Role of CD28-B7 Interactions in Generation and Maintenance of CD8 T Cell Memory

M. Suresh, Jason K. Whitmire, Laurie E. Harrington, Christian P. Larsen, Thomas C. Pearson, John D. Altman and Rafi Ahmed

*J Immunol* 2001; 167:5565-5573; doi: 10.4049/jimmunol.167.10.5565

http://www.jimmunol.org/content/167/10/5565

---

**References**

This article cites 42 articles, 26 of which you can access for free at:

http://www.jimmunol.org/content/167/10/5565.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Role of CD28-B7 Interactions in Generation and Maintenance of CD8 T Cell Memory

M. Suresh, Jason K. Whitmire, Laurie E. Harrington, Christian P. Larsen, Thomas C. Pearson, John D. Altman, and Rafi Ahmed

Although the role of CD28-B7 interaction in the activation of naive T cells is well established, its importance in the generation and maintenance of T cell memory is not yet well understood. In this study, we examined the requirement for CD28-B7 interactions in primary T cell activation and immune memory. Ag-specific CD8 T cell responses were compared between wild-type (+/+ ) and CD28-deficient (CD28−/− ) mice following an acute infection with lymphocytic choriomeningitis virus (LCMV). During the primary response, there was a substantial activation and expansion of LCMV-specific CD8 T cells in both +/+ and CD28−/− mice. However, the magnitude of the primary CD8 T cell response to both dominant and subdominant LCMV CTL epitopes was ~2- to 3-fold lower in CD28−/− mice compared with +/+ mice; the lack of CD28-mediated costimulation did not lead to preferential suppression of CD8 T cell responses to the weaker subdominant epitopes. As seen in CD28−/− mice, blockade of B7-mediated costimulation by CTLA4-Ig treatment of +/+ mice also resulted in a 2-fold reduction in the anti-LCMV CD8 T cell responses. Loss of CD28/B7 interactions did not significantly affect the generation and maintenance of CD8 T cell memory; the magnitude of CD8 T cell memory was ~2-fold lower in CD28−/− mice as compared with +/+ mice. Further, in CD28−/− mice, LCMV-specific memory CD8 T cells showed normal homeostatic proliferation in vivo and also conferred protective immunity. Therefore, CD28 signaling is not necessary for the proliferative renewal and maintenance of memory CD8 T cells.

C

urrently, there is a general consensus that effective activation of naive T cells requires two signals: one dependent on the engagement of the TCR by peptide-MHC complexes, and the second costimulatory signal that is provided by interactions between cell surface molecules on the T cell and the APC (1–4). Numerous studies have indicated that the CD28 molecule, expressed on T cells, provides a potent costimulatory signal following engagement with its ligands, B7-1 and B7-2. Costimulation via CD28 can regulate both proliferation and apoptosis of activated T cells. It has been demonstrated that CD28/B7 interactions are dispensable for initiating early T cell proliferation, but are necessary to sustain late T cell proliferation (5). The lack of late proliferation in the absence of CD28 signaling was associated with increased T cell apoptosis (6). These data suggested that CD28/B7 interactions, in addition to facilitating the initiation of T cell responses, may be important in regulating long-term T cell survival. This notion is supported by studies showing that CD28 ligation increased T cell survival by up-regulating the antiapoptotic gene, Bcl-xL, and absence of CD28-mediated signaling was associated with low levels of Bcl-xL (7, 8). Taken together, these findings establish a precedent that CD28 signaling may play an important role in the survival of memory T cells and in the generation of protective immunity.

Many acute viral infections in humans and mice elicit potent CD8+ T cell responses that are instrumental in clearing virus from the tissues. The requirement for CD28/B7 interactions in eliciting anti-viral CD8+ CTL responses has been examined in several models (9–14). Interactions between CD28 and B7 are obligatory to generate optimal CD8+ CTL responses following influenza and vesicular stomatitis virus (VSV) infections (10, 11). In contrast, CD28/B7 interactions are not essential to elicit primary CD8+ CTL responses following vaccinia virus (VV) and lymphocytic choriomeningitis virus (LCMV) infection (9–10, 12–13). CD8+ T cell responses during viral infections are directed against dominant and subdominant epitopes. It has been reported that CD28 deficiency did not affect the activation of CD8 T cells directed against the dominant CTL epitopes during LCMV infection in mice (9, 13). However, the role of CD28/B7 interactions in activation and expansion of subdominant vs dominant epitope-specific CD8 T cells is not known. Also, the requirement for CD28 signaling in the generation and maintenance of long-term T cell memory is unclear.

