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CD4$^+$ T Cell Help Is Dispensable for Protective CD8$^+$ T Cell Memory against Mousepox Virus following Vaccinia Virus Immunization

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ABSTRACT

It has been shown in various infection models that CD4$^+$ T cell help (T_H) is necessary for the conditioning, maintenance, and/or recall responses of memory CD8$^+$ T cells ($CD8_M$). Yet, in the case of vaccinia virus (VACV), which constitutes the vaccine used to eradicate smallpox and is a candidate vector for other infectious diseases, the issue is controversial because different groups have shown either T_H dependence or independence of $CD8_M$ conditioning, maintenance, and/or recall response. In agreement with some of these groups, we show that T_H plays a role in, but is not essential for, the maintenance, proliferation, and effector differentiation of polyclonal memory CD8$^+$ T cells after infection with wild-type VACV strain Western Reserve. More important, we show that unhelped and helped anti-VACV memory CD8$^+$ T cells are similarly efficient at protecting susceptible mice from lethal mousepox, the mouse equivalent of human smallpox. Thus, T_H is not essential for the conditioning and maintenance of memory CD8$^+$ T cells capable of mounting a recall response strong enough to protect from a lethal natural pathogen. Our results may partly explain why the VACV vaccine is so effective.

IMPORTANCE

We used vaccinia virus (VACV)—a gold standard vaccine—as the immunogen and ectromelia virus (ECTV) as the pathogen to demonstrate that the conditioning and maintenance of anti-VACV memory CD8$^+$ T cells and their ability to protect against an orthopoxvirus (OPV) infection in its natural host can develop in the absence of CD4$^+$ T cell help. Our results provide important insight to our basic knowledge of the immune system. Further, because VACV is used as a vaccine in humans, our results may help us understand how this vaccine induces protective immunity in this species. In addition, this work may partly explain why VACV is so effective as a vaccine.

Following primary viral infection or vaccination, naive antiviral CD8$^+$ T cells ($CD8_N$) contribute to virus control by expanding and becoming effectors ($CD8_E$) that kill infected cells and produce antiviral cytokines such as gamma interferon (IFN-γ) (1). If the virus is eliminated, most $CD8_E$ die but many survive to become resting memory CD8$^+$ T cells ($CD8_M$) that remain at higher frequencies than the original $CD8_N$ population (2). If a secondary infection occurs, the $CD8_M$ rapidly expand and become secondary $CD8_E$. $CD8_M$ can contribute to reduce the severity of a secondary viral infection by achieving high numbers of effectors more rapidly than $CD8_N$ would. Moreover, the efficient generation of $CD8_M$ may be important for the effectiveness of some vaccines.

The genus *Orthopoxvirus* (OPV) comprises highly conserved DNA viruses that are antigenically highly cross-reactive. Vaccinia virus (VACV) is an OPV that can infect multiple species but is poorly pathogenic and highly immunogenic. Because of this, it was exploited as the vaccine that eliminated human smallpox, a highly lethal disease caused by the human-specific OPV variola virus (VARV). Thus, VACV remains as the gold standard of a highly effective vaccine, and VACV recombinants are currently being tested as vaccines for other infectious diseases and cancer (3, 4). In addition to preventing smallpox, VACV is also effective as a vaccine against lethal mousepox, a disease caused by the mouse-specific OPV ectromelia virus (ECTV) (5–9). Hence, VACV and ECTV can be paired as a unique model to understand the mechanisms of highly effective vaccination that is likely translatable to humans. Using this model, we have previously shown that in addition to antibodies (Abs), $CD8_M$ induced by VACV immunization can fully protect immunocompetent but susceptible mice from lethal mousepox (10, 11). Yet, how these protective $CD8_M$ are induced and maintained is not fully understood.

For some but not all infections, the transition of $CD8_N$ to $CD8_E$ requires CD4$^+$ T cell help (T_H) in the form of cytokines and/or costimulation (12). It has also been shown in several infectious models that T_H is required for the conditioning and/or maintenance of the $CD8_M$ pool and/or their secondary expansion and differentiation into $CD8_E$ (13–16). In the case of OPVs, however, these issues remain controversial (17–25). Given that VACV is a mildly virulent virus used as a vaccine against more-pathogenic OPVs, we thought that it was important not only to perform additional experiments to confirm or exclude the need for T_H for the establishment of anti-VACV $CD8_M$ but also, more importantly, to determine whether the absence of T_H affects the ability of $CD8_M$ to

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become $CD8_E$ protective against a highly pathogenic OPV in its natural host. Thus, we used unattenuated VACV WR as the vaccine and ECTV as the pathogen to address the role of T_H in the generation of protective $CD8_M$. Our experiments measuring polyclonal rather than transgenic CD8$^+$ T cell responses show that unhelped $CD8_M$ that expand and differentiate into $CD8_E$ are as effective as helped $CD8_M$ in their ability to protect from mousepox. Thus, T_H is not essential for the generation and maintenance of memory CD8$^+$ T cells capable of protecting against an OPV in its natural host.

MATERIALS AND METHODS

Ethics statement. All animal experiments were conducted following the eighth edition of the Guide for the Care and Use of Laboratory Animals, National Research Council of the National Academy of Sciences, as mandated by the Office of Laboratory Animal Welfare (OLAW), NIH, and in accordance with protocols approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee.

Cells and virus. The dendritic cell line DC2.4 was a gift from K. Rock (University of Massachusetts Medical Center, Worcester, MA). HeLa S3 and BSC-1 cells were obtained from the American Type Culture Collection. Initial stocks of VACV virus strain Western Reserve (WR) were obtained from B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD) and amplified in HeLa S3 cells as described previously (26). Initial stocks of the wild-type (WT) ECTV Moscow were obtained from ATCC (number VR-1374), and virus production and determination of virus titers were done as described previously (27).

