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The Role of CD80/CD86 in Generation and Maintenance of Functional Virus-Specific CD8+ T Cells in Mice Infected with Lymphocytic Choriomeningitis Virus

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Lymphocytic choriomeningitis virus (LCMV)–specific CD8+ T cell responses are considered to be independent of CD28–B7 costimulation. However, the LCMV-specific response has never been evaluated in B7.1/B7.2-/- mice. For this reason, we decided to study the T cell response in B7.1/B7.2-/- mice infected with two different strains of LCMV, one (Traub strain) typically causing low-grade chronic infection, and another (Armstrong clone 53b) displaying very limited capacity for establishing chronic infection. Using Traub virus we found that most B7.1/B7.2-/- mice were unable to rid themselves of the infection. Chronic infection was associated with a perturbed CD8+ T cell epitope hierarchy, as well as with the accumulation of cells expressing markers of terminal differentiation and being unable to respond optimally to Ag restimulation. Examination of matched CD28-/- mice revealed a similar albeit less pronounced pattern of CD8+ T cell dysfunction despite lack of virus persistence. Finally, analysis of B7.1/B7.2-/- mice infected with Armstrong virus revealed a scenario quite similar to that in Traub infected CD28-/- mice; that is, the mice displayed evidence of T cell dysfunction, but no chronic infection. Taken together, these results indicate that B7 costimulation is required for induction and maintenance of LCMV-specific CD8+ T cell memory, irrespective of the LCMV strain used for priming. However, the erosion of CD8+ T cell memory in B7.1/B7.2-/- mice was more pronounced in association with chronic infection. Finally, virus-specific T cell memory was more impaired in the absence of B7 molecules than in the absence of the CD28 receptor, supporting earlier data suggesting the existence of additional stimulatory receptors for B7. *The Journal of Immunology, 2010, 185: 1730–1743.

Besides recognition of Ag through the TCR, complete T cell activation also requires a series of other signals commonly grouped together under the heading of “costimulation” (1). A key costimulatory molecule is CD28, which binds to B7.1 (CD80) and B7.2 (CD86) expressed on the surface of professional APCs (2, 3). Although many studies have addressed the importance of these molecular interactions in primary T cell activation, the exact role of CD28–B7 signaling in vivo is still not quite clear, particularly when it comes to the role of this interaction in memory T cell differentiation and maintenance. One recent confounding factor in this respect is the realization that different states of T cell differentiation (effector, memory) may require different costimulatory signals (1), and that appropriate development of stable T cell memory may be substantially more demanding than induction of a primary effector T cell response (4–7).

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Initial studies on CD28 knockout (CD28-/-) mice revealed that the importance of CD28 for induction of a primary CD8 T cell response depended very much on the pathogen and its ability to replicate in the host. Thus, whereas expression of CD28 was absolutely essential for induction of CD8 T cell responses toward viruses with localized tropism (e.g., influenza, HSV, and vesicular stomatitis virus) (8–11), systemic infection with lymphocytic choriomeningitis seemed to bypass any requirements for CD28–B7 interaction (9, 12, 13). This led to the hypothesis that extensive viral replication was associated with prolonged TCR signaling, eliminating the need for CD28 ligation (14–16). However, subsequent, more detailed studies suggested several deficiencies regarding the primary lymphocytic choriomeningitis virus (LCMV)-specific T cell response in CD28-/- mice. Thus, the CD8 T cell response was found to be somewhat delayed in mice lacking expression of CD28, and cell numbers never completely matched those in wild-type (WT) mice (17). More importantly, the expansion of virus-specific CD4 T cells was reduced by about a factor of 10, and the capacity of the virus-specific CD8 T cells to produce IL-2 was markedly reduced (17), parameters often associated with rapidly deteriorating CD8 T cell memory. Nevertheless, the first simple studies on T cell memory in CD28 knockout mice (using LCMV infection as a model) indicated that CD8 T cell memory was appropriately induced and maintained in the absence of signaling through CD28, except when extremely high doses of rapidly invasive virus were used for the infection (13, 17). Interestingly, more recent studies in other viral models have indicated that not only is CD28–B7 interaction critical for early priming of T cell memory, but also for the efficient induction of recall responses (18). In view of the fact that these issues had not been optimally dealt with in earlier studies on LCMV infection, we...
considered it important to revisit the role of CD28–B7 in inducing and maintaining LCMV-specific CD8 T cell memory, with the reason being that it would be very important to know if certain viruses would have the capacity to induce not only initial T cell activation, but also completely normal memory differentiation in the absence of this key costimulatory interaction.

