Direct CD28 Costimulation Is Required for CD8+ T Cell-Mediated Resistance to an Acute Viral Disease in a Natural Host

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Direct CD28 Costimulation Is Required for CD8\(^+\) T Cell-Mediated Resistance to an Acute Viral Disease in a Natural Host\(^1\)

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Previous studies have suggested that, differing from model Ags, viruses that replicate extensively in the host still induce normal CD8\(^+\) T cell responses in the absence of CD28 costimulation. Because these studies were performed with viruses that do not normally cause acute disease, an important remaining question is whether CD28 costimulation is required for CD8\(^+\) T cell-mediated resistance to widely replicating but pathogenic viruses. To address this question, we studied the role of CD28 costimulation in CD8\(^+\) T cell-mediated resistance to mousepox, a disease of the mouse caused by the natural mouse pathogen, the ectromelia virus (ECTV). C57BL/6 (B6) mice are naturally resistant to mousepox, partly due to a fast and strong CD8\(^+\) T cell response. We found that B6 mice deficient in CD28 (CD28 knockout (KO)) are highly susceptible to lethal mousepox during the early stages of ECTV infection but can be protected by immunization with the antigenically related vaccinia virus (VACV) or by adoptive transfer of CD28 KO anti-VACV memory CD8\(^+\) cells. Of interest, a thorough comparison of the CD8\(^+\) T cell responses to ECTV and VACV suggests that the main reason for the susceptibility of CD28 KO mice to mousepox is a reduced response at the early stages of infection. Thus, while in the absence of CD28 costimulation the end point strength of the T cell responses to nonpathogenic viruses may appear normal, CD28 costimulation increases the speed of the T cell response and is essential for resistance to a life-threatening acute viral disease. The Journal of Immunology, 2006, 177: 8027–8036.

In addition to Ag recognition through the TCR, the priming of T cells may also require additional “costimulation” signals. The best-studied T cell costimulatory molecule is CD28, which binds to B7-1 and B7-2 (CD80 and CD86) on the surface of professional APC (1–12). Although CD28-B7 signaling has been found to be required for the induction of CD4\(^+\) and CD8\(^+\) T cell responses and for the prevention of unresponsiveness to many model Ags both in vitro and in vivo (13–17), the exact role of CD28 costimulation in T cell responses to live viruses is not clear. Initial work with mice deficient in CD28 (CD28 knockout (KO))\(^3\) showed that the requirement for costimulation varied with the pathogen and correlated with its ability to replicate in the host. For example, the CD8\(^+\) T cell responses to the influenza virus, which replicates poorly in the mouse and is restricted to the respiratory lining, were absent or very reduced (18–20). In contrast, i.v. infection of CD28 KO mice with lymphocytic choriomeningitis virus (LCMV), which replicates very efficiently in the mouse and causes a generalized infection but no disease, resulted in CD8\(^+\) T cell responses that were indistinguishable from those of wild-type mice (21, 22). Moreover, during the acute phase of LCMV infection, CD28 KO mice were protected from intracerebral challenge with recombinant vaccinia virus (VACV) expressing LCMV epitopes (21). Conversely, during acute infection of CD28 KO mice with vesicular stomatitis virus (VSV), which replicates poorly in the mouse, CD8\(^+\) T cells proliferated in vitro but did not kill in ex vivo cytotoxicity assays, did not respond to further Ag restimulation in the absence of exogenous IL-2, and did not protect mice from intracerebral challenge with recombinant VACV expressing VSV epitopes (21–24). In addition, it has been reported that CD28 KO mice mount normal CD8\(^+\) T cell responses to wild-type VACV (strain WR) but not to attenuated (thymidine kinase-deficient) VACV inoculated i.v. as measured in ex vivo cytotoxicity assay 6 days postinfection (PI) (21). Based on these and other data with synthetic peptides as Ags, it was proposed that the costimulatory requirement of T cells during viral infections depended on the ability of the virus to replicate in the host. In this model, viruses that replicate extensively induce strong and prolonged TCR signaling, bypassing the need for CD28 costimulation. In addition, it was proposed that the inflammation produced by widely replicating viruses could be sufficient to induce alternative costimulation pathways. The model also suggested that for viruses that replicate poorly, the strength of the TCR signal alone would not achieve the signaling threshold or persist sufficiently to induce CD8\(^+\) independent activation (21, 22, 25). As a corollary, it was proposed that the induction of antiviral T cell responses may require CD28 costimulation only for those viruses that replicate abortively in the host (25). Yet, it is difficult to imagine that CD28 costimulation evolved under the pressure of microorganisms of limited capacity to replicate or cause disease. It should also be noted that contrary to some of the studies described above (21, 22, 25), our own experiments have shown impaired CD8\(^+\) T cell responses to wild-type VACV (WR) in the absence of B7-CD28 costimulation (26). However, differing from those studies, our route of inoculations was i.p. and not i.v. Also, work with LCMV in two other laboratories found a ~2-fold decrease in the CD8\(^+\) T