Mice, immunizations, and infections. Mice were bred at the Fox Chase Cancer Center Laboratory Animal Facility in specific-pathogen-free rooms from homozygous mice obtained from commercial vendors as follows: C57BL/6-Tg(Thy1-Sncg)HvP36Putt/J mice (B6.Thy1.1) and B6.D2-(D6Mit149-D6Mit15)/LusJ (B6.D2-D6) mice were originally purchased from Jackson Laboratories. B6 (CD45.2$^+$) mice, B6.SJL (CD45.1$^+$) mice, B6.129-H2-Ab1^{tm1Gru}N12 (major histocompatibility complex class II-deficient [MHC-II$^{0/0}$]) mice, and B6.SJL (129)^{Ptprca}/BoyAiTac *H2-Ab1^{tm1Gru}* N7$^+$N6 (CD45.1$^+$-MHC-II$^{0/0}$) mice were from Taconic Farms. Genotyping was according to the vendor's protocols. Before VACV immunization or ECTV infection, sex-matched animals 8 to 12 weeks old were transferred to a biosafety level 3 room. For VACV immunizations, mice were infected intraperitoneally (i.p.) with 500 μl phosphate-buffered saline (PBS) containing 5×10^6 PFU VACV WR. For ECTV infections, mice were infected in the left footpad with 3×10^3 PFU ECTV, approximately 9,000 LD_{50} (50% lethal dose) (28), in 25 μl PBS. All the infected mice were observed daily during the course of the experiments. In survival experiments, when death was imminent as evidenced by ruffled fur, hunched posture, lethargy, and unresponsiveness to touch, mice were euthanized according to the guidelines of the Institutional Animal Care and use Committee (IACUC) of the Fox Chase Cancer Center.

Adoptive transfers. Adoptive transfers were performed as described previously (10, 11, 19). Briefly, lymph nodes (LN) and spleens of indicated donor mice were aseptically collected, and red blood cells (RBC) were lysed with 0.84% NH_4Cl. The remaining cells were washed and labeled with rat anti-mouse CD8 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec), and CD8$^+$ cells were magnetically purified using an Automacs magnetic cell sorter (Miltenyi Biotec). The efficiency of the purification was monitored by fluorescence-activated cell sorter (FACS). Normally, the purity of CD8 T cells is >95%. In some experiments, purified cells were labeled with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) according to published procedures (29). Indicated amounts of purified CD8$^+$ T cells were resuspended in 0.5 ml PBS and inoculated intravenously (i.v.) into the recipient mice.

Flow cytometry. Determination of cytokine production by intracellular staining was done as described previously (10, 11, 27, 30). Briefly, lymphocytes from spleens were obtained from mice at different days postinfection (days p.i.) and made into single-cell suspensions. Following osmotic lysis of RBC with 0.84% NH_4Cl, lymphocytes were washed, and 10^6 cells were cultured at 37°C in 96-well plates in the presence of 10 U/ml interleukin-2 (IL-2) and 2×10^5 VACV-infected DC2.4 cells or uninfected DC2.4 cells as control. After 5 h, brefeldin A (Sigma-Aldrich) was added to block the secretory pathway and to allow for the accumulation of cytokines inside the cells. Following an additional 1.5 h of incubation, Ab 2.4G2 was added to block nonspecific binding of labeled Ab to FcR. The cells were then stained for cell surface molecules, fixed, permeabilized, and stained for intracellular molecules using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. To stain for TSYKFESV epitope-specific T cells, H-2Kb:Ig recombinant fusion protein complexes (Mouse DimerX Kb dimers; BD) were incubated with synthetic TSYKFESV (GenScript) and used as recommended by the manufacturer. TSYKFESV is the dominant CD8$^+$ T cell determinant of VACV and ECTV in the B6 background. At least 100,000 cells were analyzed by flow cytometry using the LSR II system (BD Biosciences).

***In vivo* cytotoxicity assay.** The *in vivo* cytotoxicity assay was done as described previously (10, 11, 19). Briefly, single-cell suspensions of lymphocytes from naive B6 mice were split into two populations. One population was labeled with a high concentration (4 μM) of CFSE (CFSE^{high}) and pulsed with 10 μg/ml of TSYKFESV (31). The second population of lymphocytes was labeled with a low concentration (0.8 μM) of CFSE (CFSE^{low}) and was not pulsed with peptide. The two cell populations were mixed together at a 1:1 ratio, and 2×10^7 total cells were injected i.v. into the indicated mice. Six hours later, recipient mice were euthanized, and the presence of CFSE^{low} and CFSE^{high} cells was determined by flow cytometry in cell suspensions of spleens. To calculate specific lysis, the following formula was used: % specific lysis = [1 − (ratio unprimed/ratio primed) × 100], where "ratio" is (% CFSE^{low}/% CFSE^{high}).

Data displayed and statistical analysis. Unless indicated, all displayed data correspond to one representative experiment from at least three similar experiments with groups of three to five mice. Statistical analysis was performed using Prism software (GraphPad Software, Inc.). All statistical analyses were performed using an unpaired two-tailed Student's *t* test or the Mann-Whitney test as applicable. When applicable, data are displayed as means ± standard errors of the means (SEM).