Our interest was further fueled by a few recent reports suggesting that there might be other stimulatory receptors for B7 than CD28 (19–21) and that earlier studies of the LCMV infection might have failed in finding a key role for B7 costimulation, because these studies had been performed in CD28−/− mice, not B7.1/B7.2 knockout (B7.1/B7.2−/−) mice. In particular, the study by Lyon and Sarawar on murine gammaherpesvirus 68 (MHV68) attracted our attention because it in many ways reproduced earlier results obtained in the LCMV model, but in addition it revealed certain distinctive deficiencies in the immune response of B7.1/B7.2−/− mice compared with CD28−/− mice (19). Thus, while CD28−/− mice have no obvious phenotype following infection with MHV68, T cell–dependent immune surveillance eventually completely collapses in MHC class II–deficient mice, and, interestingly, analysis of B7.1/B7.2−/− mice revealed that these mice lay between these two extremes. This is similar to the situation in mice infected with moderate doses of rapidly invasive strains of LCMV, where the infection is efficiently controlled in CD28-deficient mice (17) but leads to late recrudescence of viremia and severe disease in MHC class II–deficient mice (22).

Consequently, we decided to study T cell memory in B7.1/ B7.2−/− mice infected with moderate doses of two different strains of LCMV. One strain was the rapidly invasive Traub strain, which in WT mice induces an acute infection followed by low-grade virus persistence for several months (22); in general, the level of infection during the chronic phase is below the sensitivity of in vitro titration assays, but virus may be detected by sensitive in vivo infectivity assays. The behavior of this virus and the accompanying immune response have been extensively studied in both CD28 and MHC class II–deficient mice (12, 17, 22, 23), and the results have been briefly mentioned above. The other LCMV strain applied in this study was Armstrong virus clone 53b, which has little capacity for inducing persistent infection except under very extreme conditions (12, 24). Our results indicate that B7 costimulation is essential for induction as well as maintenance of LCMV-specific T cell memory, irrespective of which LCMV strain is used for priming; however, the erosion of T cell memory in B7-deficient mice was significantly more marked following infection with the persisting virus strain, that is, in the case of chronic antigenic stimulation. Additionally, virus-specific T cell memory was more impaired in the absence of B7 molecules than in the absence of CD28, supporting the hypothesis that there may be additional stimulatory receptors for B7.

**Materials and Methods**

**Mice**

C57BL/6 WT mice were obtained from Taconic Farms (Germantown, NY), B7.1/B7.2−/−, CD28−/−, and C57BL/6.SJL mice were bred locally from breeder pairs originally obtained from The Jackson Laboratory (Bar Harbor, ME). TCR transgenic mice (TCR318 and SMARTA) expressing a TCR for either LCMV GP 33–41 or GP 61–80 were provided by H. Pircher, A. Oxenius, and R. M. Zinkernagel (University of Zürich, Zürich, Switzerland). For the purpose of identifying the transgenic cells following adoptive transfer, the transgenic TCR has been transferred onto a B6.SJL (B6.SJL-PepRg/Boa3.1, CD45.1; Taconic Farms) background to generate TCR318 mice with a CD45.1−/− genotype. Alternatively, TCR transgenic mice were crossed with B6.SJL to generate an F1 generation, this approach was used for SMARTA mice, with the result being CD4−/− TCR transgenic cells on a mixed B6 × B6.SJL (CD45.2−/−CD45.1−) background. Seven- to 10-wk-old mice were used in all experiments, and animals from outside sources were always allowed to acclimatize to the local environment for at least 1 wk before use. All animals were housed under specific pathogen-free conditions as validated by screening of sentinel mice. All animal experiments were conducted according to national guidelines on animal experiments.

**Viruses**

LCMV of the rapidly invasive Traub strain or the slowly invasive Armstrong strain was used. Mice to be infected received a dose of 200 PFU of LCMV Traub or 104 PFU of LCMV Armstrong in an i.v. injection of 0.3 ml. Virus stocks were produced and stored as previously described (12, 24). For production of adenovirus encoding LCMV nucleoprotein (NP), an NP construct kindly provided by M. B. Oldstone (The Scripps Research Institute, La Jolla, CA) was amplified by single PCR and cloned into a pacCMV-based shuttle vector. From the shuttle plasmid, human type 5 recombinant adenoviral vector was then produced from homologous recombination by standard methods (25). Adenoviral stocks encoding LCMV NP were produced and stored as previously described (26). Mice to be infected were anesthetized and injected with 2 × 107 HEK293 infectious units in the right hind footpad.