Infection of immunocompetent mice with the Armstrong strain of LCMV (LCMV-Arm) elicits a potent CD8 T cell response that eliminates virus within 8–10 days (15–17). The generation of primary anti-LCMV CD8 T cell response is not dependent either on the presence of CD4 T cells (15, 18) or B cells (16). The CD8 T

© 2001 by The American Association of Immunologists

Copyright © 2001 by The American Association of Immunologists

0022-1767/01/$02.00

*Emory Vaccine Center and Department of Microbiology and Immunology and †Department of Surgery, Emory University School of Medicine, Atlanta, GA 30322; and ‡Department of Pathological Sciences, University of Wisconsin, Madison, WI 53706

Received for publication January 30, 2001. Accepted for publication September 4, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by Public Health Service Grant AI-30048 from the National Institutes of Health (to R.A.). M.S. was supported by an Advanced Postdoctoral Fellowship from the National Multiple Sclerosis Society.

2 M.S. and J.K.W. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Rafi Ahmed, Department of Microbiology and Immunology, Emory Vaccine Center, G211 Rollins Research Center, 1510 Clifton Road, Atlanta, GA 30322. E-mail address: ra@microbio.emory.edu

4 Abbreviations used in this paper: VSV, vesicular stomatitis virus; VV, vaccinia virus; LCMV, lymphocytic choriomeningitis virus; LCMV-Arm, the Armstrong CA 1371 strain of LCMV; BrdU, 5-bromo-2'-deoxyuridine; PL, postinfection; NP, nucleoprotein; i.c., intracranial.
CD28-mediated costimulation and T cell memory

Materials and Methods

Mice

C57BL/6 (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The CD28-/- mice (C57BL/6 background) used in these experiments were created by targeted gene disruption, which abrogates surface expression of the CD28 (12). The C57BL/6 LCMV carrier colony was established and bred at Emory University (Atlanta, GA) as described previously (20). Spleen cell preparations from these mice contain LCMV-infected cells that present viral Ag with both MHC class I and II molecules, but lack LCMV-specific T cells.

Virus

LCMV-Arm was used in these studies for infection of mice (21). Mice were infected with LCMV-Arm by i.p. injection (2 x 10^5 PFU/mouse). To measure protective immunity, LCMV-immune mice were challenged with 10^5 PFU of LCMV-Arm (intracranial [i.c.]) or 2 x 10^6 PFU of LCMV-clone 13 (i.v. injection; 15-28). Influenza virus in serum and tissues was quantitated by plaque assay on Vero cell monolayers as previously described (21).

CTL assay

MHC class I-restricted LCMV-specific CTL activity was determined by 51 Cr release assay as previously described (21).

Determination of the LCMV-specific CTLp frequency

CTLp frequency was determined by a limiting dilution assay as described previously (15-17). Spleen cells from LCMV-immunized mice were cultured in graded doses in 96-well flat-bottom plates (12 wells per dose). Syngeneic feeder spleen cells (8 x 10^4) from uninfected mice and syngeneic stimulator spleen cells (2 x 10^4) from LCMV carrier mice were irradiated (1200 rad) and added to each well. Recombinant human IL-2 was purchased from BD PharMingen (La Jolla, CA) and was added at a final concentration of 50 U per ml. After 8 days, the contents from each well were split to test CTL activity against LCMV-infected and uninfected MC57 targets in a 6-h 51 Cr-release assay.

Flow cytometry

The number of CD8 T cells in the spleen and lymph nodes was determined by staining with specific mAbs followed by FACS analysis, as previously described (16, 20). For FACS analysis, PE-conjugated anti-CD8a (53-6.7), and FITC-conjugated anti-mouse CD44 (IM7) were purchased from BD PharMingen and were used at concentrations recommended by the manufacturer.

Visualization of LCMV-specific CD8 T cells by MHC I tetramer staining

Construction of the MHC I Db tetramers that contain the LCMV CTL epitope peptides nucleoprotein (NP) 396–404 or gp33–41 has been described previously (20). Spleen cells were surface stained with either FITC- or PE-conjugated anti-CD8 (BD PharMingen) and fluorochrome-labeled MHC I tetramer for 1 h at 4°C. Spleen cells from uninfected mice were always stained in parallel with cells from infected mice as a negative control. To analyze CD28 expression on LCMV-specific memory CD8 T cells, single cell suspensions of splenocytes were stained with anti-CD8, anti-CD28 (BD PharMingen), and MHC I tetramers, followed by three-color flow cytometry.

Quantitation of T cell responses by intracellular staining for IFN-γ

Intracellular staining for IFN-γ in CD8 T cells following stimulation with the specific peptide was done as described previously (20). Spleen cells were incubated in vitro for 5 h at 37°C in medium containing brefeldin A and recombinant human IL-2 (50 U/ml). Cells were either left unstimulated during the culture period or were stimulated with MHC class I-restricted epitope peptides. After the incubation period, the cells were surface stained for CD8 using allophycocyanin- or PE-conjugated Abs and were then stained intracellularly with anti-IFN-γ-FITC using the Cytofix/Cytoperm kit from BD PharMingen. Generally, detection of LCMV-specific CD8 T cells by either intracellular cytokine staining or MHC class I tetramers gives comparable results (20).