RESULTS

T_H plays a role, but is not critical, in generating anti-VACV $CD8_E$ and/or the maintenance of anti-VACV $CD8_M$. To determine whether T_H is required for the primary endogenous anti-VACV CD8$^+$ T cell response, we compared the endogenous responses of 6- to 12-week-old WT B6 and MHC-II-deficient (MHC-II$^{0/0}$) mice to VACV strain WR at 7 days postpriming (dpp). The frequency of CD8$^+$ T cells expressing granzyme B (GzB), which for $CD8_E$ does not require *in vitro* restimulation (19, 32), was similar for MHC-II$^{0/0}$ and B6 mice, albeit the absolute numbers of GzB$^+$ CD8$^+$ T cells were slightly reduced in MHC-II$^{0/0}$ mice. Yet, the frequency and absolute numbers of CD8$^+$ T cells expressing IFN-γ, which requires *ex vivo* restimulation) or that stained with H-2K^b dimers (Dimer-X; BD) loaded with the immunodominant determinant TSYKFESV were similar in B6 and MHC-II$^{0/0}$ mice (Fig. 1A). Thus, T_H is not essential to generate large numbers of endogenous anti-VACV $CD8_E$.

Despite being similar during the effector phase and as described for other models, the frequency of K^b-TSYKFESV-specific $CD8_M$ in MHC-II$^{0/0}$ mice declined faster and was about one-half that of B6 mice at various times after immunization. Nevertheless, MHC-II$^{0/0}$ mice still maintained a relatively large population of TSYKFESV-specific $CD8_M$ as late as 180 dpp (Fig. 1B). Side-by-side comparison of TSYKFESV-specific $CD8_M$ in B6 and MHC-II$^{0/0}$ mice at 60 dpp showed that fewer $CD8_M$ cells expressed the IL-7 receptor alpha chain CD127 and the memory cell marker

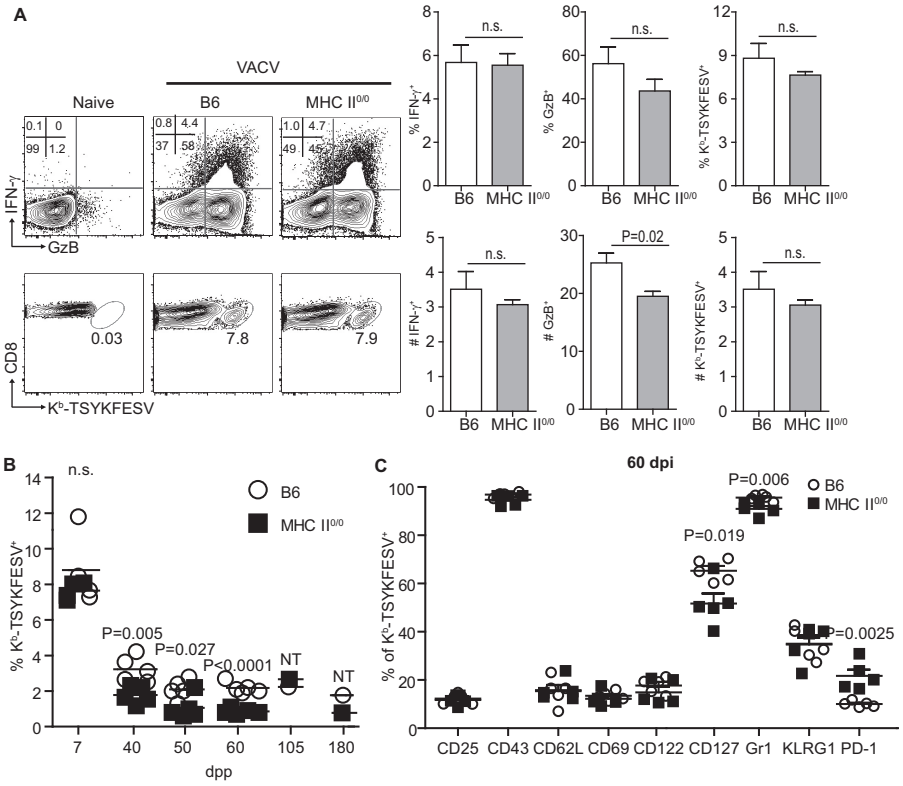

FIG 1 T_H plays a role but is not critical in generating anti-VACV $CD8_E$ and/or the maintenance of anti-VACV $CD8_M$. B6 and MHC-$II^{0/0}$ mice were primed i.p. with 5×10^6 PFU VACV strain WR. (A) CD8 T cell responses in spleens were determined at 7 dpp. Representative flow cytometry plots on the left show IFN-γ and GzB expression after *in vitro* restimulation with VACV-infected DC2.4 cells (upper row) and staining with K^b-TSYKFESV dimers (bottom row) by gated $CD8^+$ cells. The summary graphs on the right show the frequency (top row) and number in millions (bottom row) of IFN-γ^+, GzB^+, and K^b-TSYKFESV$^+$ $CD8^+$ T cells for three mice/group. (B) Frequency of K^b-TSYKFESV$^+$ $CD8_M$ at different dpp. NT, not tested because the data are from spleens pooled from 2 or 3 mice. (C) Frequency of K^b-TSYKFESV$^+$ $CD8_M$ expressing the indicated cell surface molecules at 60 dpp. Data are representative of results from two or three similar experiments. n.s., not significant.

Gr-1(Ly6G/C) while more expressed the inhibitory molecule PD-1 in MHC-$II^{0/0}$ mice. Yet, while statistically significant, it is arguable that these differences were minor because the vast majority of $CD8_M$ in MHC-$II^{0/0}$ and B6 mice had similar mean fluorescence intensity (MFI) for these markers (not shown). Moreover, the expression of the activation and memory markers CD25, CD43, CD69, CD122, and KLRG1 were indistinguishable by frequency (Fig. 1C) and MFI (not shown). Thus, in the absence of T_H, the frequency of anti-VACV $CD8_M$ is reduced compared to that of the wild type but still high compared to that of naive $CD8_M$; and while some unhelped $CD8_M$ are phenotypically altered, the vast majority of the unhelped $CD8_M$ have a phenotype that is indistinguishable from that of helped $CD8_M$.