**Virus titration**

Virus stocks and organ virus titers were assayed by an immune focus assay as previously described (27).

**Cell preparations, CFSE labeling, and adoptive transfer experiments**

For flow cytometric analysis, spleens from mice were aseptically removed and transferred to HBSS. Single-cell suspensions were obtained by pressing the organs through a fine sterile metal mesh. The cells were subsequently washed twice with HBSS, and cell concentration was adjusted in RPMI 1640 containing 10% FCS, supplemented with 2-MS, l-glutamine, and a penicillin-streptomycin solution.

For adoptive transfer and subsequent evaluation of secondary expansion of donor LCMV-specific CD8+ T cells, a single-cell suspension of the splenocytes of LCMV-infected WT or gene-modified (CD28−/− or B7.1/B7.2−/−) was washed twice with PBS and filtered through a 70-μm nylon cell strainer from BD Biosciences (Two Oak Park, Redford, MI). Cell concentration was adjusted in PBS, and 30 × 106 cells in a volume of 0.4 ml were adoptively transferred into B6.SJL (CD45.1+) recipients.

To determine proliferation of donor TCR transgenic lymphocytes in WT or B7.1/B7.2−/− mice, spleen cells from naive TCR318 transgenic (CD45.1+) or SMARTA (CD45.1+/CD45.2+) F1 mice were labeled with CFSE prior to adoptive transfer, as previously described (28). Briefly, splenocytes that had been washed with PBS were adjusted to 1 × 107 cells/ml and mixed with CFSE to a final concentration of 1 μM. After incubation for 10 min at 37°C, 1/10 volume of FCS was added to stop the reaction, and the cells were subsequently washed with RPMI 1640 plus 10% FCS. Additional washing of CFSE-labeled splenocytes was performed with PBS before cell concentration was finally adjusted in PBS. Then, 15 × 106 (TCR318 transgenic; CD45.1+) or 5 × 106 (SMARTA; CD45.1+/CD45.2+) splenocytes were adoptively transferred into B7.1/B7.2 knockout mice and WT mice (both CD45.2+).

**In vivo cytotoxicity assay**

Splenocytes from naive C57BL/6 and B6.SJL mice were incubated with LCMV-derived peptides (GP33–41, GP276–386, and NP396–404) or irrelevant peptide for control (1 μg/106 cells/ml). Following incubation for 30 min at 37°C, the cells were washed and labeled with CFSE to 2, 0.2, or 0.02 μM. Following another washing step, the labeled cells were mixed, and an equal number of each population (106 cells) was injected i.v. into LCMV-infected recipients, being either WT or gene-modified (CD28−/− or B7.1/B7.2−/−) mice; 2 h later lymphocytes from the recipients’ spleens were isolated. Target cells were distinguished by the expression of CD45.1 and/or the intensity of CFSE staining. The percentage of killing was calculated using the following equation: 100 – [(percentage of LCMV peptide-labeled cells in infected mice/percentage of cells labeled with irrelevant peptide in infected mice)/(percentage of LCMV peptide-labeled cells in uninfected mice/percentage of cells labeled with irrelevant peptide in uninfected mice) × 100].

**mAbs for flow cytometry**

The following mAbs were purchased from BD Pharmingen (San Diego, CA): PE-CyChrome 5 (Cy5), PerCP-Cy5.5-, PE-, or FITC-conjugated anti-CD8a; PE-Cy5-conjugated anti-CD4; FITC-conjugated anti-CD44; PE-
conjugated anti-Ve2; allophycocyanin- and PE-conjugated anti-CD45.1; PerCP-Cy5.5-conjugated anti-CD45.2; PE-conjugated anti-CD127; PE-conjugated anti-CD43; FITC-conjugated anti-CD27; PE-conjugated anti-CD25; allophycocyanin- and FITC-conjugated anti–IFN-γ; PE-conjugated anti–TNF-α; allophycocyanin-conjugated anti-IL-2; and matched isotype controls. Hamster anti-mouse R-phycocerythrin-conjugated KL6R-1 was purchased from SouthernBiotech (Birmingham, AL), and rat anti-mouse PE-conjugated anti-IL-2 was from eBioscience (San Diego, CA). For evaluation of apoptosis, FITC-conjugated annexin V from BioLegend (San Diego, CA) was used.