Quantitation of virus-specific IFN-γ-secreting T cells

Virus-specific T cell responses were measured by ELISPOT assay as described previously (20). The capture Ab, anti-mouse IFN-γ (clone R4-6A2) and detection Ab, biotinylated anti-mouse IFN-γ (clone XMG1.2) were purchased from BD PharMingen. The ELISPOT plates were purchased from Millipore (Bedford, MA). MHC I-restricted peptides LCMV NP_53–62, gp33–41, or gp276–286 were used to stimulate CD8 T cells. Uninfected spleen cells contain IFN-γ-producing cells at a frequency of <2 per 10^6 cells with or without stimulation. Quantitation of LCMV-specific CD8 T cells by intracellular cytokine staining or ELISPOT gives comparable results (20).

5-Bromo-2′-deoxyuridine (BrdU) incorporation studies

To monitor proliferation of LCMV-specific memory CD8 T cells in vivo, LCMV-immune mice were given BrdU in drinking water (0.8 mg/ml) for 8 days. After the BrdU pulse, splenocytes were isolated and stained with anti-CD8 and anti-CD44 or fluorochrome-labeled MHC I tetramers. Following surface staining, cells were stained with anti-BrdU Abs, as described previously (20). Flow cytometry and data analysis were performed as described above. Spleen cells from mice that were not exposed to BrdU were always used as negative controls for BrdU staining.

CTLA4-Ig treatment in vivo

The CTLA4-Ig fusion protein used in this study was provided by Bristol-Myers Squibb (New York, NY) and has been described elsewhere (22-24). CTLA4-Ig is a fusion protein that contains the extracellular domain of CTLA4 and is fused to the Cy region of human IgE. The human IgE control Ab was purchased from BD PharMingen. CTLA4-Ig was injected i.p. at a dose of 200 μg/mouse/injection on days 0, 2, 4, and 6 relative to infection with LCMV-Arm.

Results

Primary CD8 T cell responses in the absence of CD28-B7 interaction

We determined the role of CD28/B7 interactions in generating primary CD8 T cell responses by comparing the CD8 CTL responses between +/+ and CD28-/- mice following an acute infection with LCMV-Arm. On day 8 postinfection (PI), we quantitated the direct ex vivo MHC class I-restricted LCMV-specific CD8 CTL-mediated cytotoxicity in the spleens of +/+ and CD28-/- mice. Consistent with previous reports (10, 12), the LCMV-specific cytotoxic activity in the spleens of CD28-/- mice was comparable with that of +/+ mice (Fig. 1). These data show that generation of LCMV-specific effector CD8 CTL response is not dependent upon CD28/B7 interactions. CD28-/- mice cleared LCMV by day 8 PI similar to +/+ mice (data not shown).

Activated/memory CD8 T cells express elevated levels of CD44 (CD44high). The activation and expansion of CD8 T cells following...
LCMV infection was determined by flow cytometry after staining spleen cells from HBO1/H11001 and CD28/H11002/H11002 mice for CD8 and CD44. As seen in the HBO1/H11001 mice, there was a strong activation of CD8/H11001 T cells in the spleens of CD28/H11002/H11002 mice. The total number of activated CD8 T cells in the spleens of CD28−/− mice was ~2-fold lower as compared with +/+ mice (Fig. 2a). Uninfected +/+ and CD28−/− mice showed no differences in the total number of naive or activated CD8 T cells (data not shown). We next performed more precise analysis of the activation and expansion of LCMV-specific CD8 T cells using MHC class I tetramers bearing the LCMV NP CTL epitope peptide, NP396–404. On the day 8 PI, spleen cells from LCMV-infected +/+ and CD28−/− mice were stained with anti-CD8 Abs and fluorochrome-labeled MHC class I (D^b) tetramers followed by flow cytometric analysis. Representative FACS profiles of staining for CD8^+ tetramer-binding cells are shown in Fig. 2b. As shown in Fig. 2b, the relative proportions of NP396–404-specific cells in the spleens of CD28−/− mice was lower compared with +/+ mice. On day 8 PI, the spleens of +/+ and CD28−/− mice had 12–18 × 10^6 and 6–9 × 10^6 NP396–404-specific CD8 T cells, respectively. Although high numbers of NP396–404-specific CD8 T cells were generated in CD28−/− mice, the overall magnitude was ~2-fold lower compared with +/+ mice. Similar results were obtained following staining with MHC class I tetramers specific to the other LCMV immunodominant epitope in the viral glycoprotein, gp33–41 (data not shown). The effect of B7-CD28 blockade was also examined in +/+ mice treated with CTLA4-Ig. Treatment of +/+ mice with CTLA4-Ig blocks B7 interactions, and this reagent has been shown to block alloantigen-induced T cell responses and certain allograft reactions (22–24). The total number of

