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Antibodies and CD8$^+$ T Cells Are Complementary and Essential for Natural Resistance to a Highly Lethal Cytopathic Virus[1]

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It is believed that CD8$^+$ T lymphocytes or Abs can independently clear many primary viral infections, including those caused by *Orthopoxviruses* (OPV), a genus that includes the human pathogens variola and monkeypox and the vaccine species vaccinia virus. However, most experiments addressing the role of Abs and CD8$^+$ T cells in protection have used viruses that are not specific for the host. In the present study, we used the mouse-specific OPV ectromelia virus and mice deficient in CD40, B cells, or CD8$^+$ T cells and adoptive transfers of CD8$^+$ T or B lymphocytes to show that the protection afforded by CD8$^+$ T cells is incomplete. Despite sustained CD8$^+$ T cell responses, in the absence of Ab responses ectromelia virus persists. This results in delayed disease and inexorably leads to death. Therefore, CD8$^+$ T lymphocytes and Abs are not redundant but complementary and essential to survive infections with a highly pathogenic viruses in the natural host. ***The Journal of Immunology,*** **2005, 175: 6829–6836.**

It is well known that CD8$^+$ T cells have an important role in the control of several primary viral infections (1). Following activation, they differentiate into CTL that kill infected cells and secrete antiviral cytokines to decrease viral replication. The CTL activity during most viral infections in the mouse peaks 6–8 days postinfection (p.i.).[3] Thereafter, the frequency of Ag-specific CD8$^+$ T cells rapidly declines concomitant with the decrease in virus loads (2–4). B cells produce Abs that may directly neutralize viral particles or that can act indirectly by triggering other effector mechanisms such as phagocytosis, activation of the complement cascade, or inducing Ab-dependent cellular cytotoxicity (5–7). The peak of the Ab response normally occurs several days to weeks after the CD8$^+$ response, and Ab titers remain high in serum for months to years after infection (2). Abs have a well-established function in preventing secondary infections and in the transmission of passive immunity from mother to offspring (2, 4, 5, 8). However, their role in controlling primary viral infections is not clear (7). Indeed, it has been proposed that Abs may not be needed to control primary viral infections (4).

Viruses can be broadly subdivided into noncytopathic and cytopathic. Noncytopathic viruses are in general poorly pathogenic by themselves and have a tendency to cause persistent infections. In contrast, cytopathic viruses can cause acute disease and death and are in general nonpersistent (8, 9). Accumulating evidence with the noncytopathic, mouse-specific lymphocytic choriomeningitis virus (LCMV) suggests that CTL and Abs are both required for the long-term control of this persistent virus. Although CD8$^+$ T cell responses are essential to control LCMV, Abs are also critical because in the absence of CD4$^+$ T cells, B cells, or CD40 costimulation, all of which are essential for Ab production, LCMV re-emerges within 200–300 days and exhausts the CTL response due to overstimulation (10–13). More recently, it was shown that the exhaustion of the anti-LCMV CTL response is a direct consequence of the absence of Abs (14). Because LCMV is noncytopathic, the infected animals lacking Ab responses become lifelong carriers without serious consequences.

The mechanisms that mediate clearance of cytopathic viruses, in particular those that replicate optimally in the host and can cause acute disease and death, are less well understood. Experiments with influenza virus and vaccinia virus (VACV) in the mouse appear to indicate that CD8$^+$ T cells and Abs can independently clear primary infections with cytopathic viruses (Refs. 11, 15–19 and our unpublished results). However, these viruses do not replicate or spread efficiently in the mouse, probably because they are not mouse-specific.

Ectromelia virus (ECTV), the agent of mousepox, is an *Orthopoxvirus* (OPV) that has host specificity for the mouse. It is genetically very similar to VACV, the human pathogens variola virus (the agent of smallpox) and monkeypox virus (20), which sporadically infects people in Africa and produced a recent outbreak in the Midwestern United States (21, 22). Although all mouse strains can be infected with ECTV, the outcome of the infection following footpad inoculation varies. Some strains such as BALB/c develop mousepox and endure very high mortality during the first 2 wk p.i. (23), while other strains such as C57BL/6 (B6) do not become sick. The resistance of B6 mice to disease is not due to an inherent decreased ability of the virus to replicate in this strain but to the combined action of the innate and adaptive immune systems as indicated by experiments where B6 mice depleted of NK cells or Mϕ (24) or severely immunodeficient animals in a B6 background such as those deficient in the recombinase activating gene 1 (data not shown) readily succumb to footpad infection and bear virus titers in organs as high as those of susceptible strains. Therefore, ECTV infection of B6 mice and syngeneic immunodeficient strains constitutes an excellent model

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[3] Abbreviations used in this paper: p.i., postinfection; LCM1V, lymphocytic choriomeningitis virus; VACV, vaccinia virus; ECTV, ectromelia virus; OPV, *Orthopoxvirus*; GzB, granzyme B.

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to study the immune mechanisms responsible for resistance to highly pathogenic viruses.

For the OPV VACV, it has been shown recently that T cells or Abs can independently clear the infection in the mouse (25). However, VACV replicates very poorly in this species. In the case of ECTV, several lines of evidence have indicated that T cells and, in particular CD8$^+$ T cells, are essential and sufficient to control primary infections. First, depletion of T cells resulted in loss of resistance (26). Second, adoptive transfer of immune T cells was more effective than hyperimmune sera at reducing virus titers in infected hosts during the early phase of the infection (27). Third and most important, B6 mice depleted of CD4$^+$ T cells do not generate Ab responses. However, when these mice were infected with a mildly pathogenic strain of ECTV, they raised strong CD8$^+$ responses and, 11 days p.i., had virus titers in organs that were similar to those of undepleted controls (28). However, whether these mice would develop mousepox or whether virus burden would re-emerge at later times postinfection was not analyzed. Interestingly, a subsequent study by Karupiah et al. (24) found that thymectomized B6 mice depleted of CD4$^+$ T cells or MHC class II-deficient B6 mice survived up to 27 days p.i. At this time, the animals were sacrificed, and it was found that they had not cleared the virus. Although the reason for this virus persistence was not investigated, the authors suggested as an explanation the loss of an undefined direct-effector function of CD4$^+$ T cells. Alternatively, as with LCMV, it is possible that clearance of highly pathogenic viruses in natural hosts cannot be accomplished in the absence of Th-dependent Ab responses.