Flow cytometric analysis
For visualization of proliferated donor lymphocytes (CD4+CD45.1+ or CD8+CD45.1+V2+) or to determine the extent of cell killing in vivo, 1 × 10^6 splenocytes were stained with mAbs in FACS medium (PBS containing 10% rat serum, 1% BSA, and 0.1% NaN_3) for 20 min in the dark and at 4°C. The cells were washed twice and fixed with 1% paraformaldehyde in PBS.

For visualization of virus-specific cytokine-producing (IFN-γ and/or IL-2) CD8+ and CD4+ T cells, 2 × 10^6 splenocytes were resuspended in 0.2 ml of complete RPMI 1640 medium supplemented with 10 U of murine recombinant IL-2 (R&D Systems, Abingdon, U.K.), 3 μM monensin (Sigma-Aldrich, St. Louis, MO), and 1 μg/ml relevant peptide and incubated for 5 h at 37°C. The following peptides were used: GP33–41, GP276–286, and NP396–404. After incubation, cells were surface stained, washed, fixed, permeabilized, and stained with anti-cytokine Ab as described previously (28). Isotype-matched Ab served as control for nonspecific staining, and splenocytes from naive WT mice were used to set the cut-off for marker expression on T cells.

Cells were analyzed using a FACSCalibur (BD Biosciences, San Jose, CA), and at least 5 × 10^4 cells obtained after surface staining, or 10^5 cells after intracellular staining, were gated using a combination of low angle and side scatter to exclude dead cells and debris. Data analysis was conducted using CellQuest software (BD Biosciences).

Detection of GPC-specific IgG by ELISA
The ELISA for analysis of LCMV-GP-specific Abs was performed as previously described (29), with the sole modification that a GPC-Fc fusion construct produced in a eukaryotic system was used. In this construct, amino acids 1–440 (i.e., the GP1 and GP2 extracellular domains) of the LCMV-WE glycoprotein gene were fused to the Fc portion of human IgG1 as described (30). In brief, recombinant GPC-Fc was immobilized on goat anti-human Fc coupled 96-well plates. Serum samples were prediluted at 1:8, and serial 3-fold dilutions were performed. A total of 50 μl per well of the diluted serum samples was transferred to the GPC-coated plates, which were subsequently incubated for 1 h at room temperature. Following washing, the plates were incubated for 1 h at room temperature with HRP-coupled goat anti-mouse IgG Ab (1/1000; Sigma-Aldrich). HRP activity was detected using an ABTS color reaction and was read at 450 nm. The ELISA titer was defined as the lowest serum dilution yielding a value equal to or above twice the background level.

Statistical evaluation
Results were compared using the Mann-Whitney U test. A p < 0.05 was taken as evidence of statistical significance.

Results
Infection with LCMV Traub induces low-grade persisting infection in most B7.1/B7.2−/− mice
Several studies have shown that upon infection with viruses causing systemic infection, primary CD8+ T cell responses can develop even in the absence of costimulatory molecules (12, 13, 31). The generated virus-specific CD8+ T cells in turn eliminate or control the viral infection. Whether the ligands B7.1/B7.2 of the costimulatory receptor CD28 are also dispensable for the induction of the antiviral Ab response

To examine the importance of B7.1/B7.2 costimulatory signals for the appropriate generation of a primary LCMV-specific T cell response, the development of virus-specific cytokine producing (IFN-γ and TNF-α) CD8+ and CD4+ T cells was determined in LCMV Traub-infected B7.1/B7.2−/− and matched WT mice. To this end, splenocytes harvested at different time points p.i. were first in vitro stimulated with MHC class I (GP33–41, GP142–150, and NP396–404) and MHC class II (GP13–26)–restricted LCMV peptides, and they were subsequently stained for cell surface markers CD8/CD4 together with CD44, permeabilized, and stained for intracellular IFN-γ or TNF-α.

First, looking at the LCMV-specific CD8+ T cell response, the analysis revealed that the development of this response was delayed in B7.1/B7.2−/− mice. Thus, at day 6 p.i. the spleens of commences around day 7 postinfection (p.i.), the initially matching virus loads in both groups are rapidly reduced, and by day 28 little or no virus could be detected in the spleen of the mice by standard titration, irrespective of their genotype (Fig. 1). In contrast to the situation in the spleen, the virus persisted in the lungs of B7.1/B7.2−/− mice (Fig. 1), and low virus loads were detected in the knockout mice even 10 mo p.i. The fact that B7.1/B7.2−/− mice had some difficulty in controlling the LCMV Traub infection was also supported by analysis of the kidneys, which revealed a couple of B7.1/B7.2−/− mice with high amounts of the virus in this organ several months p.i. (Fig. 1).