---

**FIGURE 1.** LCMV-specific CTL responses in CD28-deficient mice. Spleen cells were isolated from LCMV-infected +/+ and CD28−/− mice on day 8 PI, and MHC class I-restricted CTL activity was measured in a ^51Cr release assay using uninfected (Uninf.) and LCMV-infected (Inf.) MC57 cells as targets.

**FIGURE 2.** Expansion of LCMV-specific CD8^+ T cells in CD28−/− mice. a, To monitor activation of CD8 T cells, 8 days following infection with LCMV, spleen cells from +/+ and CD28−/− mice were stained with anti-CD8 and anti-CD44 Abs and were analyzed by flow cytometry. Naive CD8 T cells are CD44^low and activated/memory CD8 T cells are CD44^high. b, On the eighth day after infection, the number of LCMV-specific CD8 T cells among splenocytes was determined by staining with anti-CD8 Abs and D^b NP396–404 tetramers. The flow cytometry plots are gated on spleen cells based on forward and side scatter. The numbers represent percentages of tetramer-binding CD8 T cells among total spleen cells. Numbers in parentheses represent percentages of naive CD8 T cells. c, +/+ and −/− mice were infected with LCMV-Arm, and B7-mediated costimulatory interactions in +/+ mice were blocked by treatment with CTLA4-Ig as described in Materials and Methods. On day 8 PI, the total number of NP396–404-specific cells in the spleens of LCMV-infected +/+ and −/− mice was determined by staining with D^b NP396–404 tetramers.
Role of CD28-B7 interactions in activation of CD8 T cells specific to dominant and subdominant epitopes

The MHC class I-restricted LCMV-specific CD8 T cell epitopes can be divided into dominant and subdominant epitopes. In H-2b mice, the dominant epitopes are NP\textsubscript{396–404} and gp33–41, and the subdominant epitopes are gp276–286, NP\textsubscript{205–212}, and gp92–101 (19–20). It was of interest to determine the requirement for CD28-mediated costimulation for optimal activation of CD8 T cells, specific to dominant epitopes vs the weaker subdominant epitopes. The epitope-specific CD8 T cell responses in +/+ and CD28\textsuperscript{−/−} mice were analyzed by intracellular staining for IFN-γ on day 8 PI (Fig. 3). Upon LCMV-Arm infection, CD28\textsuperscript{−/−} mice mounted readily detectable CD8 T cell responses to both dominant and subdominant epitopes. At the peak of immune response, the total number of CD8 T cells specific to all the epitopes was ~2-fold lower in the CD28\textsuperscript{−/−} mice compared with +/+ mice. A previous study has indicated that CD8 T cells specific to the immunodominant epitopes undergo cell division ~15 times during the expansion phase of the anti-LCMV T cell response (20). Therefore, a 2-fold reduction in the number of LCMV-specific CD8 T cells in CD28\textsuperscript{−/−} mice may reflect one less cell division as compared with +/+ mice during the proliferation phase of the CD8 T cell response. In summary, these data suggested that following an acute LCMV infection, the activation of CD8 T cells specific to both dominant and subdominant epitopes is largely independent of CD28-mediated costimulation. Furthermore, these data show that the development of CD8 T cell effector functions, namely cell-mediated cytotoxicity (Fig. 1) and production of IFN-γ (Fig. 3), did not require CD28 signaling.

Generation and maintenance of CD8 T cell memory in the absence of CD28-B7 interactions