Unhelped $CD8_M$ expand and generate secondary $CD8_M$ when maintained and boosted in an MHC-II-deficient environment. Next, we compared the abilities of helped and unhelped $CD8_M$ to expand and generate secondary $CD8_M$ after booster VACV immunization within their respective WT or MHC-$II^{0/0}$ environments. B6 and MHC-$II^{0/0}$ mice were immunized with VACV, rested for 5 weeks, and boosted with VACV. Sixty days postboost (dpb), the frequency of $CD8_M$ as determined by IFN-γ production and K^b-TSYKFESV dimer staining increased sharply in both B6 and MHC-$II^{0/0}$ mice (Fig. 2A), demonstrating that unhelped $CD8_M$ can be recalled into secondary effectors. Still, the frequency of secondary $CD8_M$ cells in B6 mice was significantly higher than in MHC-$II^{0/0}$ mice ($P < 0.001$ for IFN-γ production and $P < 0.001$ for K^b-TSYKFESV dimer staining). Yet, because MHC-$II^{0/0}$ mice had a lower frequency of $CD8_M$ than B6 mice before boosting, it cannot be concluded that the differences in the frequency of secondary $CD8_M$ were due to intrinsic defects of the unhelped $CD8_M$. Yet, consistent with results from Fuse et al. (20), the MFI for IFN-γ in the IFN-γ^+ gates were significantly lower in MHC-$II^{0/0}$ than in B6 mice (not shown), suggesting an altered effector function of recalled $CD8_M$ after VACV.

We also compared the phenotypes of secondary helped and unhelped $CD8_M$. Compared with B6 mice, boosted MHC-$II^{0/0}$ mice had significantly larger frequencies of $CD8_M$ that expressed PD-1 and lower frequencies that expressed KLRG1 (Fig. 2B). Nevertheless, in MHC-$II^{0/0}$ and B6 mice, most secondary $CD8_M$ did not express PD-1 and 40 to 60% of $CD8_M$ were KLRG1$^+$. Furthermore, all other markers analyzed were indistinguishable. Thus, as with primary unhelped $CD8_M$, most unhelped secondary $CD8_M$ have a normal phenotype.

We next compared the effector function of helped and unhelped primary and secondary $CD8_M$ in *in vivo* cytotoxicity assays at 60 dpp or dpb (10, 19, 33). Similar to secondary $CD8_M$ in B6 mice, secondary $CD8_M$ killed splenocytes loaded with TSYKFESV with high efficiency in MHC-II mice (Fig. 2C).

Thus, when maintained in an MHC-$II^{0/0}$ environment, unhelped primary $CD8_M$ proliferate after booster immunization to