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\(^1\) Abbreviations used in this paper: KO, knockout; DC, dendritic cell; D-LN, draining lymph node; ECTV, ectromelia virus; GrB, granzyme B; LCMV, lymphocytic choriomeningitis virus; PI, postinfection; VACV, vaccinia virus; VSV, vesicular stomatitis virus.

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cell response of CD28 KO mice to LCMV (23, 27). However, because CD28 KO mice eliminated LCMV with normal kinetics, this 2-fold difference was considered of minimal significance and the final conclusion of both studies was that the CD8+ T cell response to LCMV does not require CD28 costimulation.

A limitation of previous reports, including ours (21–24, 26–28), was that they measured CD8 T cell responses following infections with viruses that do not infect mice in nature (VSV and VACV) or that infect mice naturally but do not normally cause apparent disease (LCMV). Therefore, an important remaining question was whether or not CD28 costimulation is required for CD8+ T cell-mediated resistance to diseases caused by infections with host-specific viruses that replicate widely and cause major inflammation but also can cause a life-threatening disease.

The ectromelia virus (ECTV), the agent of mousepox, is an orthopoxvirus that has host specificity for the mouse. It is genetically and antigenically very similar to VACV and to the human pathogen variola virus (the agent of smallpox) (29). In fact, recent evidence shows that VACV and ECTV share at least some of their immunodominant CD8+ T cell determinants (30). Although all mouse strains can be infected with ECTV, the outcome of primary infection through the natural route of entry (i.e., the footpad) varies. Susceptible strains such as BALB/c develop mousepox, which is characterized by massive replication of the virus in many organs, including the liver and spleen. Most susceptible mice die due to acute liver failure within the first 2 wk PI, and those that survive develop the typical skin rash of poxvirus infections. Of interest, mousepox in sensitive strains can be prevented by immunization with live VACV.

Resistant strains such as C57BL/6 (B6) do not show any symptoms of mousepox due to the strong innate and T cell responses during early infection and the Ab response at the later stages (31–34). In this report we show that CD28 KO mice (in a B6-resistant background) are sensitive to mousepox. Importantly, we demonstrate that the loss of resistance is mostly due to a delayed kinetics of the primary antiviral CD8+ T cell response. In addition, we show that CD28 costimulation is not required for the establishment of CD8+ T cell memory and the acquired resistance to mousepox following VACV immunization.

Materials and Methods

Cells

The B6-derived dendritic cell (DC) line DC2.4 (35) was a gift from Dr. K. Rock (University of Massachusetts Medical Center, Worcester, MA). As out standard tissue culture medium we used RPMI 10, which consisted of RPMI 1640 tissue culture medium (Invitrogen Life Technologies) supplemented with 10% FCS (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen Life Technologies), 10 mM HEPES buffer (Invitrogen Life Technologies), and 0.05 mM 2-ME (Sigma-Aldrich). Where indicated, RPMI 2.5 (as described above but with 2.5% FCS) was used instead. When required, 10 U/ml IL 2 (IL2) was added to RPMI 10 (RPMI 10-IL2). All cells were grown at 37°C and 5% CO2.