Memory CD8 T cells in humans exhibit heterogeneity with respect to CD28 expression; only a subpopulation of memory CD8 T cells in humans express CD28 on their surface (25). Although it has been shown that all murine T cells express CD28 constitutively (3), the expression of CD28 on Ag-specific memory CD8 T cells has not been studied. We examined the expression of CD28 on the surface of LCMV-specific memory CD8 T cells by flow cytometry. As illustrated in Fig. 4, LCMV-specific memory CD8 T cells expressed readily detectable levels of CD28 on their surface with little or no heterogeneity. Currently, it is not known whether CD28-B7 interactions are necessary to maintain CD8\textsuperscript{+} T cell memory. To examine the role of CD28-B7 interactions in generating LCMV-specific CD8 T cell memory, CD28\textsuperscript{−/−} mice were immunized with LCMV-Arm, and memory CD8 T cell responses were analyzed by IFN-γ ELISPOT assay at various time points after infection. Fig. 5 shows the kinetics of LCMV-specific CD8 T cell response in +/+ and CD28\textsuperscript{−/−} mice. As shown in Fig. 5, the peak of LCMV-specific CD8 T cell response was attained on day 8 PI, which was followed by a contraction/down-regulatory phase (days 8–15 PI). During the contraction phase of the T cell response, the magnitude of loss of LCMV-specific CD8 T cells in the CD28\textsuperscript{−/−} mice was comparable with that of +/+ mice. Approximately 90% of the LCMV-specific CD8 T cells were lost (20), presumably by apoptosis (30) during the contraction phase in both +/+ and CD28\textsuperscript{−/−} mice. There is evidence that activation in the absence of CD28-mediated costimulation may lead to T cell apoptosis in vitro (6). However, the data shown in Fig. 5 suggested that in vivo, lack of CD28-mediated costimulation did not lead to an exaggerated death phase of LCMV-specific CD8 T cell response. The contraction phase of the CD8 T cell response is followed by the phase of memory, when a stable pool of memory T cells is maintained indefinitely. Analysis of LCMV-specific CD8 T cell responses revealed that the number of memory cells was

![CD8 T cell responses to dominant and subdominant epitopes in CD28-deficient mice. On day 8 PI with LCMV-Arm, splenocytes from +/+ and CD28\textsuperscript{−/−} mice were stimulated with CTL epitope peptides, and number of CD8 T cells specific to dominant and subdominant epitopes were determined by intracellular staining for IFN-γ. Data are the means of three to five mice/group.](http://www.jimmunol.org/)

![CD8 Expression on LCMV-specific memory CD8 T cells. Groups of +/+ and CD28\textsuperscript{−/−} mice were infected with LCMV-Arm. Sixty days following infection, splenocytes were stained with anti-CD8, anti-CD28, and MHC class I tetramers (specific to the LCMV CTL epitopes NP\textsubscript{396–404} and gp33–41), and were analyzed by flow cytometry. The histograms shown in the figure are gated on tetramer-binding CD8 T cells. The bold and the broken lines in the histograms represent CD28 staining on LCMV-specific memory CD8 T cells from +/+ and CD28\textsuperscript{−/−} mice, respectively. Data are representative of studies using six mice/group.](http://www.jimmunol.org/)
~2-fold lower in CD28−/− than in +/+ mice. However, this did not change over time and the lower number of CD8 memory T cells in CD28−/− mice most likely reflected the 2- to 3-fold lower expansion seen in these mice during the acute phase of the CD8 response (day 8). These data were confirmed by quantifying the number of LCMV-specific CTL precursors in the spleens of LCMV-immune +/+ and CD28−/− mice by limiting dilution analysis (data not shown).

We also analyzed CD8 T cell memory in +/+ and CD28−/− mice (292 days PI) using MHC class I tetramers. As shown in Fig. 6a, consistent with published data, the spleens of LCMV-immune +/+ mice contained high frequencies of memory CD8 T cells specific to the immunodominant epitopes, NP396–404 and gp33–41 (20). LCMV-specific memory CD8 T cells were also readily detected in the spleens of LCMV-immune CD28−/− mice (Fig. 6a), and the frequencies were comparable with those in +/+ mice. Memory CD8 T cells specific to dominant and subdominant epitopes were also quantitated in the spleens of LCMV-immune +/+ and CD28−/− mice by staining for intracellular IFN-γ. Data in Fig. 6b illustrate that irrespective of epitope specificity, the percentages of memory CD8 T cells in the spleens of LCMV-immune CD28−/− mice were comparable with +/+ mice. Thus, B7-CD28 interaction is not necessary to generate and maintain memory CD8 T cells following an acute LCMV infection.

Activation threshold of LCMV-specific memory CD8 T cells in CD28-deficient mice

We examined the effect of CD28 deficiency on the activation threshold of memory CD8 T cells in LCMV-immune mice. The activation threshold of memory CD8 T cells (specific to both dominant and subdominant epitopes) was compared between LCMV-immune +/+ and CD28−/− mice by measuring IFN-γ production as a function of the concentration of antigenic peptide. As shown in Fig. 7, in both +/+ and CD28−/− mice, the number of IFN-γ-producing LCMV-specific memory CD8 T cells varied in a peptide dose-dependent fashion. As shown in Fig. 7, memory CD8 T cells in CD28−/− mice exhibited a slight difference in the activation threshold at one peptide dilution for three of the four epitopes as compared with +/+ mice; 0.0001 μg/ml for NP396–404, 0.001 μg/ml for gp276–286, and 0.0001 μg/ml for NP205–211. Taken together, these data suggest that lack of CD28/B7 interactions did not significantly affect the sensitivity of LCMV-specific memory CD8 T cells to antigenic stimulation in vitro. Further, these data show that memory CD8 T cells generated under conditions of CD28 deficiency may not be qualitatively different as compared with memory CD8 T cells that were generated in the presence of CD28/B7 interactions.