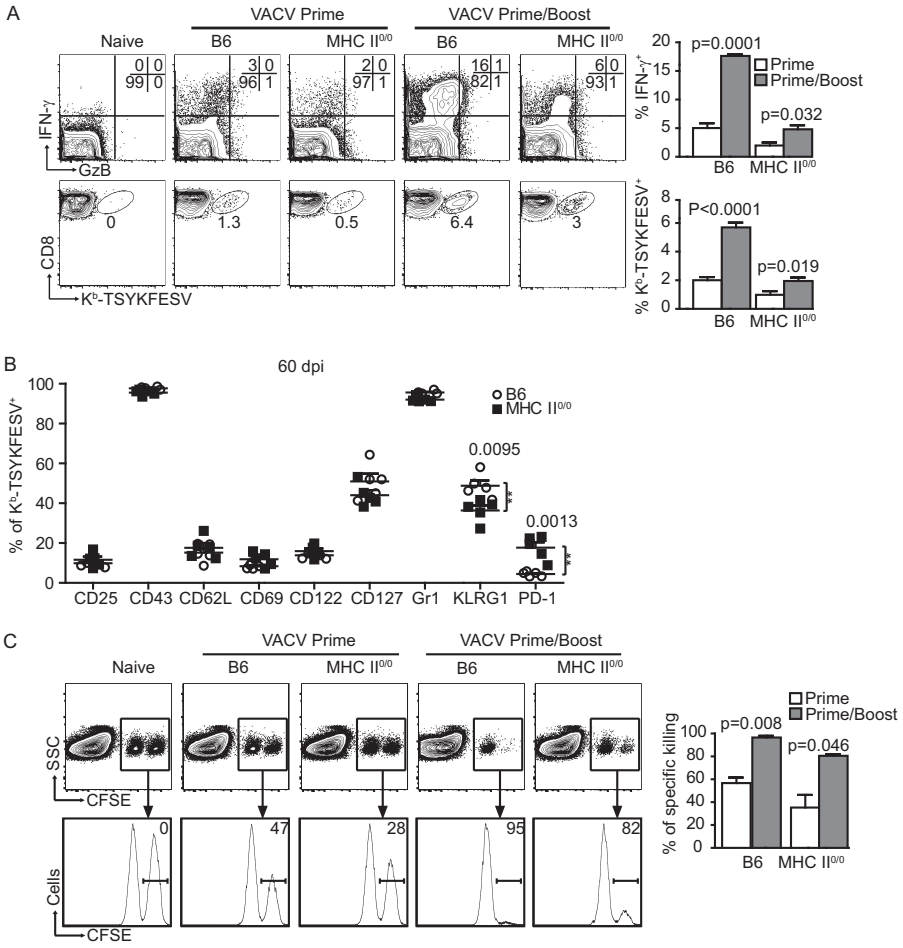

FIG 2 Unhelped $CD8_M$ expand and generate secondary $CD8_M$ cells when maintained and boosted in an MHC-II-deficient environment. B6 mice and MHC-II$^{0/0}$ mice were primed i.p. with 5×10^6 PFU VACV and, when applicable, similarly boosted 5 weeks later. (A) $CD8_M$ in spleen were determined at 60 dpp or dpb; the cells were gated on CD8$^+$ T cells. Representative flow cytometry plots on the left show IFN-γ and GzB expression after *in vitro* restimulation with VACV-infected DC2.4 cells (top row) and staining with K^b-TSYKFESV$^+$ dimers (bottom row). The summary graphs on the right show the frequency of IFN-γ$^+$ and K^b-TSYKFESV$^+$ $CD8_M$. (B) Frequency of K^b-TSYKFESV$^+$ $CD8_M$ expressing the indicated cell surface molecules at 60 dpp or dpb. (C) *In vivo* killing of target cells by $CD8_M$. Splenocytes from naive B6 mice were labeled with CFSE at 3.0 μM (CFSE^{High}) or 0.8 μM (CFSE^{Low}). CFSE^{High} cells were pulsed with TSYKFESV, and CFSE^{Low} were not. Mixtures (1/1) of CFSE^{High} and CFSE^{Low} cells were injected i.v. into primed or primed/boosted B6 and MHC-II$^{0/0}$ mice at 60 dpp or dpb. Killing was determined 6 h later in spleen. Representative flow cytometry plots are shown on the left. Plots in the upper row are gated on CD8$^+$ cells. The transferred cells can be distinguished by their CFSE expression. The histograms in the lower row show the peaks of CFSE^{Low} and CFSE^{High} cells. The number inside the plot indicates the percentage of specific killing of CFSE^{High} (TSYKFESV-pulsed) cells. Data correspond to groups of three to five mice and are representative of results from two or three experiments.

generate an expanded secondary $CD8_M$ pool. Most of these secondary $CD8_M$ maintain a normal phenotype and are effective killers *in vivo*. However, with this experimental setting, it is difficult to quantitatively compare the secondary response of helped and unhelped $CD8_M$ because they start from different numbers of primary $CD8_M$ precursors and are maintained in an immunodeficient environment.

T_H is not essential during priming for the conditioning of anti-VACV $CD8_M$. The data above show that MHC-II$^{0/0}$ mice have a reduction in their polyclonal anti-VACV primary $CD8_M$ pools and somewhat decreased expansion during the secondary response. Why unhelped $CD8_M$ are deficient remains controversial. Using tumor cells and lymphocytic choriomeningitis virus (LCMV), Janssen et al. (34) showed that in the absence of help, the responding polyclonal CD8$^+$ T cells upregulated proapoptotic molecules such as TRAIL. As a consequence, when the unhelped $CD8_M$ reencountered antigen, they suffered antigen-induced cell death (AICD). This indicated that, in this system, the unhelped phenotype is programmed during priming. On the other hand, Sun et al. used adoptive transfer of T cell receptor (TCR) transgenic CD8$^+$ T cells during LCMV and *Listeria monocytogenes* infections to show that the defect of the unhelped $CD8_M$ was acquired during the maintenance phase and not during priming (15). To test whether the quantitative differences that we observed in our model were acquired during priming, we immunized B6 and MHC-II$^{0/0}$ mice (CD45.2) with VACV to prime in the presence or the absence of T_H. At 7 dpp, 5×10^6 $CD8_E$ from these mice were transferred into naive B6-CD45.1 mice. Seven days later, both CD45.2 B6 and MHC-II$^{0/0}$ donor cells represented 3 to 4% of the total CD8$^+$ T cells and ~2% of these were TSYKFESV specific (Fig. 3A). Hence, the early contractions of helped and unhelped $CD8_E$ were similar in a WT environment. At later time points, the