CD40, a member of the TNF family, is present on the surface of B cells, professional APC, and, recently described, CD8$^+$ T cells (29). CD40 interacts with CD154 on CD4$^+$ T cells playing an essential role in the induction of Ab responses (30, 31). CD40-CD154 interactions are also known to play a role for the priming and maintenance of memory CD8$^+$ T cell responses to some Ags and viruses but not others (29, 32–36). In the present study, we show that CD40-deficient (CD40$^{0/0}$) mice (37) initially resist ECTV without signs of mousepox but become sick and succumb several weeks later. Analysis of the T and B cell responses of CD40 mice, studies of the pathogenesis of ECTV infection in B cell-deficient μMT mice (38), and adoptive transfer experiments of CD8$^+$ T and B cells demonstrate that the susceptibility of CD40$^{0/0}$ mice is due to the absence of Ab and not to an intrinsically defective CD8$^+$ T cell response. Thus, CD8$^+$ T cell and Abs are not redundant or self-sufficient but play complementary roles that enable survival to infections with highly pathogenic OPV.

Materials and Methods

Cells

The dendritic cell line DC2.4 was a gift from Dr. K. Rock (University of Massachusetts Medical Center, Worcester, MA). Hela S3, MC57G and BSC-1 cells were obtained from American Type Culture Collection. As standard tissue culture medium, we used RPMI 10 that consisted of RPMI 1640 tissue culture medium (Invitrogen Life Technologies) supplemented with 10% FCS (Sigma-Aldrich), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies), 10 mM HEPES buffer (Invitrogen Life Technologies), and 0.05 mM 2-ME (Sigma-Aldrich). When indicated, RPMI 2.5 (as above but with 2.5% FCS) was used instead. When required, 10 U/ml IL-2 was added to RPMI 10 (RPMI 10-IL2). All cells were grown at 37°C and 5% CO_2.

Viruses

We obtained the initial stocks of ECTV Moscow (39), a highly pathogenic strain, from American Type Culture Collection. New stocks were expanded by infecting BALB/c mice with 3000 PFU of ECTV in the footpad. Seven days p.i., the spleen and liver were removed and homogenized in 20 ml of RPMI 2.5 using a tissue homogenizer. The solid material was pelleted by centrifugation, and the supernatant was stored in aliquots at −80°C. Initial stocks of VACV virus Western Reserve were obtained from Dr. B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD) and amplified in Hela S3 cells as described previously (40). Virus titers in ECTV and VACV stocks were determined by plaque assays on BSC-1 cells. Briefly, 10-fold serial dilutions of the stocks in 0.5 ml of RPMI 2.5 were used to infect confluent BSC-1 cells in 6-well plates (2 wells/dilution) for 1 h. Two milliliters of fresh RPMI 2.5 were then added, and the cells were incubated at 37°C for 3 days (VACV) or 5 days (ECTV). After this time, the medium was aspirated and the cells fixed and stained for 10 min with 0.1% crystal violet and 20% ethanol in water. The fix/stain solution was subsequently aspirated, the cells air-dried, the plaques counted, and the PFU per milliliter in stocks were calculated accordingly.

To titer virus, weighed portions of different organs were homogenized in medium and titers determined as above. The titers were calculated as PFU/100 mg of tissue. To determine virus titers in spleens, we used small aliquots of single-cell suspensions prepared by disrupting the spleens between two frosted glass slides. In this case, we calculated the PFU per spleen. The remainder of the splenocytes was used for flow cytometry analysis and cell counts.

Mice

All mice were bred at the Fox Chase Cancer Center Laboratory Animal Facility in specific pathogen-free rooms (all the experimental protocols were approved by the Fox Chase Cancer Center Institutional Animal Use and Care Committee). B6 mice were from Fox Chase Cancer Center stocks. CD40$^{0/0}$, μMT, and CD8$^{0/0}$ breeders were originally purchased from The Jackson Laboratory. Experimental CD154$^{0/0}$ animals were purchased directly from The Jackson Laboratory. For experiments, sex-matched animals 8–12 wk old were transferred to a biosafety level 3 room. For blood collection, mice were placed in a restrainer and 50 μl of blood collected through a small incision in the tail. For ECTV infection, anesthetized mice were infected in the left footpad with 50 μl of PBS containing 3×10^3 PFU of ECTV. VACV was inoculated via the i.p. route with 500 μl of PBS containing 5×10^6 PFU of VACV. Following infections, mice were observed daily for signs of disease (lethargy, ruffled hair, weight loss, skin rash, and eye secretions) and imminent death (unresponsiveness to touch and lack of voluntary movements). Moribund mice were euthanized by halothane inhalation and counted as dead. For antisera collection, mice were infected twice with ECTV 1 mo apart and exsanguinated under deep anesthesia 1 mo after the second infection. Blood was allowed to clot at 37°C, refrigerated for 1 h, and the sera separated from the clot by centrifugation. Sera was stored in aliquots at −80°C.

ELISA

To determine the Ab response to ECTV, we used an ECTV-based ELISA where 2×10^6 PFU of ECTV in 100 μl of PBS were used to coat the wells of high protein binding ELISA plates (Costar) at 4°C overnight. The plates were blocked for 2 h at 37°C with 1% BSA in PBS, washed, and different dilutions of serum from immune or naive mice in 0.5% BSA in PBS-T (0.05% Tween 20 in PBS) were added to duplicate wells and incubated for 90 min at room temperature. The plates were washed three times with PBS-T, incubated for 30 min with isotype-specific goat anti-mouse Ab labeled with HRP, washed five times with PBS-T, developed using tetramethylbenzidine substrate (KPL), and the reaction was stopped after 15 min using 0.5 M HCl. Plates were read in a 96-well spectrophotometer (μQuant; Bio-Tek Instruments) at 450 nM absorbance.