Homeostatic proliferation of LCMV-specific memory CD8 T cells in the absence of CD28/B7 interactions

It is well established that memory T cells undergo homeostatic proliferation, which is believed to be an important mechanism promoting survival by avoiding cell attrition over time. Studies have indicated that cytokines IL-15 and IL-7 may be important for homeostatic proliferation of memory T cells (31–33). Although homeostatic proliferation of memory CD8 T cells is not dependent upon TCR/MHC interactions (34), the role of costimulatory molecules has not been examined. In this study, we determined the requirement for CD28/B7 interactions in the homeostatic proliferation of LCMV-specific memory CD8 T cells. At 60 days PI with LCMV, +/+ and CD28−/− mice were given BrdU in drinking water for 8 days. At the end of the BrdU pulse, the percentage of BrdU+ cells among LCMV-specific memory CD8 T cells was determined by flow cytometry. As shown in Fig. 8, 23% and 20% of CD8+ CD44high T cells (activated/memory) incorporated BrdU in +/+ and CD28−/− mice, respectively. About 1.5–2% of naive CD8 T cells (CD44low) in both +/+ and CD28−/− mice incorporated BrdU over a period of 8 days (data not shown). Importantly, the percentages of BrdU+ memory CD8 T cells specific to two different LCMV CD8 CTL epitopes were comparable between +/+ and CD28−/− mice. Further, blocking interactions between B7 and CD28/CTLA-4 molecules using CTLA-4-Ig fusion proteins did not affect the homeostatic proliferation of LCMV-specific memory CD8 T cells (data not shown). Taken together, these data suggest that CD28/B7 interactions are not obligatory for homeostatic proliferation of memory CD8 T cells. Also, the proliferation rate of memory CD8 T cells was not affected by antigenic specificity; within the same mouse, memory CD8 T cells specific to two different epitopes had similar proliferation rates in both +/+ and CD28−/− mice.

Protective immunity in CD28-deficient mice

T cell memory to LCMV infection is characterized by the ability of memory CD8+ T cells to generate an accelerated response upon re-exposure, leading to viral clearance more rapidly than they do during the primary exposure (17, 20). We examined protective immunity in CD28−/− mice using two challenge models: 1) protection against a lethal i.c. infection and 2) protection against a lethal i.p. infection with LCMV-Arm. To evaluate protection against lethal choriomeningitis, 35 days after immunization, these mice were challenged with LCMV-Arm by i.c. injection. Naïve +/+ and CD28−/− mice were infected with LCMV-Arm (i.c.) as controls. As shown in Fig. 9a, all of the naive +/+ and CD28−/− mice succumbed to an i.c. LCMV-Arm infection by 7–8 days postchallenge. In striking contrast, all of the LCMV-immune +/+ and CD28−/− mice were completely protected against a lethal LCMV challenge, and survived at least up to 4 mo. The clone 13 strain of LCMV establishes persistent infections in naive immunocompetent mice, which is characterized by low CTL responses and disseminated infection of several tissues (15, 21). However, wild-type mice that have recovered from an
acute infection with LCMV-Arm are protected against a persistent infection with LCMV-clone 13 (17). To examine protective immunity against a persistent LCMV infection, +/+ and CD28−/− mice that were previously immunized with LCMV-Arm (100 days PI) were challenged with 2 × 10^6 PFU LCMV-clone 13 by i.v. injection. Naïve +/+ and CD28−/− were also infected with LCMV-clone 13 for comparison. LCMV titer in the serum was determined 5 days after challenge with LCMV clone-13. Data in Fig. 9b show that both +/+ and CD28−/− LCMV-immune mice had undetectable levels of infectious virus in the serum; serum from naïve +/+ and CD28−/− mice infected with LCMV-clone 13 contained high levels of infectious virus. In summary, these

FIGURE 6. Analysis of CD8 T cell memory in CD28−/− mice using MHC I tetramers and intracellular cytokine staining. a, Two hundred and ninety-two days following infection with LCMV-Arm, LCMV-specific memory CD8 T cells in the spleens of +/+ and CD28−/− mice were quantitated by staining with MHC I tetramers. The flow cytometry profiles are gated on total splenocytes based on forward and side scatter properties. The numbers represent percentages of tetramer-binding CD8 T cells among splenocytes. Numbers in parentheses represents percentages of tetramer-binding CD8 T cells of total CD8 T cells in the spleen. b, Two hundred and ninety-two days following infection with LCMV-Arm, LCMV-specific memory CD8 T cells in the spleens of +/+ and −/− mice were quantitated by intracellular staining for IFNγ as described in Materials and Methods. The numbers represent percentages of IFN-γ-producing CD8 T cells among splenocytes.