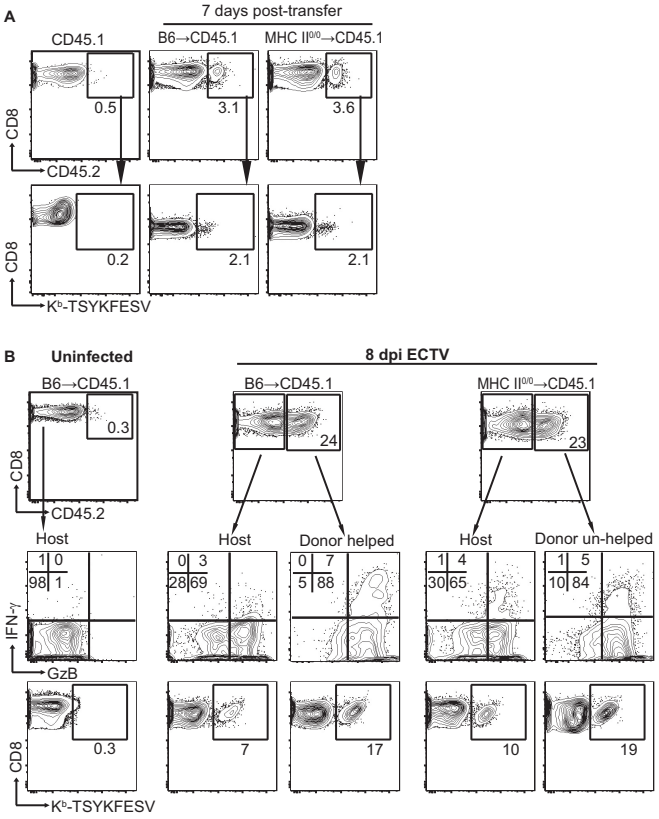

FIG 3 T_H is not required during priming for the conditioning of anti-VACV $CD8_M$. B6 and MHC-$II^{0/0}$ mice were infected i.p. with 5×10^6 PFU VACV. At 7 days p.i., 5×10^6 magnetically purified $CD8^+$ T cells from these mice were transferred i.v. into B6.CD45.1 mice. (A) Seven days after transfer, the donor cells were detected in the spleens of the recipient mice. Representative flow cytometry plots show the frequency of $CD45.2^+$ transferred cells in the $CD8^+$ gate (top row) and the frequency of K^b-TSYKFESV$^+$ cells in the CD45.2 gate (bottom row). Data correspond to three pooled spleens, and the experiment was repeated three times. (B) Four months after transfer, the recipient mice were infected with 3×10^3 PFU ECTV in the footpad, and the $CD8^+$ T cells responses were determined at 8 days p.i. Flow cytometry plots show the frequency of host and transferred cells in the $CD8^+$ gate (top row), expression of IFN-γ and GzB after restimulation with VACV-infected DC2.4 cells (middle row), and the frequency of K^b-TSYKFESV$^+$ cells in the CD45.2 gate (bottom row). The data correspond to two pools of two spleens and are representative of results from three experiments that were similar but had some differences in the methods (for example, less time between transfer and infection).

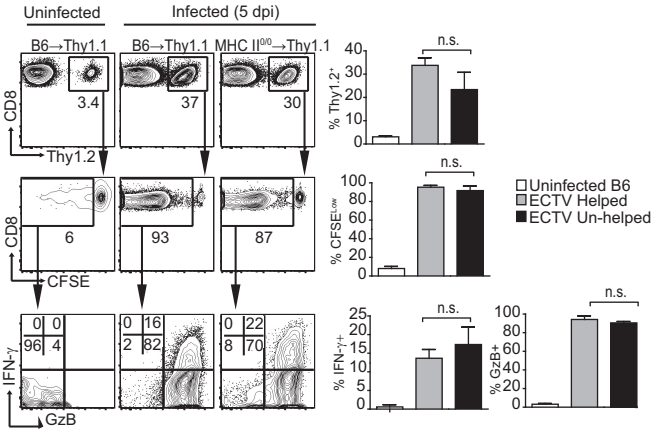

FIG 4 Quantitatively normal response to ECTV by unhelped secondary anti-VACV $CD8_M$ maintained in the absence of help. B6 mice and MHC-$II^{0/0}$ mice were primed and boosted 5 weeks later with 5×10^6 PFU VACV i.v. At 60 dpp or dpb, the splenocytes were obtained and labeled with CFSE, and the $CD8^+$ T cells were magnetically purified, equalized for similar numbers of K^b-TSYKFESV$^+$ $CD8^+$ T cells, and inoculated i.v. into B6.Thy1.1 mice. One day later, the mice were infected with 3×10^3 PFU ECTV in the footpad or left uninfected as a control. Cells in the draining popliteal lymph node were analyzed at 5 days p.i.. Representative flow cytometry plots on the left show the frequencies of transferred cells within the $CD8^+$ gate (top row), the frequencies of cells within the transferred cells that diluted their CFSE (middle row), and the frequencies of cells that diluted CFSE and express GzB and IFN-γ. Summary graphs for the same parameters are shown on the right. Data correspond to three mice/group and are representative of results from three similar experiments. n.s., not significant.

VACV-specific donor $CD8_M$ could not be detected by flow cytometry, regardless of the donor strain (not shown). We hypothesized that if $CD8_M$ were still present, they would expand after VACV challenge and any differences would be apparent by alterations in the frequency of helped and unhelped donor $CD8_E$. Thus, we performed experiments in which groups of 2 or 3 B6-CD45.1 mice received equal numbers of helped (B6) or unhelped (MHC-$II^{0/0}$) $CD45.2^+$ $CD8_E$ and 1.5 to 4 months later were infected in the footpad with the OPV ECTV, a natural pathogen of the mouse. Of note, ECTV and VACV share most of their $CD8^+$ T cell determinants. At 8 days p.i., helped and unhelped donor $CD8^+$ T cells represented 23 to 24% of the total $CD8^+$ T cells, demonstrating extensive expansion. The vast majority of the donor cells were effectors, as ~90% expressed GzB and 6 to 7% expressed IFN-γ. Fifteen to 20% were K^b-TSYKFESV specific, whether helped or unhelped during priming (Fig. 3B). Because the analysis was performed with pooled spleens, no statistics could be calculated. Yet, comparable results were obtained in two similar experiments, indicating that if there were any differences, these are minimal. Thus, during VACV infection, T_H does not condition the polyclonal $CD8_M$ response at priming.

Quantitatively normal response to ECTV by unhelped secondary anti-VACV $CD8_M$ maintained in the absence of help. As we show (illustrated in Fig. 2), it is difficult to quantitatively compare secondary $CD8_M$ responses in immunized WT and MHC-II-deficient hosts because they contain different numbers of $CD8_M$. Also, most vaccines use prime/boost regimes. Thus, it is the secondary (or higher) $CD8_M$ that need to differentiate into tertiary (or higher) $CD8_E$ for vaccine-mediated protection. Therefore, we quantitatively compared the helped and unhelped $CD8_M$ responses primed, contracted, and maintained in their respective environments, after transfer into naive WT hosts. For this purpose, B6 and MHC-$II^{0/0}$ mice were primed/boosted with VACV. At 60 dpb, their splenic $CD8^+$ T cells were labeled with CFSE, adjusted to equal numbers of K^b-TSYKFESV$^+$ $CD8^+$ T cells, and transferred into B6.Thy1.1 mice. One day after transfer, the mice were infected in the footpad with ECTV, and the $CD8^+$ T cell responses were determined in the draining lymph node (D-LN) at 5 days p.i. We found no differences in the abilities of helped and unhelped secondary $CD8_M$ in becoming $CD8_E$ as determined by frequency of the expanded donor cells (Fig. 4, first row), proliferation by CFSE dilution (Fig. 4, second row), or expression of the effector molecules GzB and IFN-γ (Fig. 4, third row). Thus, when transferred in equal numbers to WT hosts, unhelped anti-VACV $CD8_M$ primed, contracted, and maintained in a T_H-deficient en-