B7.1/B7.2 costimulatory molecules are of minor and major importance for the initial expansion of LCMV-specific CD8+ or CD4+ T cells, respectively, and profoundly influence the antiviral Ab response

For visualization of virus-specific cytokine-producing (IFN-γ and/or IL-2) CD8+ and CD4+ T cells, 2 × 10^6 splenocytes were resuspended in 0.2 ml of complete RPMI 1640 medium supplemented with 10 U of murine recombinant IL-2 (R&D Systems, Abingdon, U.K.), 3 μM monensin (Sigma-Aldrich, St. Louis, MO), and 1 μg/ml relevant peptide and incubated for 5 h at 37°C. The following peptides were used: GP33–41, GP276–286, and NP396–404. After incubation, cells were surface stained, washed, fixed, permeabilized, and stained with anti-cytokine Ab as described previously (28). Isotype-matched Ab served as control for nonspecific staining, and splenocytes from naive WT mice were used to set the cut-off for marker expression on T cells.

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Several studies have shown that upon infection with viruses causing systemic infection, primary CD8+ T cell responses can develop even in the absence of costimulatory molecules (12, 13, 31). The generated virus-specific CD8+ T cells in turn eliminate or control the viral infection. Whether the ligands B7.1/B7.2 of the costimulatory receptor CD28 are also dispensable for the induction of the efficient control of infection with the rapidly invasive Traub strain of LCMV was addressed in the present study. In B7.1/B7.2−/− and matched WT mice an acute type of infection was induced with 200 PFU of LCMV Traub, and the rate of virus clearance from lymphoid (spleen) and nonlymphoid (lungs and kidneys) organs was studied. Analysis of spleen virus loads revealed that with the appearance of virus-specific CD8+ T cells, which...
B7.1/B7.2−/− mice contained ∼5-fold less epitope-specific CD8+ IFN-γ+ T cells compared with matched WT mice (Fig. 2A). At day 8 p.i. the difference in numbers of virus-specific CD8+ T cells between the two strains had diminished, but the knockout mice still only harbored about half as many GP276–286- and NP396–404-specific IFN-γ+CD8+ T cells in their spleens. However, at the peak of the primary CD8+ T cell response (day 10 p.i.), B7.1/B7.2−/− and WT mice contained approximately equal numbers of epitope-specific CD8+IFN-γ+ T cells (Fig. 2A). Furthermore, according to intensity of staining for IFN-γ (measured as mean fluorescence intensity [MFI]) of CD8+IFN-γ+ T cells), cytokine expression at this time point was not affected by the absence of costimulatory signals provided through B7.1/B7.2 molecules (regarding this point, please see Fig. 9A). Similarly, the level of TNF-α in virus-specific B7.1/B7.2−/− CD8+ T cells, which was coexpressed with IFN-γ in ∼70% of virus-specific CD8+IFN-γ+ T cells (results not shown), was not reduced either.

In contrast to the LCMV-specific CD8+ T cell response, the development of virus-specific CD4+ T cells was markedly impaired in LCMV Traub-infected B7.1/B7.2-deficient mice. First, LCMV-specific CD4+IFN-γ+ T cells became detectable 2 d (day 8 p.i.) later in B7.1/B7.2−/− mice compared with WT mice, and, second, maximal numbers were reduced by about a factor of 10 (Fig. 2B). Based on our earlier studies showing that CD28-deficient mice, which lack the stimulatory receptor for B7.1/B7.2, generate significantly less LCMV-specific CD4+ T cells than do WT mice (17), this result was anticipated. However, to determine whether the lack of B7.1/B7.2 ligands induced a more marked phenotype than did the absence of CD28, we included in a repeat experiment CD28-deficient mice for a direct comparison. We enumerated LCMV-specific CD4+IFN-γ+ T cells in the spleen on day 10 p.i. in mice of all three genotypes. Consistent with our preliminary observation, we recovered ∼2-fold less virus-specific CD4+ T cells in B7.1/B7.2−/− mice compared with CD28−/− mice (data not shown), a result that could be interpreted to suggest the existence of an additional stimulatory receptor for B7.1/B7.2 ligands.