FIGURE 7. Activation threshold of LCMV-specific memory CD8 T cells in CD28−/− mice. Sixty days after infection with LCMV-Arm, the activation threshold of memory CD8 T cells was analyzed by intracellular staining for IFN-γ following stimulation with various LCMV CTL epitope peptides at the indicated concentrations. The results are expressed as a percentage of maximum response attained at a peptide concentration (saturating) of 1.0 μg/ml. Data are the means of three mice/group and represents one of two independent experiments.
data provide convincing evidence that memory CD8 T cells in LCMV-immune CD28−/− mice confer protective immunity in both peripheral (i.c. infection with LCMV-Arm) and systemic (i.v. infection with LCMV-clone 13) challenge experiments.

Discussion

In this study, we examined the requirement for CD28-mediated costimulation in the activation and expansion of Ag-specific CD8 T cells and maintenance of T cell memory during an acute viral infection. We show that the activation and expansion of LCMV-specific CD8 T cells to both dominant and subdominant epitopes is largely independent of CD28-mediated costimulation. We also show that maintenance of LCMV-specific CD8 T cell memory and protective immunity does not require CD28-B7 interactions.

Previous studies have documented that CD28-B7 interactions are not essential for generating primary LCMV-specific CTL responses (9–13). These studies primarily examined CD8 T cell responses against dominant epitopes of LCMV. The present study confirms and extends these findings. It is well established that CD8 T cell responses during an acute LCMV infection are directed against dominant and subdominant epitopes (19, 20). It is believed that the strength of TCR signaling determines the requirement for costimulation during T cell activation (3, 35). Dominant epitopes most likely are presented at higher densities on the cell surface, thereby delivering a potent signal through the TCR, obviating a need for costimulation. In contrast, activation of T cells by weaker subdominant epitopes may be costimulation dependent. To address this issue, we compared the CD8 T cell responses to dominant and subdominant LCMV CTL epitopes for their dependence on CD28-mediated costimulation. Interestingly, CD28−/− mice generated high numbers of CD8 T cells specific to both dominant and subdominant epitopes, albeit 2-fold lower in magnitude compared with +/+ mice (Fig. 3). Furthermore, the hierarchy of immunodominance among LCMV CTL epitopes was not altered in the absence of CD28-B7 interactions. Although the mechanistic basis of LCMV CTL epitope hierarchy is not known, these data suggested that CD8 T cell responses against dominant and subdominant epitopes are largely independent of CD28-mediated costimulation. These data are in agreement with the studies done by Tan et al. (36), who blocked CD28/CTLA-4-B7 interactions by treating mice with CTLA-4-Ig fusion proteins during an acute LCMV infection. Similar to LCMV infection, CTL responses to VV are not...
significantly altered by abrogation of CD28 signaling (10). However, B7-CD28 interactions are essential for generating effector CD8 CTL following VSV and influenza virus infection in mice (10, 11). In contrast to LCMV and VV, which replicate efficiently in the lymphoid system, the replication of influenza virus and VSV is very limited in lymphoid tissues. Thus, one common theme that has emerged from these studies is that the CD28-mediated costimulation seems to be dispensable for CTL responses against viruses that replicate to very high levels in the lymphoid system. The rules defining the costimulatory requirements of CD8 T cells may also depend on whether the infection is localized or systemic, and the ability of virus to infect APC, particularly the dendritic cells. Primary CD8 T cell responses to LCMV were normal in the absence of CD40-CD40L interactions (9, 37, 38). In the absence of 41BB-41BBL interactions, LCMV-specific CD8 T cell responses were reduced by ~2-fold (36). It remains to be determined whether CD40-CD40L- and 41BB-41BBL-mediated costimulatory interactions play redundant and/or compensatory roles in activating CD8 T cells under conditions of CD28 deficiency.

The resolution of an acute LCMV infection is dependent upon CD8<sup>+</sup> CTLs (39–40) and does not require CD4 T cells (15, 18). Nonetheless, mice acutely infected with LCMV mount a strong CD4 T cell response and develop humoral immunity (41, 42). In contrast to the development of a potent CD8 T cell response, the induction of LCMV-specific CD4 T cell response was compromised in CD28<sup>−/−</sup> mice (Ref. 13 and M. Suresh, J. K. Whitmire, J. D. Altman, and R. Ahmed, manuscript in preparation). It is likely that lack of CD4 T cell activation in CD28<sup>−/−</sup> mice reduced the CD8 T cell response by one-half compared with +/+ mice. CD4-deficient mice also exhibit a similar phenotype: the magnitude of LCMV-specific CD8 T cell response was ~2-fold lower in comparison with what was generated in +/+ mice.