TABLE 1 Unhelped secondary $CD8_M$ protect susceptible mice from mousepox[a]

No. of K^b-TSYKFESV$^+$ cells transferred	No. dead/total no. of mice receiving:		
	Helped $CD8_M$[b]	Unhelped $CD8_M$[c]	No cells
1.8×10^5	0/5**	0/5**	
9×10^4	0/5**	0/5**	
4.5×10^4	0/5**	0/5**	
2.25×10^4	4/5	0/5**	
0			5/5

[a] B6 mice and MHC-II$^{0/0}$ mice were primed and then boosted 5 weeks later with 5×10^6 PFU VACV i.v. Two months later, the splenocytes of these mice were obtained, the CD8$^+$ T cells were magnetically purified, the frequency of Kb-TSYKFESV$^+$ cells was determined by flow cytometry, and the indicated numbers of Kb-TSYKFESV$^+$ cells from B6 (helped $CD8_M$) and MHC II$^{0/0}$ (unhelped $CD8_M$) mice were inoculated i.v. into mousepox-susceptible B6.D2-D6 mice. One day later, the mice were infected with 3×10^3 PFU ECTV in the footpad and observed for lethality as detailed in Materials and Methods. **, $P < 0.01$ compared with mice receiving no cells (control).
[b] Helped $CD8_M$ contained 2.25% Kb-TSYKFESV$^+$ cells.
[c] Unhelped $CD8_M$ contained 1% Kb-TSYKFESV$^+$ cells.

vironment responded as efficiently as helped $CD8_M$ to pathogenic viral challenge.

Unhelped secondary $CD8_M$ protect susceptible mice from mousepox. The results above show that $CD8_M$ generated by VACV immunization in the absence of T_H can mount strong recall responses to secondary and tertiary ECTV challenge whether maintained in the presence or absence of T_H. Because the goal of vaccination is to protect from disease, we next determined whether unhelped $CD8_M$ generated by VACV prime/boost immunization can protect susceptible mice from lethal mousepox.

B6.D2-D6 is a B6 congenic mouse strain that is susceptible to mousepox (35) but can be protected by $CD8_M$ after vaccination with dendritic cells pulsed with TSYKFESV (36) or adoptive transfer of CD8$^+$ T cells from B6 mice primed/boosted with VACV (10). Helped and unhelped $CD8_M$ were generated by VACV prime/boost immunization of B6 and MHC-II$^{0/0}$ mice, respectively. At 60 dpb, purified CD8$^+$ T cells from these mice were adjusted to contain similar numbers of K^b-TSYKFESV$^+$-specific $CD8_M$ and graded numbers were adoptively transferred into groups of five B6.D2-D6 mice, which were subsequently infected with ECTV. As shown in Table 1, all control (untransferred) mice succumbed to mousepox. However, all B6.D2-D6 mice transferred with as little as 4.5×10^4 K^b-TSYKFESV$^+$ $CD8_M$ from VACV-primed/boosted B6 or MHC-II$^{0/0}$ mice were fully protected. Consistently, mice receiving B6 or MHC-II$^{0/0}$ $CD8_M$ did not have the splenic lymphopenia characteristic of lethal mousepox (Fig. 5A). Moreover, the virus titers at 7 days p.i. were significantly lower in mice that received helped or unhelped $CD8_M$ than in untransferred mice, while there were no significant differences in virus titers between B6 and MHC-II$^{0/0}$ $CD8_M$ recipients (Fig. 5B). Because in these experiments we did not use mice with congenic markers, we were unable to analyze the $CD8_M$ response. Yet the fact that helped and unhelped $CD8_M$ are similarly responsive was already demonstrated, as illustrated in Fig. 4.

DISCUSSION

We have previously shown that $CD8_M$ induced by VACV immunization become $CD8_E$ and protect mice from mousepox, strongly suggesting that the establishment of a pool of $CD8_M$ cells is one of the mechanisms whereby the smallpox vaccine protects from pathogenic OPVs (10, 11, 36). Given the importance of $CD8_M$ induced by VACV in protection from lethal OPVs, we thought that it was important to determine the impact of T_H in the establishment of this form of protective immunity. For this purpose, we used nonattenuated VACV WR as the vaccine and ECTV as the pathogen. Using this model, we showed that in MHC-II$^{0/0}$ mice, which lack T_H, $CD8_N$ generate potent $CD8_E$ that transition into long-lived $CD8_M$. We also found that unhelped $CD8_M$ can be activated to become secondary $CD8_E$ in a WT as well as in an MHC-II-deficient environment and transition into secondary $CD8_M$. Moreover, when transferred into mousepox-resistant WT mice, unhelped secondary $CD8_M$ expand and become effectors as efficiently as their helped counterparts in response to ECTV. More importantly, unhelped secondary $CD8_M$ are as effective as helped $CD8_M$ at protecting susceptible mice from lethal mousepox.