^{51}Cr release assays

Mouse spleens were disrupted between two frosted glass slides, washed with RPMI 10, and filtered to eliminate cell aggregates. For ex vivo assays, splenocytes were used immediately. For this purpose, serial dilutions of spleen cells in 100 μl of RPMI 10 were added in triplicate to round-bottom 96-well plates. For assays that required in vitro restimulation, 5×10^6 spleen cells were placed in 24-well plates in 2 ml in RPMI 10-IL2 with 2×10^5 MC57G cells that had been infected for 4 h with 10 PFU/cell VACV and treated for an additional hour with 50 μg/ml mitomycin C. After 5 days, the restimulation cultures were harvested, washed, and serial dilutions were added in triplicate to round-bottom 96-well plates. Target cells were prepared by infecting 10^6 MC57G cells with 10 PFU/cell VACV virus in 100 μl of RPMI 2.5 for 1 h, followed by incubation with 200 μCi of ^{51}Cr in 100 μl of FCS for an additional hour. Cells were thoroughly washed, resuspended in RPMI 10, and 50 μl (5×10^3 targets) were added to the wells containing effector cells. The plates were incubated at 37°C for 5 h, and 50-μl supernatants were transferred to white 96-well plates Microscint-40 scintillation fluid (PerkinElmer). Controls included wells with target cells alone for spontaneous release and wells with target cells and

1% Triton-X for full release. Radioactivity was determined using a Packard Topcount instrument (PerkinElmer). Specific lysis was determined with the formula ((experimental release − spontaneous release)/(full release − spontaneous release)) × 100. These experiments were repeated three times using two or three animals per experimental group analyzed either individually or as a pool.

Flow cytometry

Spleens were obtained from mice at different times postinfection and made into single-cell suspensions. Following osmotic lysis of RBC with 0.84% NH_4Cl, splenocytes were washed and 10^6 cultured at 37°C in 96-well plates in the presence of 2×10^5 VACV-infected DC2.4 cells or uninfected DC2.4 cells as control. (In preliminary experiments (data not shown), we found that stimulation with VACV or ECTV-infected DC2.4 cells produces similar results when measuring anti-ECTV responses. For simplicity, we used restimulated with VAVC in all experiments.) 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 (anti-Fcγ II/IIIR; American Type Culture Collection) 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. The following Abs were used: for cell surface staining, anti-CD4 (RM4-5; BD Biosciences) and anti-CD8α (53-6.7; BD Biosciences) for intracellular staining; anti-IFN-γ (clone XMG1.2; BD Biosciences); anti-TNF-α (clone MP-XT22; BD Biosciences); anti-IL-2 (clone JES6-5H4, BD); anti-IL-4 (11B11; BD Biosciences); an isotype control (clone A95-1; BD Biosciences); and anti-human granzyme B (GzB; Caltag Laboratories) that is cross-reactive with mouse GzB (41). One thousand to 200,000 cells were analyzed by flow cytometry at the Fox Chase Cell Sorting Facility using a LSR II system (BD Biosciences).

These experiments were repeated a minimum of three times, and the experimental groups included two to four mice per group that in some experiments were analyzed individually and in others as pools.

Adoptive transfer of B cells and CD8$^+$ T cells

Single-cell suspensions of pooled lymph nodes and spleens from donor mice were labeled with anti-mouse CD19 (for B cells) or anti-CD8 (for CD8$^+$ T cells) microbeads (Miltenyi Biotec), according to manufacturer's instructions, and purified by two passages over an Automacs magnetic sorter (Miltenyi Biotec) the first time with a normal and the second time with a sensitive setting. Final purity was >97%. Cells were washed twice with PBS and 2×10^7 injected in the tail veins of recipient mice. These experiments were repeated three times and involved four mice per group.

Antisera treatment

Six days after infection, CD40$^{0/0}$ mice were inoculated i.p. with 500 μl of antisera. Treatment was repeated weekly for 4 mo by inoculation of 250 μl of antisera i.p.

Results

Delayed mousepox and death in the absence of CD40 costimulation

As part of our effort to understand the mechanisms of natural resistance to highly pathogenic viruses, we investigated whether resistance to ECTV was impaired in the absence of CD40 costimulation. CD40$^{0/0}$ animals inoculated in the footpad with ECTV did not show any signs of disease during at least 3 wk p.i. However, within 20–50 days p.i., all CD40$^{0/0}$ animals developed mousepox and succumbed with an average time to death of 40.75 ± 16.1 days p.i. (n = 43). Because infection of CD154$^{0/0}$ mice (42) resulted in a similar outcome, it was most likely that the loss of natural resistance of CD40$^{0/0}$ mice was due to a lack of CD40-CD154 interaction. The death of CD40$^{0/0}$ mice at later stages of the infection did not seem to be due to lack of CD8$^+$ T cell responses because CD8$^{0/0}$ mice lacking CD8$^+$ T cells (43) succumbed to mousepox during the early and not the late stage of the infection, as previously reported by others (44). As expected, control B6 mice survived without any symptoms of mousepox throughout several months of observation. Survival curves for CD40$^{0/0}$, CD154$^{0/0}$, CD8$^{0/0}$, and B6 mice inoculated in the footpad are shown in Fig. 1*A*. Also of note, CD40$^{0/0}$ mice also succumbed to delayed mousepox when infected naturally by mouse-to-mouse transmission (Fig. 1*B*).