In addition to providing costimulatory signals necessary for activation of naïve T cells, CD28-B7 interaction has also been shown to enhance survival of activated T cells by inducing the expression of the antiapoptotic gene Bcl-x<sub>L</sub> (6, 7). Furthermore, Bcl-x<sub>L</sub> induction prevented Fas- and anti-CD3-induced apoptosis in activated T cells (7, 8). These data suggested that CD28-mediated signaling may be important in the survival of memory T cells. It was of interest to examine the role of CD28-B7 interactions in the generation and maintenance of LCMV-specific memory CD8 T cells. In this study, the maintenance of LCMV-specific CD8 T cell memory was unaffected by lack of CD28/B7 interactions (Figs. 5 and 6). The initial expansion of CD8 T cells during the primary response (clonal burst size) has been shown to be one of the determinants of the magnitude of T cell memory (20). Therefore, a slight reduction in the total number of LCMV-specific memory CD8 T cells in CD28<sup>−/−</sup> mice most likely reflect a ~2-fold lower expansion of virus-specific CD8 T cells during the primary response.

Studies have shown that Ag dose, duration of TCR stimulation, and number of TCRs engaged can determine the requirement for costimulation during T cell activation. During activation of naive T cells, CD28-mediated costimulation reduces the number of TCRs that need to be triggered by the Ag (43). It was of interest to examine the activation thresholds of memory CD8 T cells in the absence of CD28/B7 interactions. To this end, we compared the activation thresholds of memory CD8 T cells specific to multiple epitopes between +/+ and CD28<sup>−/−</sup> mice. These experiments revealed that loss of CD28/B7 interactions did not significantly affect the activation thresholds of memory CD8 T cells (Fig. 7). One intriguing finding was that the activation threshold of memory CD8 T cells specific to the subdominant epitope NP<sub>205–212</sub> was lower as compared with memory CD8 T cells specific to the dominant epitopes NP<sub>396–404</sub> and gp33–41 (even in the presence of CD28/B7 interactions in +/+ mice). The increased sensitivity of NP<sub>205–212</sub>-specific memory CD8 T cells to peptide stimulation cannot be explained based on the differences in the binding affinities of peptides to the MHC I molecule. This is because the MHC-binding affinity of peptide NP<sub>396–404</sub> is substantially greater than for NP<sub>205–212</sub> (19). One possibility is that during the primary response, low-level presentation of NP<sub>205–212</sub> subdominant epitope by the APCs may selectively activate CD8 T cells with high affinity. Alternatively, the repertoire of CD8 T cells that recognize NP<sub>205–212</sub> are inherently of high affinity and/or avidity. Nevertheless, taken together, these data suggested that CD28-deficient memory CD8 T cells may be qualitatively similar to +/+ memory CD8 T cells.

According to the current axiom, maintenance of memory T cells is dependent upon homeostatic proliferation, which prevents T cell attrition over time (31–33). We examined whether homeostatic proliferation of LCMV-specific memory CD8 T cells is affected in the absence of CD28-B7 interactions. In vivo BrdU labeling studies indicated that the rate of homeostatic proliferation of LCMV-specific memory CD8 T cells in LCMV-immune CD28<sup>−/−</sup> mice was comparable with that of immune +/+ mice. These data are consistent with normal maintenance of CD8 T cell memory in CD28<sup>−/−</sup> mice.

Protective immunity is a definitive marker of T cell memory. +/+ mice that have recovered from an acute LCMV infection are protected against lethal choriomeningitis resulting from an i.c. LCMV challenge. Akin to +/+ mice, memory CD8 T cells in LCMV-immune CD28<sup>−/−</sup> mice successfully protected against a lethal i.c. infection with LCMV (Fig. 9a). LCMV-clone 13 is a highly virulent strain of LCMV that establishes persistent infections in immunocompetent mice (21). However, accelerated CD8 T cell responses in LCMV-immune mice promptly controls LCMV-clone 13 infection, preventing viral persistence (17). LCMV-immune CD28<sup>−/−</sup> mice were completely protected against a persistent infection with LCMV-clone 13, presumably due to memory CD8 T cell-dependent accelerated viral clearance (Fig. 9b). In summary, generation and maintenance of CD8 T cell memory, as assessed by quantitation of virus-specific CD8 T cells by phenotypic and functional assays and protective immunity, is not dependent on CD28/B7 interactions.

Acknowledgments
We thank Morry Hsu, Mary Katherine Large, and Kaja Madhavi Krishna for excellent technical assistance.

References