Viruses, mice, and infections

The production of ECTV and VACV stocks and the determination of titers in stocks and organs were done as described previously (33, 36). All mice were bred at the Fox Chase Cancer Center Laboratory Animal Facility in specific pathogen free rooms or purchased from Jackson Laboratories. For infections, sex-matched animals 8–12 wk old were transferred to a mouse infection room. For all ECTV infections, anesthetized mice were infected in the left footpad with 50 µl of PBS containing 3 × 106 PFU of ECTV. For all VACV infections, mice were inoculated i.p. with 500 µl of PBS containing 5 × 106 PFU of VACV. Following infection, 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. All of the experimental protocols involving animals were approved by the Fox Chase Cancer Center Institutional Animal Use and Care Committee. Disease was scored as follows: grade 1, eye secretion, mild rash of the tail (five lesions or less); grade 2, eye secretion, moderate rash of the tail (6–20 lesions); grade 3, extensive rash of the tail with coalescing lesion and moderate rash of skin and ears; grade 4, as grade 3 but more serious rash of tail, skin, and ears and sometimes with loss of tissue. In addition, all grades of disease are characterized by lethargy, ruffled hair, a hunched position, and apparent weight loss.

In vivo depletion of CD4+ T cells

To deplete CD4+ T cells, mice were injected i.p. with 200 µg of the anti-CD4 mAb GK1.5 1 day before and 3 days PI. The efficiency of depletion was verified by flow cytometry using RM4-5, a mAb that recognizes a different epitope than GK1.5 (<1% CD4+ T cells were detected).

Determination of T cell responses by flow cytometry

Spleens and draining lymph nodes (D-LN) were obtained from mice at different times PI and made into single cell suspensions. RBCs were lysed with 0.84% NH4Cl, the lymphocytes were washed, and the live cells were counted by standard trypan blue exclusion. To determine overall anti-VACV or ECTV CD8+ T cell responses, 106 lymphocytes were cultured at 37°C in 96-well plates in the presence of 2 × 105 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 allow for the accumulation of IFN-γ inside the cells. Following an additional 1.5 h of incubation, Ab 2.4G2 (anti-FcγRII/III receptor; American Type Culture Collection) was added to block nonspecific binding of labeled Ab to Fc receptors. The cells were then stained for the cell surface CD8a mAb 53-6.7 (BD Biosciences), fixed and permeabilized using the Cytofix/Cytperm kit (BD Biosciences, according to the manufacturer’s instructions), and stained for intracellular IFN-γ (XMG1.2; BD Biosciences) or an isotype control Ab (A95-1; BD Biosciences) and the anti-human granzyme B (GzB; Caltag), which is cross-reactive with mouse GzB (37). To stain for TSFKFESV-specific T cells, H-2Kb/Ig recombinant fusion protein complexes (mouse DimerX K8 dimers; BD Biosciences) were incubated with synthetic TSFKFESV (GenScript) and used as recommended by the manufacturer. TSFKFESV is the dominant CD8+ T cell determinant of VACV.
and ECTV in the B6 background (30). 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 2–4 mice/group that in some experiments were analyzed individually and in others as pools.

In vivo proliferation assay

The D-LNs and spleens of naive or VACV-immune B6 or CD28 KO mice were aseptically collected and RBC lysed with 0.84% NH4Cl. For the adoptive transfer of naive cells, we used whole spleen suspensions. For the adoptive transfer of immune cells, CD8⁺ T cells were purified using the autoMACS system (Miltenyi Biotec) according to the manufacturer’s instructions. Purity (>98%) was assessed by flow cytometry. In both cases, the cells were labeled with CFSE (BD Biosciences) according to published procedures (14), resuspended in PBS, and inoculated i.v. into recipient Thy.1 mice (5 × 10⁶ cells per mouse for naive unpurified cells and 5 × 10⁷ for purified immune CD8⁺ T cells). The next day, recipient mice were infected with ECTV in the footpad. Five (memory transfer) or 7 (naive transfer) days PI the mice were sacrificed; D-LNs and spleens were made into single-cell suspensions, restimulated, stained, and analyzed by flow cytometry as described above.