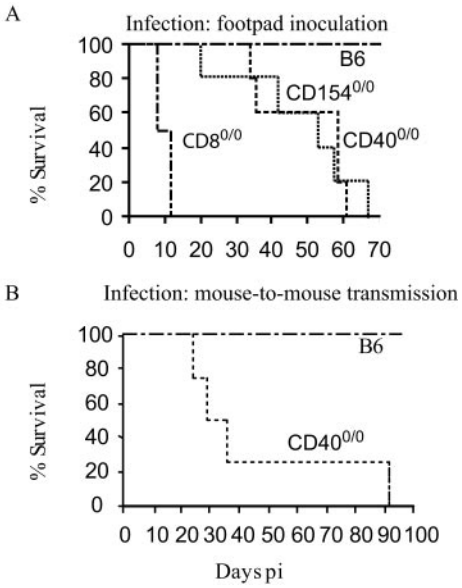

FIGURE 1. Delayed mousepox and death in the absence of CD40 costimulation. *A*, Survival of B6 (n = 5), CD40$^{0/0}$ (n = 5), CD154$^{0/0}$ (n = 4), and CD8$^{0/0}$ (n = 5) mice infected with 3000 PFU of ECTV Moscow. *B*, Survival of B6 (n = 4) and CD40$^{0/0}$ (n = 4) when infected BALB/c mice were introduced into their cages.

CD40$^{0/0}$ mice can control ECTV replication at early but not late stages of infection

To determine the reason for the delayed onset of mousepox in CD40$^{0/0}$ mice, we measured ECTV titers in the spleen at different times postinfection. The results showed that CD40$^{0/0}$ mice initially controlled ECTV virus because titers in their spleens were similar to those of B6 and 100–500 lower than in CD8$^{0/0}$ mice 7 days p.i. (Fig. 2*A*). Moreover, during the next few weeks and before the signs of mousepox emerged, ECTV titers in the spleens of CD40$^{0/0}$ mice were below the limit of detection of our plaque assays (data not shown). However, after the signs of mousepox appeared and CD40$^{0/0}$ mice were moribund (i.e., between 5 and 10 wk p.i.),

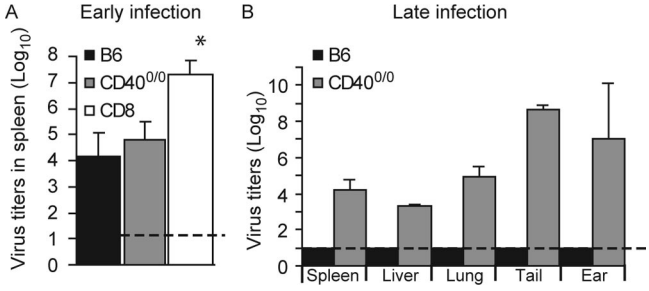

FIGURE 2. Early control and late persistence of virus in CD40$^{0/0}$ mice. *A*, The indicated mice were infected with ECTV in the footpad and virus loads in different organs determined at early stages of infection (7 days p.i.) (n = 10 for CD40$^{0/0}$ and B6 and n = 3 for CD8$^{0/0}$ mice). *B*, Virus titers were determined in the indicated organs of CD40$^{0/0}$ mice (n = 3) that had advanced symptoms of mousepox (typically 5–9 wk p.i.). The exact time of analysis varied for individual mice. Matching B6 controls were infected at the same time as the experimental CD40$^{0/0}$. Horizontal stapled lines mark detection limits of the assay. *, Significant statistical difference (p = 0.0008; ANOVA single factor).

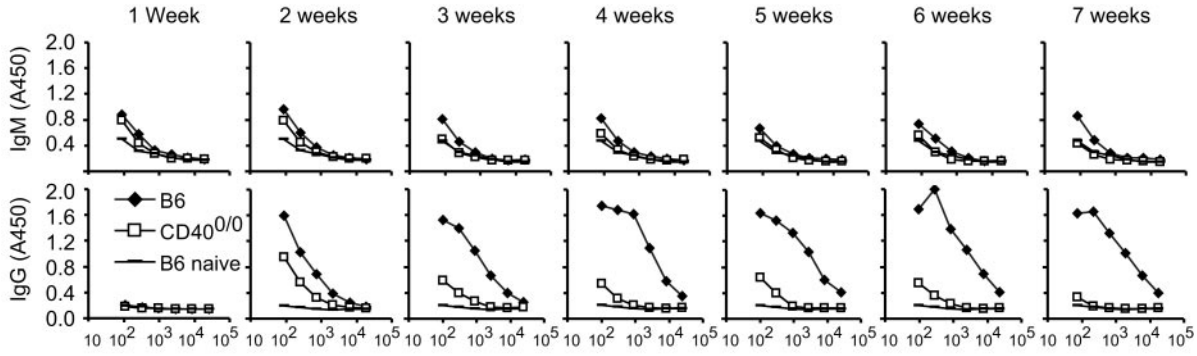

FIGURE 3. Stunted IgG Ab response in CD40$^{0/0}$ mice. B6 and CD40$^{0/0}$ mice were infected in the footpad with ECTV Moscow. Mice were bled at the indicated times, and anti-ECTV IgM (*top panels*) or IgG (*bottom panels*) were determined by ELISA. Data are from pooled sera from four animals per group.

ECTV was found in their spleen and also in the liver, lungs, and, in very high titers, in the skin of tail and ears (Fig. 2*B*). This indicated that the virus was not eliminated and that disease and death was due to a loss in the ability to control the virus at later stages of the infection. Such recurrence of virus was not detected in control B6 mice. The observed pathogenesis was not due to an inability of CD40$^{0/0}$ mice to control OPV in general because 5-mo p.i., VACV-infected CD40$^{0/0}$ mice had not developed any symptoms of disease and had no virus in ovaries, the major site of VACV replication (data not shown).