To date, the role of T_H in primary and secondary CD8$^+$ T cell responses to VACV remains controversial. While some have shown that T_H is necessary for the induction of anti-VACV primary CD8$^+$ T cell responses (21–24, 37), others, including us, have shown the opposite (17–20). Similarly, disparate results have been published regarding the role of T_H in the generation, maintenance, and recall responses of anti-VACV $CD8_M$. Some have shown that T_H is required (14, 20–22, 37), and others have shown that it is not (24, 25). A possible explanation for these discrepancies may lie in the replicative capacity of the VACV strain used. In our experiments, we used VACV WR, which is not attenuated. Recently, Salek-Ardakani et al. (25) showed that MHC-II$^{0/0}$ mice were protected by $CD8_M$ from intranasal VACV WR challenge when preimmunized with the nonattenuated VACV WR but were not protected after immunization with the attenuated VACV strain Lister. Moreover, several of the publications showing T_H requirement were performed using VACV isolates with deletion of the thymidine kinase gene (14, 21–23, 37, 38), which results in attenuation (39). This suggests that the generation, maintenance, and recall response of $CD8_M$ to nonattenuated VACV may be less dependent on T_H than strains with poorer replicative potential. Another reason for the differences might be that, similar to Salek-Ardakani et al. (25), we determined polyclonal responses against a natural epitope shared by VACV and ECTV (31, 36) rather than TCR transgenic responses to ectopic determinants (14, 21–23, 37,

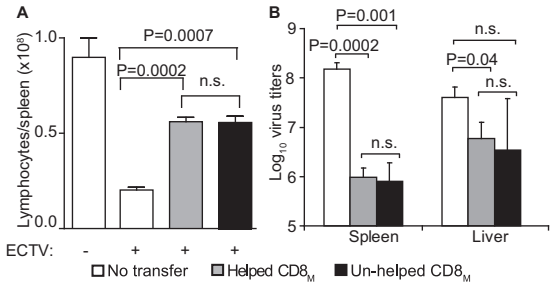

FIG 5 Unhelped secondary $CD8_M$ protect susceptible mice from mousepox. B6 mice and MHC-II$^{0/0}$ mice were primed and then boosted 5 weeks later with 5×10^6 PFU VACV i.v. At 60 dpb, the splenocytes of these mice were obtained, the CD8$^+$ T cells were magnetically purified, the frequency of K^b-TSYKFESV$^+$ cells was determined by flow cytometry, and 4.5×10^4 K^b-TSYKFESV$^+$ cells were inoculated i.v. into mousepox-susceptible B6 mice. One day later, the mice were infected with 3×10^3 PFU ECTV in the footpad. (A) Total number of lymphocytes in the spleen. (B) Virus titers in spleen and liver. n.s., not significant.

38). In addition to attenuation, other factors that may affect the need for T_H and protective capacity are the dose of VACV and the route of inoculation (20, 25).

It is also important to note that in our experiments we used MHC-II-deficient mice because they guarantee the absence of MHC-II-restricted T_H. $CD4^+$ T cells recognize antigen as peptides bound to MHC class II molecules. Peptide-MHC-II recognition is also required for their development in the thymus. Hence, mice deficient in MHC-II lack MHC-II-restricted cells. This is different for CD4-deficient mice, which have MHC-II-restricted $CD4^-$ $CD8^-$ helper T cells (40–42), and a large fraction of their $CD8^+$ T cells are also restricted by MHC-II (43, 44), resulting in poorer overall MHC-I-restricted $CD8^+$ responses (43). This can account for some of the deficits in the anti-VACV $CD8_M$ previously observed by others (38). MHC-$II^{0/0}$ mice also lack possible complications arising from incomplete and/or prolonged $CD4^+$ T cell depletion with the anti-$CD4^+$ monoclonal antibody (MAb) GK1.5 (45), which we and others previously used (19, 38, 46, 47). Yet, experiments with MHC-$II^{0/0}$ mice, which have a major immunodeficiency, may still have caveats. In our experiments, we have found significantly reduced numbers of $CD8_M$ in MHC-$II^{0/0}$ mice and low but significant differences in the frequency of primary and/or secondary $CD8_M$ expressing CD127, Gr1, KLRG1, and PD-1. While it is possible that these differences are due to the lack of T_H, it is also possible that they are a consequence of the imbalance of the immune system in MHC-$II^{0/0}$ mice. For example, it is very likely that their interaction with the microbiome is different from that in immunocompetent mice, and this can affect their health and the quality of their immune responses, particularly as the mice age. In this regard, we have observed that naive aged MHC-$II^{0/0}$ mice have poor health and tend to die earlier than age-matched B6 mice. Further, we have found that aged naive MHC-$II^{0/0}$ mice have fewer cells with the $CD8_M$ phenotype, suggesting an overall dysregulation of the $CD8^+$ T cell compartment with aging (our unpublished experiments). These defects may contribute to the faster decline in the frequency of $CD8_M$ in MHC-$II^{0/0}$ than in the frequency of $CD8_M$ in B6 mice that we (Fig. 1) and others (16) have observed, an issue deserving of further exploration. To overcome this caveat, we have used adoptive transfer of polyclonal anti-VACV $CD8_M$. Using this approach, we have shown that when cell numbers are adjusted, polyclonal anti-VACV $CD8_M$ generated in the absence or presence of help are similarly potent at protecting mice from a lethal viral disease.

In summary, our experiments confirm in the VACV model that $CD8_M$ in MHC-$II^{0/0}$ mice are not completely normal, as they contracted more than in WT mice and a minority of them had an altered phenotype. Thus, our work does not dispute that T_H contributes to the optimal generation and maintenance of $CD8_M$. Yet, our work clearly shows that T_H is not crucial for the establishment of $CD8_M$ or essential to confer $CD8_M$ the capacity to protect from a lethal infection. Because VACV is used as a vaccine in humans, our results may have implications for our understanding on how this vaccine induces protective immunity in this species.

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