In vivo cytotoxicity assay

This assay was adapted from Oehen and Brduscha-Riem (38) and Coles et al. (39). Briefly, single cell suspensions of lymphocytes from naive B6 mice were split into two populations. One population was labeled with a high concentration of CFSE (4 μM) (CFSEhigh) and pulsed with 10 μg/ml TSYKFESV (30). The second population of lymphocytes was labeled with a low concentration of CFSE (0.8 μM) (CFSElow) and was not pulsed with peptide. The two populations were mixed in a 1:1 ratio and 2 × 10⁷ were injected i.v. into recipient mice. At the indicated times, the recipient mice were sacrificed and the presence of CFSElow and CFSEhigh cells was determined by flow cytometry in cell suspensions of lymph nodes and spleens. To calculate specific lysis, the following formula was used: percentage of specific lysis = [1 - (ratio unprimed/ratio primed)] × 100, where ratio = (percentage of CFSElow/percentage of CFSEhigh) (39).

In vivo protection assays

Wild-type B6 or CD28 KO mice were immunized with VACV i.p. One month after immunization, mice were sacrificed, spleens and lymph nodes pooled, and CD8⁺ T cells were purified with autoMACS. Pure CD8⁺ T cells (5 × 10⁶) were injected i.v. into naive CD28 KO mice that were then infected with ECTV. Seven days PI, spleen cell counts and virus titers were determined in some mice. Other mice were not sacrificed to determine disease.

FIGURE 2. Increased virus loads and organ pathology in CD28 KO mice following ECTV infection. A, Mice were infected with ECTV and virus loads were determined in the indicated organs on day 7 PI using plaque assays. Open bars, B6 mice; gray bars, CD28 KO mice. Data for LN and spleen (original magnification, ×10) and liver (original magnification, ×20) 7 days PI. Arrows indicate areas of necrosis in livers of B6 and CD28 KO mice. Note the heavy infiltration of lymphocytes in the B6 but not in the CD28 KO sample. Data are representative of two experiments (two or three mice per experiment). Three sections were taken from each organ.

FIGURE 3. Detection of virus-specific CD8⁺ T cells by intracellular staining and flow cytometry. B6 mice were infected or not infected with ECTV, and 7 days PI the CD8⁺ T cell responses in spleen were determined as described in Material and Methods. A, Upper panels, Intracellular staining of cells using infected DC2.4 cells as stimulators; lower panels, uninfected DC2.4 cells as control. Dot plots are gated on CD8⁺ cells. B, Cells were stimulated with infected DC2.4 cells. The left and center panels are gated on CD8⁺ cells and show K⁺-TSYKFESV reactivity (a dominant VACV/ECTV determinant) and GzB expression. The right panel is gated on the K⁺-TSYKFESV reactive cells of the infected mice and shows that the majority of these cells express GzB but not IFN-γ, mirroring the global response. Data are from pools of cells from three mice and are representative of a large number of experiments.
Results

CD28 costimulation is required for resistance to primary mousepox

To determine whether and when CD28 costimulation is required for resistance to mousepox, CD28 KO mice in a mousepox-resistant B6 background were infected in the footpad, which is the natural route of infection for this virus. Strikingly, 40% died 7–14 days PI (Fig. 1A) and all remaining CD28 KO mice developed mousepox (Fig. 1B and C). As expected, all control wild-type B6 mice survived the infection without any symptoms of disease. Importantly, the time of disease or death in CD28 KO mice was similar to that of CD8 KO mice (Fig. 1A and also Ref. 40) and much earlier than in B cell KO mice (Fig. 1A and also Refs. 33 and 34). Together, these data show that disease and death in CD28 KO mice were not due to defective humoral immunity and suggests that their defect was in the CD8 T cell response. Also of interest, all CD28 KO mice that survived past 14 days PI gradually recovered from the disease and survived indefinitely. These results demonstrate that CD28 costimulation is essential for resistance to mousepox and provides substantial survival advantage against an acute viral disease but is not consistently necessary for survival and clearing of the infection. Similar results were obtained when mice were infected with a dose of virus 2- or 5-fold lower (1500 and 600 PFU; not shown), indicating that CD28 KO mice are susceptible to mousepox over a wide dose range. The data also suggest that those animals that survive the acute period of the infection eventually mount an effective immune response capable of controlling ECTV.