CD40$^{0/0}$ mice have stunted Ab responses and strong but sustained T cell responses

To establish the basis for the loss of virus control, we determined Ab and T cell responses at different times following ECTV

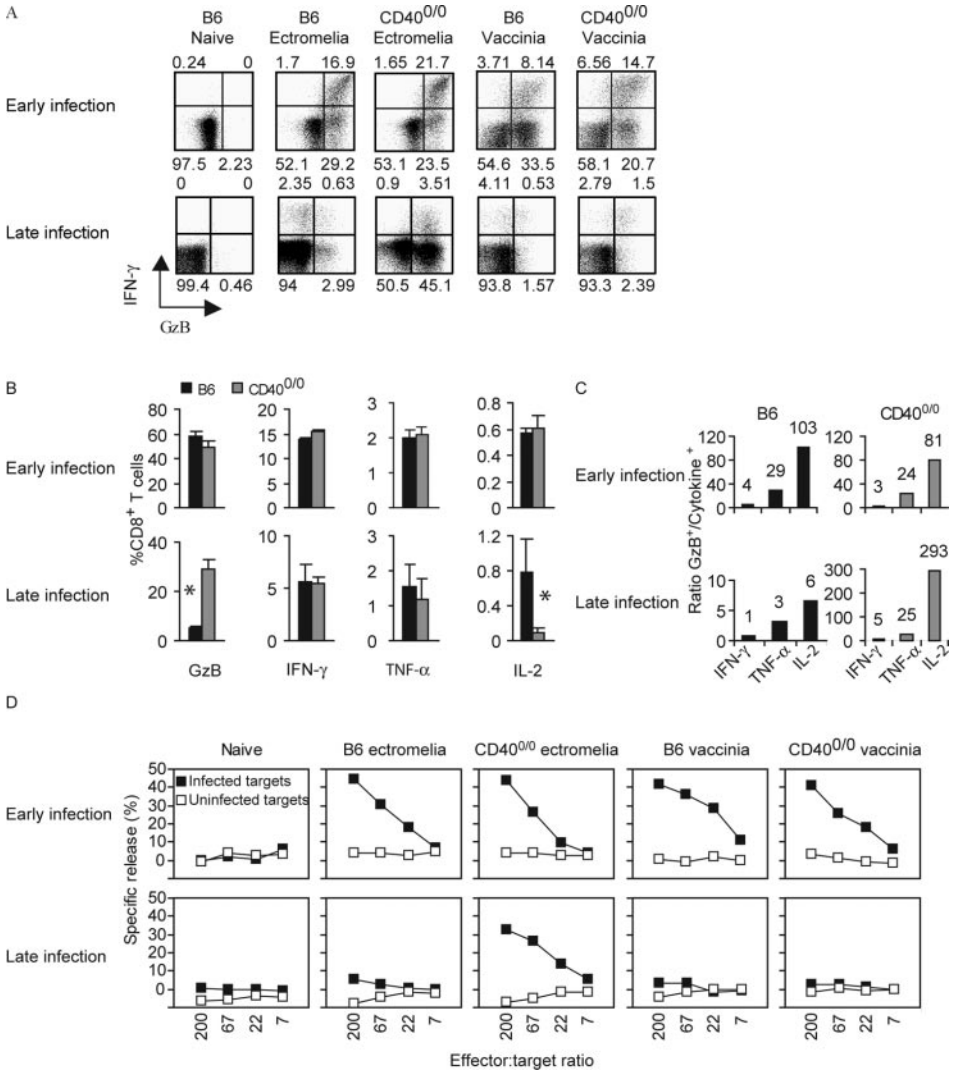

FIGURE 4. Sustained CD8$^+$ T cell responses in CD40$^{0/0}$ mice. *A*, Representative flow cytometry analysis of lymphocytes from pools of two or three spleens from B6 and CD40$^{0/0}$ mice infected with ECTV or VACV during early infection (7 days p.i.) or late infection (8 wk p.i. for ECTV and 4 mo p.i. for VACV). Graphs are gated on CD8$^+$ T cells. Numbers indicate the proportion of CD8$^+$ T cells in the nearest quadrant. Stains as indicated. *B*, Average ± SE of CD8$^+$ T cells that express the indicated molecules during early (7 days p.i., CD40$^{0/0}$, $n = 14$; B6, $n = 12$) or late infection (5–8 wk, CD40$^{0/0}$, $n = 9$; B6, $n = 10$). *, Significant statistical difference (in both cases $p = 0.008$, two tailed t test). *C*, Ratio of GzB$^+$/cytokine$^+$CD8$^+$ T cells derived from the data in *B*. Numbers indicate the actual ratio. Cytokines as indicated. *D*, ^{51}Cr release assay with fresh spleen cells from B6 and CD40$^{0/0}$ mice during early infection (7 days p.i.) or late infection (8 wk p.i. for ECTV and 4 mo p.i. for VACV). Data correspond to pooled spleens from two or three mice per group.

infection. Consistent with the role of CD40 in helper-dependent Ab responses (30, 31), $CD40^{0/0}$ mice produced a stunted Ab response while the response of B6 mice was strong and long-lasting (Fig. 3). In contrast, 7 days p.i., the $CD8^+$ (Fig. 4) and $CD4^+$ (data not shown) T cell responses of $CD40^{0/0}$ and B6 mice were similar as measured by expression of IFN-γ, IL-2, TNF-α, and GzB (Fig. 4, *A* and *B*, *upper panels*). In addition, the T cell responses in B6 and CD40 mice were also similar when infected with VACV (Fig. 4*A*, *upper panels*). Furthermore, CTL assays using freshly isolated spleen cells revealed that virus-specific CTL activity was similar in B6 and CD40 mice infected with either virus (Fig. 4*C*, *upper panels*). Also, no differences were found in the absolute number of spleen cells and $CD4^+$ or $CD8^+$ T cells (data not shown). This indicated that $CD40^{0/0}$ mice mount normal T cell responses to ECTV and also to VACV during the early phase of infection. However, when the T cell responses were determined in ECTV-infected $CD40^{0/0}$ mice after the appearance of mousepox, we found they had a sustained T cell response, as revealed by a large proportion of $CD8^+$ T cells that expressed GzB (Fig. 4, *A* and *B*, *lower panels*) and the ability of the fresh spleen cells to kill infected targets in CTL assays (Fig. 4*C*, *lower panels*). This is in stark contrast with the $CD8^+$ T cells of B6 mice, which, as expected, had entered into the memory phase as revealed by the attrition of Ag-specific cells (45) (compare *upper* and *lower panels* in Fig. 4, *A–C*) with significantly lower numbers of cells expressing GzB. The finding of sustained CTL responses in infected $CD40^{0/0}$ mice is surprising because for other infections viral persistence results in CTL exhaustion, which is the loss of killer activity by Ag-specific effector CTL due to deletion or anergy as a consequence of overstimulation (46). Because sustained $CD8^+$ responses were found in every moribund mouse analyzed, it is doubtful that this state of activation was the prelude of a later exhaustion. The sustained T cell response in $CD40^{0/0}$ mice did not seem due to an intrinsic defect of the $CD40^{0/0}$ T cells because we observed normal T cell attrition when $CD40^{0/0}$ mice were infected with VACV (Fig. 4, *A* and *C*, compare B6 VACV and $CD40^{0/0}$ VACV in *upper* and *lower panels*). It is interesting to note that the sustained $CD8^+$ T cell response in ECTV-infected $CD40^{0/0}$ mice is apparent when staining for the effector molecule GzB or in CTL killing assays using freshly isolated spleen cells. However, the proportion of $CD8^+$ T cells that expressed IFN-γ and TNF decreased as much as in B6 mice (compare Fig. 4*B*, *upper* and *lower panels*). Furthermore, the proportion of $CD8^+$ T cells that expressed IL-2 at late infection in $CD40^{0/0}$ mice was significantly lower than in B6 animals. Also, in $CD40^{0/0}$ mice, it can be noted that the ratio of GzB:cytokine remained the same in the case of IFN-γ and TNF-α and increased substantially for IL-2 between early and late infection. In contrast, all ratios for GzB:cytokine decreased 4- to 10-fold in B6 animals (Fig. 4*C*, *right panels*). This indicates that a decrease in the ability of Ag-specific cells to produce cytokines is not necessarily synonym of exhaustion and that also occurs in activated effectors.

