond to D-LN from mice that received memory CD8$^+$ T cells and are gated on donor-derived Thy1$^+$ cells. (*D*) *In vivo* killing of antigen-bearing targets occurs earlier in the D-LN. BALB/c mice were adoptively transferred with memory CD8$^+$ T cells and infected or not with ECTV. Three or 4 days p.i., mice were inoculated i.v. with control and antigen-loaded targets consisting of a 1:1 mixture of BALB/c splenocytes labeled with 0.8 μM CFSE (control targets, CFSE^{low}) or pulsed with a pool of three peptides corresponding to three ECTV CD8$^+$ T cell determinants and labeled with 4 μM CFSE (antigen-loaded targets, CFSE^{high}). Mice were killed 150 min after target inoculation, and the proportion of CFSE^{low} and CFSE^{high} cells was determined by FACS in D-LN and spleen. Histograms are gated on CFSE-positive cells. Numbers indicate percentage of specific killing of CFSE^{high} cells calculated as detailed in *Materials and Methods*. Data correspond to pools of three mice. The experiment was repeated four times with identical results. (*E*) Increased virus titers in spleen and liver after removal of the D-LN. Mice were subjected to surgery to remove the left popliteal LN (filled bars). Control mice were similarly operated, but the LN was not removed (open bars). Three days after surgery, mice received 3 × 10^6 memory CD8$^+$ T cells i.v. and the next day were infected with ECTV in the left footpad. Virus titers were determined in spleen and liver 4 days p.i. Data correspond to five mice per group.

protected as early as day 4 p.i. Consistent with the lack of resistance to disease, no responses were detected at any time in the donor-derived cells from mice that received naïve $CD8^+$ T cells (Fig. 3*C*), indicating an almost complete paralysis in the response of naïve $CD8^+$ T cells to ECTV in BALB/c mice.

The combined data in Fig. 3 *A–C* show that protection of the spleen and liver commenced before the memory $CD8^+$ T cell response in the spleen and the dispersal of $CD8^+$ T cell effectors became apparent. Because GzB is required for granule-mediated killing (22, 23), the data in Fig. 3 *B* and *C* were consistent with the hypothesis that armed $CD8^+$ T cells curb virus spread to liver and spleen by killing infected cells within the D-LN at the early stages of infection. We tested for this possibility by comparing the *in vivo* killing in the D-LN and spleen of targets loaded with synthetic peptides representing three ECTV $CD8^+$ T cell determinants restricted to H-2^d (24). Fig. 3*D* shows that mice that received memory $CD8^+$ T cells had incipient but detectable specific killing of targets in D-LN as early as 3 days p.i. and very strong killing (84%) on day 4. On the other hand, specific killing in spleens was absent on day 3 p.i. and was only incipient on day 4. Killing of target cells in mice recipient of naïve $CD8^+$ T cells was very limited or nonexistent at every time point (data not shown). Although our experiment used peptide-pulsed targets and not infected cells, it is reasonable to conclude that ECTV-infected cells expressing the same epitopes should also be killed within the D-LN. To further resolve the role of memory $CD8^+$ T cells in restricting the spread of ECTV from the D-LN, we determined virus titers 4 days p.i. in spleen and liver of mice that received memory $CD8^+$ T cells but had their D-LN removed 4 days before ECTV challenge. Fig. 3*E* shows that removal of the D-LN resulted in a 100-fold increase of virus loads in the spleen ($P = 0.000428$) and 28-fold increase in the liver ($P = 0.022$) as compared with mock operated mice. In summary, the data in Fig. 3 demonstrates that memory $CD8^+$ T cells proliferated rapidly and massively and became armed killers first in the D-LN and later in the spleen and that a reduction in virus titers in spleen and liver occurred even before the splenic response and the migration of armed effectors took place.