To determine whether disease and death of CD28 KO mice were associated with increased virus loads, we determined virus titers in different organs 7 days PI. As shown in Fig. 2A, the virus titers in the D-LN of B6 and CD28 KO mice were high and similar. How-ever, the virus loads of CD28 KO mice were 10^4-fold higher in spleen and 10^3 times higher in nondraining LN and liver as compared to control B6 mice.
for IFN-γ under-represents the extent of the CD8+ T cell response to this virus. It should be pointed out that the detection of GzB in activated CD8+ T cell does not require a short restimulation with virus-infected APC as the detection of IFN-γ does (Fig. 3A). However, GzB+IFN-γ+CD8+ T cells readily kill infected targets in vitro (33). Thus, although a direct demonstration that all GzB+CD8+ cells are virus specific is not possible in this context, the cytotoxicity assays strongly suggest that they are virus specific and not the result of bystander activation. Determinant-specific antiviral CD8+ T cells can be determined using DimerX Kb (BD Biosciences) complexed with the ECTV/VACV determinant TSYKFESV (Kb-TSYKFESV specific; Fig. 3B). Notably, most Kb-TSYKFESV-specific cells are also GzB+IFN-γ+, further supporting the notion that GzB is an excellent marker of anti-viral CD8+ T cells.

To test the hypothesis that CD28 costimulation is required for CD8+ T cell-mediated resistance to mousepox, CD28 KO and control B6 wild-type mice were infected with ECTV in the foot-pad, and at different times PI the CD8+ T cell responses were determined in the D-LN and spleen. Fig. 4 show that 5 days PI the CD8+ T cell responses in the D-LN of B6 mice were already very strong, with as many as ~80% of CD8+ T cells expressing GzB and ~35% expressing IFN-γ. In contrast, the D-LN of CD28 KO mice contained only 6% GzB+ cells in the CD8+ population. At this early stage, both B6 and CD28 KO mice showed only an incipient CD8+ T cell response in the spleen. On day 7 PI the CD8+ T cells responses remained similarly high in the D-LN of wild-type B6 mice and had increased in the spleen, but by day 9 they had begun to decline. In contrast, 7 days PI the proportion of virus-specific CD8+ T cells (as measured by GzB expression) in the D-LN and spleen of CD28 KO mice increased to 50 and 25%, respectively and increased further by day 9 PI. Thus, when looking at relative numbers, CD28 KO mice appear to generate a strong but delayed CD8+ T cell response. The picture is less clear, however, when looking at total number of lymphocytes. In accord with the massive necrosis of lymphoid organs evidenced in Fig. 2, the cellularity in the spleens of infected CD28 KO mice was reduced ~2-fold (from 1.45 ± 0.1 × 10^8 in uninfected mice to 0.54 ± 0.22 × 10^8 7 days PI), whereas the total number of live splenocytes almost doubled in B6 mice (2.88 ± 0.16 × 10^8) (Fig. 4B, left panel). As a consequence, 7 days PI the absolute numbers of CD8+ T cells expressing GzB (Fig. 4B, center panel) and epitope-specific CD8+ T cells (Fig. 4B, right panel) were reduced ~30-fold in infected CD28 KO mice as compared with infected B6 mice. Fig. 4C shows representative flow cytometry plots from the data in Fig. 4A at 5 and 7 days PI. Also, note that despite a relatively strong response, very few cells were recovered from the D-LN of CD28 KO mice 7 days PI, and this result is reflected in the paucity of cells in the corresponding plot of Fig. 4C.
Consistent with the results described above, 5 and 7 days PI the CD8⁺ T cell effectors in CD28 KO mice killed target cells pulsed with the immunodominant ECTV/VACV CD8⁺ T cell epitope TSYKFESV (30), but much less effectively than those in wild-type mice as measured by in vivo cytotoxicity assays 5 (D-LN) or 7 (spleen) days PI (Fig. 4).