$CD40^{0/0}$ $CD8^+$ T cells protect $CD8^{0/0}$ mice from mousepox and death

It has been shown previously that $CD8^{0/0}$ mice are susceptible to ECTV and succumb 1–2 wk p.i (24). Our data above seemed to indicate that $CD40^{0/0}$ $CD8^+$ T cells generate normal responses to ECTV. However, it has been reported that $CD8^+$ T cells express CD40 and that its ligation with CD154 on $CD4^+$ T cells is required for efficient $CD8^+$ T responses (29). Although other laboratories have later reported that CD40 expression is not required for effective responses to influenza virus, *Listeria*, and LCMV (47, 48), we thought it was important to experimentally exclude a contribution of CD40 on $CD8^+$ T cells to resistance to ECTV. For this purpose, $CD8^{0/0}$ mice were adoptively transferred with purified $CD8^+$ T cells obtained from $CD40^{0/0}$ mice (CD8-$CD40^{0/0}$→$CD8^{0/0}$). In two independent experiments, one with three and another with four mice per group, none of the CD8-$CD40^{0/}$→$CD8^{0/0}$ died or developed signs of mousepox during a period of observation of several months. In contrast, all control $CD8^{0/0}$ animals developed mousepox and died during the early acute phase of the infection. This indicated that $CD40^{0/0}$ $CD8^+$ T cells can mount protective responses and that the susceptibility of $CD40^{0/0}$ mice to mousepox was probably due to a defective response of another critical component of the immune system.

μMT mice succumb with delayed mousepox and sustained CTL responses

Based on the results above, we hypothesized that the loss of virus control by $CD40^{0/0}$ mice was due to the absence of Abs. However, because CD40 deficiency can affect other aspects of the immune response to some Ags, we thought it was important to determine whether another mouse strain unable to make Abs responded to ECTV similarly to $CD40^{0/0}$ mice. For this purpose, we used μMT mice that lack B cells and, as a consequence, do not produce Abs. Similar to $CD40^{0/0}$ mice, μMT mice challenged with ECTV controlled the infection initially but developed mousepox and died at later stages of the infection (Fig. 5*A*). Furthermore, μMT mice had virus titers during the early and late phases of the infection that were similar to those of $CD40^{0/0}$ mice (data not shown) and a sustained T cell response (Fig. 5*B*).

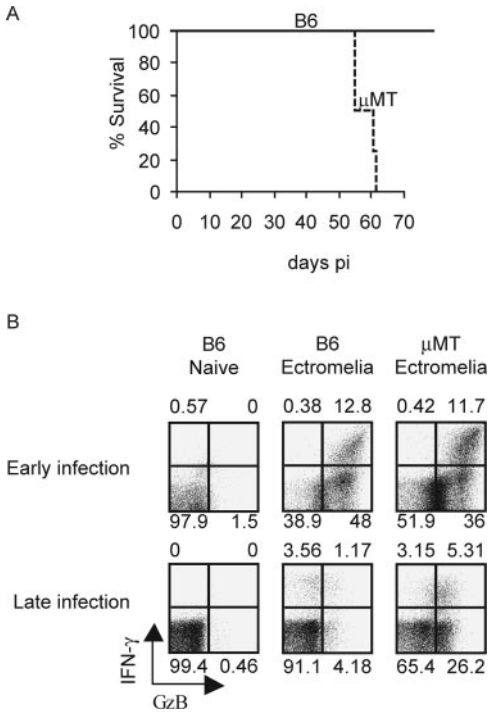

FIGURE 5. Late mousepox and sustained $CD8^+$ T cell responses in the absence of B cells. *A*, Survival of μMT ($n = 4$) and B6 ($n = 5$) mice following footpad ECTV inoculation. *B*, Representative flow cytometry analysis of lymphocytes from pools of two spleens from B6 and μMT during early infection (7 days p.i.) or late infection (56 days p.i.). Graphs are gated on $CD8^+$ T cells. Numbers indicate the proportion of $CD8^+$ T cells in the nearest quadrant. Stains as indicated.