Discussion

Immunity is the "improved survival after reexposure to a pathogen that causes acute disease" (5, 25). Understanding the components of the immune system that provide immunity to disease-causing viruses is essential for the development of new vaccines. We have used the natural route of infection with a highly pathogenic OPV in its natural host to demonstrate that both circulating antibodies and memory $CD8^+$ T cells can independently prevent acute disease and death within the context of an able immune system. Our work addresses several important aspects of viral immunity. These include a definitive and irrefutable demonstration that antigen persistence is not inevitably required for memory $CD8^+$ T cell protection from an acute systemic viral disease, and the finding that memory $CD8^+$ T cells or Abs protect from disease only when *de novo* responses by the complementary arms of the immune system can occur. However, the most important aspect of this work is the unveiling of the mechanism whereby memory $CD8^+$ T cells protect from viral disease. Our data show that protection from disease does not involve limiting viral replication at the primary site of infection but, at least in part, curbing virus spread from the D-LN to target organs. Thus, the D-LN is not only the place where lymphocytes become activated and proliferate but is also a key site where infected cells are killed by $CD8^+$ T cells that act as the gatekeepers. Theoretically, this mode of protection may be applicable not only to OPVs but also for many other viruses that spread through secondary lymphoid organs (9). Of note, protection by memory $CD8^+$ T cells within the D-LN appears to be extremely efficient on a per cell basis. In our experiments, VACV-specific memory $CD8^+$ T cells represented only 0.25% of the total $CD8^+$ T cell pool in the recipient mice, and we estimate that only ≈350 virus-specific memory $CD8^+$ T cells were present in the D-LN at the time of ECTV challenge. It should be noted, however, that neither memory $CD8^+$ T cells nor Abs could prevent systemic infection completely because low but consistent virus loads were detected in the liver and spleen of recipients of memory $CD8^+$ T cells, Abs (data not shown), or even VACV-immunized mice (Fig. 3*A*), which have memory $CD8^+$ T cells as well as high Ab titers in their serum (data not shown). Therefore, for complete protection against highly pathogenic viruses, memory $CD8^+$ T cells and Abs must also act synergistically and systemically even in the case of a peripheral infection. Thus, it is very likely that $CD8^+$ T cells also mediate protection by eliminating infected cells in vital organs such as liver. This is supported by our finding of a lymphocytic infiltrate in the necrotic foci of livers of memory $CD8^+$ cells (Fig. 1*B*). It is also interesting to note that the restriction of virus spread from the D-LN occurs without detectable decrease in virus titers in the LN. Our main hypothesis to explain this finding is that ECTV spreads from the LN to central organs not as free virus but inside cells. We envision two alternative possibilities. (*i*) If infected cells harboring mature viral particles are killed by effector $CD8^+$ T cells, the mature viruses release to the milieu would still be infective and detected in the plaque assay while they would not be able to spread. A caveat to this explanation is that cells may become targets of CTL before the viral particles mature (the eclipse phase of viral replication). (*ii*) The cell type responsible for virus spread is a minor population within the LN but is preferentially killed by effector $CD8^+$ T cells. For example, preliminary experiments in our laboratory indicate that B cells and DC are infected by ECTV in the D-LN (Ricardo Lopez and L.J.S., unpublished results). However, the number of DCs in the LN is much smaller than that of B cells (20- to 60-fold lower). Thus, if DC were the main population responsible for spread and were preferentially killed (which is possible because, different from B cells, DC reside in T cell areas), virus spread to organs could be reduced without a noticeable effect in virus titers in the D-LN as determined by the plaque assay.

Work by Lanzavecchia and colleagues has shown that memory T cells can be subdivided into effector memory (CD62L low) and central memory (CD62 high) populations. It has been proposed that effector memory T cells normally transit through tissues and are important in protecting the site of virus entry (8, 26). It is therefore important to note that in the case of OPV infections, effector memory $CD8^+$ T cells do not seem to have a major effect in protecting the site of entry because ECTV replicated extensively with only moderate reduction in titers even in the case of mice immunized with VACV where ≈50% of the memory $CD8^+$ T cells were CD62 low (data not shown). This failure of memory $CD8^+$ T cells to provide good protection at the site of entry may be general because they have been shown to protect the lung but only moderately in the case of influenza virus infection (8) and may indicate that memory $CD8^+$ T cells may not be able to prevent clinical disease in nonsystemic viral infections such as those of the respiratory tract.

Methods

Mice. All of the experimental protocols involving animals were approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee. BALB/c and B6 mice were from Fox Chase Cancer Center stocks or purchased from The Jackson Laboratory. TAP KO breeders were originally purchased from The Jackson Laboratory. B cell KO and Thy1 KO mice were gifts from Randy Hardy and Kyoko Hayakawa (Fox Chase Cancer Center), respectively.

Cells, viruses, immunization, production of antisera, infec-

tions, determination of virus titers, and histopathology were as described previously (12, 15).