Optimal primary CD8⁺ T cell responses to ECTV require direct costimulation

The results thus far demonstrate altered CD8⁺ T cell responses to ECTV in CD28 KO mice. However, the data do not distinguish whether the decrease in the early CD8⁺ T cell response is a lack of direct CD28 costimulation to CD8⁺ T cells or an indirect effect due to impaired CD4⁺ T cell help. To address this question, CD4⁺-depleted B6 and CD28 KO mice were infected with ECTV, and 7 days PI we determined their CD8⁺ T cell responses as described previously. As shown in Fig. 5A, the proportion of CD8⁺ T cells producing IFN-γ did not vary whether the mice had been depleted or not depleted of CD4⁺ T cells. Moreover, CD4⁺ T cell depletion did not affect the absolute number of IFN-γ-producing CD8⁺ T cells (not shown). This result confirms previous reports showing that optimal CD8⁺ T cell responses to primary ECTV are CD4⁺ T cell independent (32) and provides indirect evidence that the altered CD8⁺ T cell response in CD28 KO mice is due to a lack of direct CD28 costimulation.

A major outcome of Ag-induced T cell activation is the rapid expansion of naive precursors through proliferation. To further address the role of direct CD28 costimulation, we determined whether CD28 was required for the optimal expansion of naive polyclonal ECTV-specific CD8⁺ T cell precursors in vivo during the early stages of infection. Naive B6-Thy1.1 mice (Thy1.1 and Thy1.2) were adoptively transferred with CFSE-labeled naive lymphocytes from Thy1.2 CD28 KO (CD28 KO 3 Thy1.1) or wild-type B6 mice (B6 3 Thy1.1). Recipient mice were then challenged with ECTV, and the expansion and proliferation of the T cells in response to ECTV infection was determined by the loss of CFSE fluorescence. Seven days PI, ~60% of the donor-derived CD8⁺ T cells in B6→Thy1.1 mice had lower CFSE fluorescence than in the cells of control uninfected mice, and most expressed IFN-γ and/or GzB, indicating a dramatic expansion and activation of the antiviral CD8⁺ T cells from the few precursors that might have been present in the transferred cells. This finding is in agreement with the proportion of antiviral CD8⁺ T cells that is found in intact B6 mice (as shown in Fig. 4). The proportion of donor cells
with decreased CFSE florescence was reduced to ≈20% in CD28 KO mice (Fig. 5B, upper panels). Still, most of these daughter cells were effectors as determined by expression of GzB and IFN-γ (Fig. 5B, middle panels).

**Delayed CD8⁺ T cell response to VACV in the absence of CD28 costimulation**

Based on the results described above, we hypothesized that CD8⁺ T cells have the potential to mount CD8⁺ T cell responses of normal potency but with delayed kinetics. In this setting, ECTV would replicate and spread more efficiently during early infection, causing mousepox. This finding may indicate that the swiftness and not just the potency of the CD8⁺ response are essential for resistance to mousepox. Alternatively, CD8⁺ T cells may not have the potential of mounting responses of normal strength, and this could be the ultimate cause of disease following ECTV infection. Distinguishing between these two scenarios is not possible when dealing with a virus that, when causing disease, induces major damage to secondary lymphoid organs. However, this distinction could be accomplished using VACV, which is genetically very similar and shares several dominant CD8⁺ T cell determinants with ECTV (30), but is much less pathogenic and can be inoculated in
VACV resulted in an early increase in the splenic cellularity of responses of CD28 KO mice were almost as strong as those of B6 KO or untreated B6 mice (Fig. 8). CD28 KO antiviral memory CD8 cells were as effective as wild-type cells at killing targets pulsed but almost normal strength as compared with B6 controls. CD28 KO and B6 mice proliferated and differentiated into effectors with equal functional. Fig. 8A shows that CD28 KO memory CD8 T cells were as effective as wild-type cells at killing targets pulsed with TSYKFESV in a 6-h in vivo CTL assay, suggesting that they were functionally normal. This ability of memory CD8 T cells to rapidly acquire lytic activity and kill target cells in vivo has previously been described for LCMV (41) but never for poxviruses.