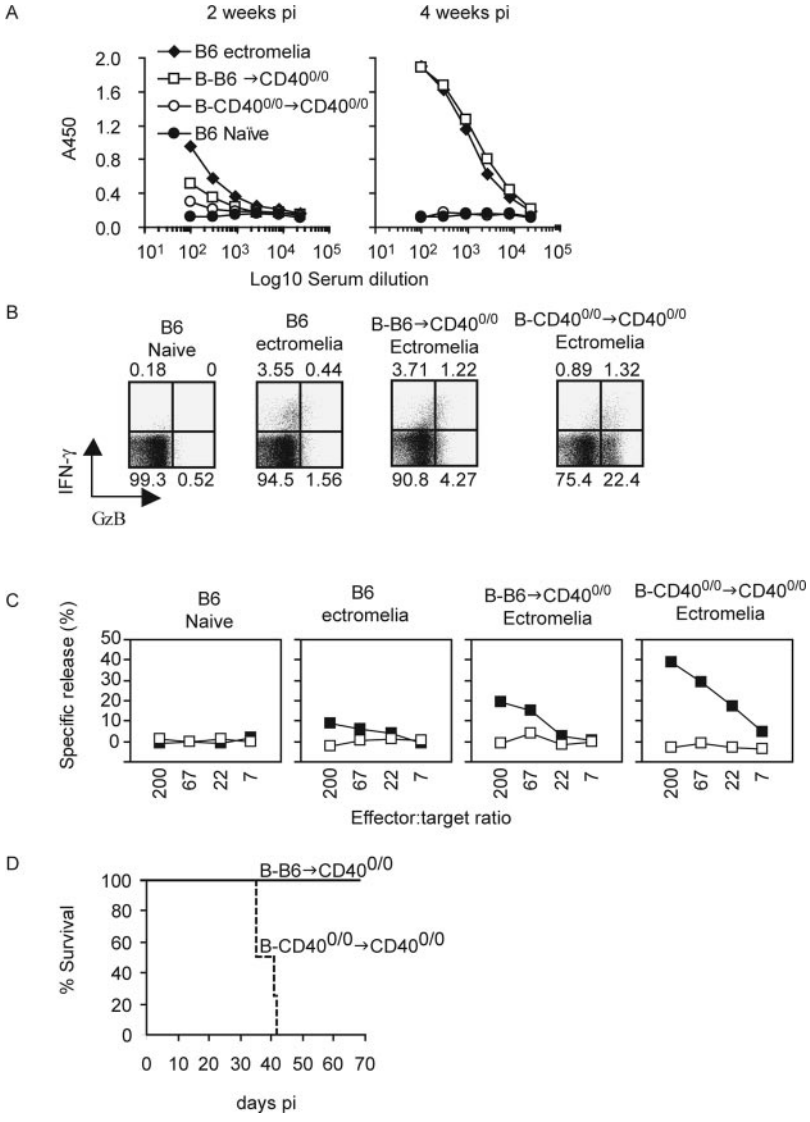

FIGURE 6. Adoptive transfer of CD40$^+$ B cells rescues CD40$^{0/0}$ mice from susceptibility to ECTV infection. *A*, B-B6→CD40$^{0/0}$ and B-CD40$^{0/0}$→CD40$^{0/0}$ mice were infected with ECTV, and anti-ECTV IgG in serum was determined by ELISA at the indicated times postinfection. Data correspond to pooled sera from four mice per group. *B*, Representative flow cytometry analysis from pools of two spleens from B6→CD40$^{0/0}$ and CD40$^{0/0}$→CD40$^{0/0}$ mice during late infection (5 wk p.i.). Graphs are gated on CD8$^+$ T cells. Numbers indicate the proportion of CD8$^+$ T cells in the nearest quadrant. Stains as indicated. *C*, ^{51}Cr release assay with pools of two freshly isolated spleen cells from B-B6→CD40$^{0/0}$ or B-CD40$^{0/0}$→CD40$^{0/0}$during late infection (5 wk p.i.). *D*, Survival of B-CD40$^{0/0}$→CD40$^{0/0}$ ($n = 4$) and B-B6→CD40$^{0/0}$ ($n = 4$) following footpad infection with ECTV.

Adoptive transfer of CD40$^+$ B cells or antiserum infusion rescues CD40$^{0/0}$ mice from susceptibility to ECTV infection

If the inability of CD40$^{0/0}$ mice to control ECTV was due to the absence of Abs, restoring their B cell responses should reinstate resistance. To test for this, we transferred B cells from B6 mice into CD40$^{0/0}$ mice (B-B6→CD40$^{0/0}$) and challenged them with ECTV. Results showed that B-B6→CD40$^{0/0}$ mice generated Ab responses that were similar to those of B6 mice (albeit with a somewhat slower kinetics) (Fig. 6*A*). Furthermore, B-B6→CD40$^{0/0}$ showed a normal attrition of the CD8$^+$ T cell response (Fig. 6*B*) and did not have active CTL in their spleens (Fig. 6*C*). More importantly, B-B6→CD40$^{0/0}$ were completely resistant to mousepox (Fig. 6*D*). In contrast, control CD40$^{0/0}$ mice that received CD40$^{0/0}$ B cells (B-CD40$^{0/0}$→CD40$^{0/0}$) did not produce Abs, had a sustained CD8$^+$ T cell response, effector CTL in their spleens, and succumbed to ECTV during late infection (Fig. 6, *A–D*, respectively). In additional experiments (two repeats), groups of three CD40$^{0/0}$ mice were treated weekly with antiectromelia antiserum starting on day 6 p.i. Similar to B cell-treated mice, these animals did not develop late mousepox and did not have sustained CD8$^+$ T cell responses during 4 mo of observation.

Discussion

In these studies, we have used the mouse pathogen ECTV as a model to determine the mechanisms of resistance to infection with a highly pathogenic and cytolytic OPV. Several conclusions can be drawn from our work.