Adoptive Transfers. LN and spleens of donor mice were aseptically collected, and red blood cells were lysed with 0.84% NH_4Cl. The remaining cells were washed and labeled with rat anti-mouse CD8 magnetic beads as per the manufacturer's instructions (Miltenyi Biotec), and $CD8^+$ cells were magnetically purified twice by using an Automacs magnetic cell sorter (Miltenyi Biotec) first in the normal setting and next in the sensitive setting. Positively selected cells were used as $CD8^+$ T cells and negatively selected cells as $CD8^-$ lymphocytes. The efficiency of the purification was monitored by FACS. Final purity of $CD8^+$ T cells was consistently >90%, and no $CD8^+$ cells were detected in the $CD8^-$ population (data not shown). In some experiments, purified cells were labeled with CFSE (Molecular Probes) according to published procedures (20). The cells were resuspended in PBS (8×10^6 per ml), and 0.5 ml was inoculated i.v. into recipient mice.

Flow Cytometry. Spleens and LNs were obtained from mice at different times p.i. and made into single-cell suspensions. After osmotic lysis of red blood cells with 0.84% NH_4Cl (for spleen), cells were washed, and 2×10^6 cells were cultured at 37°C in 5-ml culture tubes in the presence of 2×10^5 vaccinia-infected A20 cells (for BALB/c and Thy1 KO mice) or DC2.4 cells (for B6 mice). Uninfected cells were used as control. After 5 h, brefeldin A (BFA) (Sigma) was added to block the secretory pathway and allow for the accumulation of cytokines inside the cells. After an additional 1.5-h incubation, antibodies 2.4G2 (anti-Fc-γ II/III receptor; American Type Culture Collection) were added to block nonspecific binding of labeled antibodies to Fc receptors. The cells were then stained for CD4 and/or CD8, fixed, permeabilized, and stained for IFN-γ and GzB by using the Cytofix/Cytoperm kit (Becton Dickinson) according to the manufacturer's instructions. The following antibodies were used: anti-CD4 (RM4–5; Pharmingen), anti-CD8α (53–6.7; Becton Dickinson), anti-IFN-γ (clone XMG1.2; Becton Dickinson), an isotype control (clone A95–1; Becton Dickinson), and anti-human GzB (Caltag) that is cross-reactive with mouse GzB (27). Cells were analyzed by flow cytometry at the Fox Chase Flow Cytometry and Cell Sorting Facility using an LSR II system (Becton Dickinson).

***In Vivo* Cytotoxicity Assays.** Red blood cell-depleted splenocytes from naïve BALB/c mice were split into two populations (28, 29). One population was labeled with a high concentration of CFSE (4 μM) ($CFSE^{high}$) and pulsed with 10 μg/ml each of the ECTV determinants SNHAAGYDL, VGPSNSPIF, and KYGRLFNEI restricted to L^d, D^d, and K^d, respectively (24). The second population of lymphocytes was labeled with a low concentration of CFSE (0.8 μM) ($CFSE^{low}$) and was not pulsed with peptides. The two cell populations were mixed together in a 1:1 ratio, and 2×10^7 cells were injected i.v. into mice that had received memory $CD8^+$ T cells and left uninfected, or that received memory $CD8^+$ T cells followed by infection with ECTV for 3 or 4 days. The recipient mice were killed 150 min after target cell inoculation, and the presence of $CFSE^{low}$ (antigen−) and $CFSE^{high}$ (antigen+) cells was determined by flow cytometry in cell suspensions of LNs and spleens. To calculate specific lysis, the following formula was used: % specific lysis = [1 − (ratio unprimed/ratio primed) × 100], where ratio = (% $CFSE^{low}$/% $CFSE^{high}$) (29).

Surgical Removal of the LN. Mice were anesthetized by i.p. inoculation with 5 μl/g saline solution containing 8 mg/ml ketamine, 2 mg/ml xylazine, and 0.3 mg/ml Acepromazine. Additional anesthetic was used when required. After mice were under anesthesia, the left hind leg was shaved and the skin was sprayed with ethanol. A small incision of the skin was made with a sterile scalpel (#11) on the plantar side of the tibia at the level of the popliteal space. Before surgery, mice were inoculated in the footpad with 20 μl of 0.4% trypan blue in saline, which permitted the easy visualization of the LN that stained bright blue. The connective tissue surrounding the popliteal LN was opened by divulsion and the LN was removed with sterile thumb forceps. Finally, the skin was closed with silk #6. An identical procedure but without removal of the LN was performed in control mice. After surgery, the mice were allowed to recover from anesthesia at 37°C and received an i.p. inoculation of 5,000 units of penicillin and 5 mg of streptomycin (Cellgro). Mice were observed daily and fed medicated food (Harlan TD 03184) until the end of the experiment. Three days after the surgery, mice were transferred with memory $CD8^+$ T cells and infected with ECTV the following day.

Statistical Analysis. We used a two-tailed *t* test for two samples for means with a confidence level (alpha) of 0.05. The software used was the Excel Analysis Tool Pack (Microsoft).

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