Next, we tested the hypothesis that immunization with VACV in the absence of CD28 costimulation could still be effective at inducing a memory response capable of preventing mousepox. For this purpose, we immunized CD28 KO and control wild-type B6 mice with VACV and challenged them with ECTV 4 wk later. Results showed that both strains resisted mousepox and that the virus titers in the D-LN, spleen, and liver were very low or undetectable (data not shown). Moreover, 5 and 7 days following ECTV challenge, the recall CD8 T cell responses in the D-LN and spleen of CD28 KO and B6 mice previously immunized with VACV were comparable (Fig. 8B). Also, 7 days after ECTV infection the total numbers of lymphocytes/spleen in VACV-immune B6 and CD28 KO mice were similar (B6, 2.2 ± 0.04 × 10^8; CD28 KO, 2.1 ± 0.03 × 10^8) and the proportion of CD8 T cells was also similar (≈16%).

Because other immune mechanisms such as recall CD4 T cells or circulating Abs could have participated in the protection of VACV-immunized CD28 KO mice, we next performed adoptive transfer experiments to directly test the hypothesis that CD28 KO memory CD8 T cells can proliferate normally, differentiate into effectors, and protect against mousepox. Results showed that adoptively transferred anti-VACV memory CD8 T cells from CD28 KO and B6 mice proliferated and differentiated into effectors with similar efficiency following a challenge of the adoptive host with ECTV (Fig. 8C). Finally, ECTV-infected CD28 KO mice that had received CD28 KO memory CD8 T cells (mCD28 KO to CD28 KO) had virus titers in spleens that were much lower than untreated CD28 KO mice but similar to those of CD28 KO mice that had received memory CD8 T cells from B6 mice (mB6 to CD28 KO) or untreated B6 mice (Fig. 8D). Also, 7 days PI, CD28 KO mice that had received either type of memory CD8 T cell had an increase rather than a decrease in splenic cellularity (B6 to CD28 KO, 2.3 ± 0.3 cells per spleen; CD28 KO to CD28 KO, 2.3 ± 0.4 cells/spleen) roughly comparable to that of ECTV-infected B6 mice (2.88 ± 0.16 × 10^8), further demonstrating the ability of memory CD28 KO CD8 T cells to protect against pathology.

**Discussion**

The CD28-B7 axis is considered one of the most important mechanisms of costimulation and is essential for the induction of T cell responses to many Ags, including those to viruses that replicate abortively (17–23, 27, 28, 42–44). Previous work with LCMV suggested that for infections with host-specific viruses that replicate widely, the T cells could bypass the requirement for CD28 costimulation and mount responses of wild-type or almost wild-type strength (21–23, 27, 28). This conclusion was perplexing, because it carried the implication that CD28 costimulation is required for Ags that do not impose a threat, such as inert Ags and viruses in non-natural hosts, but not for viruses that can cause damage. However, because LCMV is a mouse-specific virus but normally does not cause disease unless inoculated intracerebrally (in which case it causes death due to immunopathology (45)), an important remaining question was whether the T cell responses induced in the absence of CD28 costimulation could afford resistance to an acute disease when a widely replicating virus has the potential to produce disease and death. Thus, we tested whether the natural resistance of B6 mice to mousepox following footpad challenge with ECTV was affected by the absence of CD28. Our results showed that the absence of CD28 costimulation resulted in complete loss of resistance to mousepox and high mortality, which were accompanied by vastly increased virus loads and pathology in secondary lymphoid organs and the liver and by decreased migration of lymphocytes to the latter. It should be noted that acute death during mousepox is due to liver failure (46). Therefore, our results demonstrate that CD28 costimulation is important for the induction of immune responses capable of preventing the symptoms of acute disease. Furthermore, our results suggested that the loss of resistance to mousepox in the absence of CD28 was due to a deficit in effector CD8 T cell function because CD28 KO mice became sick or died during the first 2 wk PI, a time at which CD8 T cells but not Abs are required for proper control of the virus (33).