Most important, we demonstrate that primary infection with a highly cytolytic and pathogenic OPV cannot be controlled by the T cell response alone. Although our data confirm that CD8$^+$ T cells are essential for resistance, it also shows that they can control the virus independently of Abs only at the early stage of the infection (1–2 wk p.i.) and that, at later stages, Abs become essential for virus elimination and survival. This was not obvious from previous reports, which seemed to indicate that T cells could control ECTV (24, 28). Whether CD8$^+$ T cell become dispensable after the Ab response peaks is not solved by our data and still needs to be tested. However, it is tempting to speculate that the immune system combats cytolytic viral infections using different effector mechanisms in successive phases of attack. In support of this notion, we have also found that NK cells are required during the first 5 days following ECTV infection but become dispensable afterward (our unpublished work). Alternatively, CD8$^+$ T cells could still be required at the later phases of the infection even after Abs

have already peaked. This could reflect a need for the concomitant function of mechanisms that eliminate intracellular viruses such as $CD8^+$ T cells and extracellular viruses such as Abs.

Another interesting finding is that even in the presence of strong $CD8^+$ T cell responses, a highly cytolytic, host-specific virus such as ECTV can persist for weeks without noticeable symptoms. This persistence of ECTV in the absence of Abs resembles the infection with the noncytolytic LCMV. In the absence of $CD4^+$ T cells, B cells, or CD40, LCMV is also controlled to undetectable levels at early time postinfection but re-emerges within 100–200 days. However, this re-emergence of LCMV is always accompanied by the exhaustion of the $CD8^+$ T cell response (11, 14, 49, 50). However, as we show, ECTV is not cleared and reaches high virus titers in many organs despite the presence of a sustained CTL activity that does not become exhausted. Why ECTV does not induce T cell exhaustion despite massive virus loads and whether the persistently activated $CD8^+$ T cells participate in the pathology of the disease will require further experimentation.

It is also necessary to comment on the finding that, at the later stages of infection, $CD40^{0/0}$ mice had a large proportion of GzB-expressing cells and killed infected targets, but the relative proportion of cytokine-producing T cells declined. Because loss of cytokine expression (in particular IL-2) in Ag-specific cells has been correlated with the loss of effector activity exhaustion (51), we could have mistakenly concluded that the cells in $CD40^{0/0}$ mice were exhausted if we had not looked at expression of GzB and CTL killing. Our results indicate that decreased T cell expression of cytokines, particularly IL-2, might be due to sustained activation and does not necessarily reflect exhaustion. This is consistent with a recent report indicating that T cells express IL-2 only immediately after activation (52).

Our experiments also show that CD40 costimulation is essential for survival to a highly pathogenic virus infection. However, our data show that the role of CD40 during ECTV infection is mainly in the production of Abs and not the induction of primary $CD8^+$ T cell responses. Three lines of evidence support our conclusion that $CD40^{0/0}$ $CD8^+$ T cell respond normally to OPV infections. First, the primary $CD8^+$ responses to ECTV in wild-type and $CD40^{0/0}$ mice are indistinguishable during the early phase of infection. Second, $CD40^{0/0}$ $CD8^+$ responses to VACV are identical during the early and memory phases. Third, $CD40^{0/0}$ cells can protect $CD8^{0/0}$ mice from mousepox, demonstrating that their responses to ECTV are not intrinsically defective. That $CD40^{0/0}$ mice can mount normal $CD8^+$ T cell responses is in agreement with previous reports showing that CD40 is not required to generate efficient $CD8^+$ T cells responses to LCMV (33, 49, 53).

It is also important to note that the requirements to clear ECTV differ markedly with those to clear VACV because mice with different immunodeficient backgrounds, including $CD8^{0/0}$, $CD4^{0/0}$, MHC-$II^{0/0}$, μMT, and $CD40^{0/0}$, resisted i.p. infection with 5×10^6 PFU of VACV, eliminated the virus, and did not show symptoms of disease (Refs. 14 and 25 and data not shown). In contrast, all these mice succumb to footpad infection with ECTV. Furthermore, even wild-type B6 mice that are resistant to footpad infection with ECTV readily succumb to this virus following i.p. infection with 200 PFU. The different outcomes following infection with VACV and ECTV are interesting because these two viruses are cytolytic, genetically similar, and immunologically cross-reactive. However, ECTV and VACV vary tremendously in their pathogenicity for the mouse. Although ECTV is highly infectious at very low doses, can be transmitted from mouse to mouse, and causes mousepox, VACV produces disease only at very high doses following the i.p. route (e.g., 5×10^8 i.p.) and can be used without pathology to vaccinate against ECTV. As we show here for $CD40^{0/0}$ mice, this difference in specificity and pathogenicity translates into a much more stringent requirement for controlling ECTV. Our data show that VACV can be eliminated with an impaired immune system while resistance to disease and death with the mouse-specific ECTV requires the immune system to be fully functional. Therefore, results with nonhost-specific viruses that replicate poorly in the host should be used with caution to extrapolate conclusions about the immune mechanisms involved in the clearance of viral infections in natural hosts. For example, one could mistakenly conclude from the VACV data alone that human infections with pathogenic OPV can be controlled without $CD8^+$ T cells or Abs. However, our data with ECTV demonstrate that both lymphocyte populations are essential for effective resistance to a pathogenic OPV. Nonetheless, because VACV can induce strong immune responses, this and other viruses that replicate poorly in the mouse can still yield important information regarding how immune responses are induced. For example, infection with VACV can be used successfully to study the mechanisms of vaccine-mediated protection to pathogenic OPV.

In summary, our data provide substantial insight into the mechanisms of protection to ECTV and demonstrate the complementary function of $CD8^+$ and B cell responses for its control. This study addresses important issues regarding the immune mechanisms involved in natural resistance to highly pathogenic viruses and highlights the importance of pairing pathogenic viruses with their natural hosts for studies of protective immunity. Moreover, our data may have clinical importance. Because massive vaccination against smallpox ceased, the majority of the world human population is no longer immune to OPV. This results in a risk for natural infections with monkeypox virus, a known human pathogen, or with other animal OPV that could in theory cross the species barrier. Furthermore, there is a risk of variola virus being used as an agent of bioterrorism. Thus, the experiments described here may have importance to predict the consequences of pathogenic OPV infections and perhaps other cytopathic viral infections in immunodeficient people.

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Disclosures

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