To determine the mechanism of loss of resistance to mousepox in the absence of CD28, we analyzed the kinetics of the CD8 T cell responses. Our results showed that B6 mice mounted very early responses that were notoriously strong as early as 5 days PI in the D-LN and peaked on day 7 PI in the spleen. In contrast, the responses in the D-LN of CD28 KO mice on day 5 PI were notably reduced but increased by 7 days PI. In addition, the splenic response did not peak until 11 days PI. Further analysis of surviving CD28 KO mice revealed that they had a well-developed memory pool of anti-ECTV CD8 T cells, suggesting that the major factor in the loss of resistance to mousepox in CD28 KO mice was the initial delay in the CD8 T cell response rather than an intrinsic inability to mount strong responses. Unfortunately, the extensive necrosis of lymphoid organs that ECTV causes in CD28 KO mice complicated the analysis. To solve this problem, we analyzed the kinetics of the CD8 T cell response to the antigenically cross-reactive VACV that is readily controlled by CD28 KO mice. The results confirmed that the major defect of CD28 KO mice was an intrinsically delayed response without a major decrease in its end point strength. Thus, immunization of CD28 KO mice with VACV resulted in the generation of an antiviral CD8 T cell memory pool that was numerically and functionally comparable to that of B6 mice and capable of protecting naive CD28 KO mice from ECTV.

Together, our data suggest that in the case of poxvirus infections CD28 costimulation is necessary for a swift T cell response but.
does not necessarily affect the strength of the response, the establishment of memory, or the ability of the memory cells to protect from secondary infections. Our work, therefore, is consistent with experiments demonstrating that CD28 KO generates protective memory to LCMV (27) and with the general view that memory responses may not require CD28 costimulation (7).

More importantly, our results show the importance of a very early response in protection against disease. Although a slightly slower kinetics of the T cell response in the absence of CD28 costimulation has been reported previously for LCMV (23), this difference appeared to be minimal and unimportant for the control of this virus, which does not cause disease. Similarly, in our experiments the slow kinetics of the response did not have any consequences for the elimination of the poorly pathogenic VACV. The essential role of appropriate CD28 costimulation in protection from disease became apparent only following infection with the highly pathogenic ECTV. Based on these results we propose a model where CD28 KO mice have the potential to mount strong CD8+ T cell responses but, because they are intrinsically slower, they cannot curtail the initial spread of ECTV. Thus, the final consequences of the late response are poor initial control of the virus resulting in massive necrosis of lymphoid organs and early death due to liver failure or dissemination of the virus to skin and the appearance of rash. However, because CD28 KO mice can still generate an immune response, survivors of the acute disease eventually clear the virus.

An interesting remaining question is why is the CD8+ T cell responses in the absence of CD28 costimulation are slower. One possibility is that the responding cells in CD28 KO and wild-type mice originate from similarly sized pools of precursors but that those from CD28 KO mice take more time to respond. In this case, CD28 KO cells could be intrinsically slower or could require higher antigenic load (i.e., increased virus titers) to become activated and, therefore, additional rounds of virus replication. However, our proliferation data in Fig. 5B, which show a similar intensity of CFSE fluorescence for the divided CD28 KO and B6 cells, suggest a more likely possibility that the initial pool of responding precursors in CD28 KO mice is smaller, probably because only cells with high-affinity TCRs are recruited to the response during early infection. In this case it would require additional rounds of proliferation of the same cells to reach a response of similar intensity as that of wild-type mice or the late recruitment of additional precursors. Alternatively, the number or responding cells may be similar, but the responding CD28 KO cells may have a survival defect. In future experiments we will test these possibilities by analyzing TCR usage and survival in the antiviral responses of CD28 KO and wild-type B6 mice.

In summary, our work demonstrates that CD28 costimulation is not necessarily irrelevant for the induction of primary T cell responses to viruses that replicate widely in the host. Although in the case of nonpathogenic viruses the final responses may appear to be similar, a swift response due to CD28 costimulation is essential to afford resistance to disease and provides substantial survival advantage when measured against a highly pathogenic virus. In addition, this finding also shows that even minor changes in the delicate equilibrium of the host-pathogen interaction may fundamentally alter the outcome of the infection for viruses that naturally cause disease.

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References