Finally, since the above results pointed to a pronounced defect in the generation of virus-specific helper T cells in LCMV Traub-infected B7.1/B7.2−/− mice, we also evaluated the capacity of these mice to mount an Ab response to the viral surface GP, as this could potentially affect the viral load (29). Moreover, as B7.1/B7.2−/− mice appeared to generate even fewer LCMV-specific CD4+ T cells than previously found for CD28−/− mice, we included not only WT mice, but also CD28−/− mice for comparison. As shown in Fig. 3, marginal production of GP-specific Abs could be detected in LCMV Traub-infected B7.1/B7.2−/− mice, whereas significant levels of these Abs were produced in most infected WT mice. Despite the higher CD4+ T cell response in CD28−/− mice compared with B7.1/B7.2−/− mice, no difference in ability to produce LCMV-specific Abs was observed.

**FIGURE 2.** Kinetics of the virus-specific CD8+ and CD4+ T cell response in B7.1/B7.2−/− mice infected with LCMV Traub. C57BL/6 (WT) and B7.1/B7.2−/− mice were infected i.v. with 200 PFU of LCMV Traub. On the indicated days p.i., splenocytes were harvested and stimulated for 5 h with peptides representing known LCMV-derived epitopes (GP33–41, GP276–286, GP23–35, and NP21–30). Following incubation, the cells were surface stained for CD8 or CD4 and CD44, permeabilized, and stained for intracellular IFN-γ. Numbers of epitope-specific IFN-γ+ CD44highCD8+ (A) and CD4+ (B) T cells from WT or B7.1/B7.2−/− mice are presented as averages ± SD (n = 3–5 mice/group). *p < 0.05 relative to WT mice (Mann-Whitney rank-sum test).

**Impaired proliferation of naive CD8+ and CD4+ TCR transgenic cells in B7.1/B7.2−/− mice**

Although the above results were most simply interpreted to indicate the absence of normal T cell proliferation in B7.1/B7.2−/− mice, other possibilities existed. Thus, T cells might be activated in the absence of CD28–B7 interaction but might fail to survive following initial expansion (32). Alternatively, LCMV-specific CD4+ T cells might become activated and undergo expansion but fail to complete functional development; such cells may have been missed in our analysis because detection is entirely based on cytokine production. To investigate these possibilities we performed adoptive transfer of CFSE-labeled splenocytes from TCR transgenic F1 C57BL/6.SJL × SMARTA (CD45.2+/CD45.1+) mice expressing a TCR for LCMV GP31–41 on ∼90% of their CD4+ T cells into WT and B7.1/B7.2−/− recipients (both only CD45.2+). The day after cell transfer, the recipients were infected with LCMV Traub, and the dilution of CFSE on donor CD4+ TCR transgenic cells was followed by flow cytometry from day 3 to day 5 p.i. Additionally, total numbers of donor CD4+ TCR transgenic cells in recipient spleens were determined. Similarly, to follow the proliferation of WT virus-specific CD8+ T cells in the knockout mice, we transferred CFSE-labeled splenocytes from TCR318 mice (expressing a TCR for LCMV GP33–41...
on ∼60% of their CD8+ T cells) on a B6.SJL background (CD45.1+). As expected, assuming that B7 costimulation is essential to induce normal division of Ag-stimulated T cells, the expansion of donor-derived LCMV-specific CD4+ and CD8+ T cells was significantly impaired in B7.1/B7.2−/− recipients compared with WT recipients (Fig. 4A). It is also evident that impaired expansion correlated with a significantly reduced rate of cell division, as indicated by a less pronounced dilution of CFSE staining on donor cells stimulated in the absence of B7 molecules (Fig. 4B).

Given that the impairment of T cell expansion in B7.1/B7.2−/− mice was most pronounced for CD4+ T cells, we looked further at the phenotype of the CD4+ T cells present in either type of recipients. To determine whether increased apoptosis contributed significantly to the reduced CD4+ T cell expansion in B7.1/B7.2−/− recipients, we evaluated the binding of annexin V to donor CD4+ T cells present in infected recipients of either genotype. Neither on day 4 nor on day 5 p.i. did we observe an increased binding of annexin V to donor CD4+ T cells in B7.1/B7.2−/− recipients compared with WT recipients (Fig. 4C).

FIGURE 3. Kinetics of production of GP-specific Abs in CD28- and B7.1/B7.2-deficient mice infected with LCMV Traub. C57BL/6 (WT) and gene-modified (CD28−/− and B7.1/B7.2−/−) mice were infected with 200 PFU of LCMV Traub i.v. At the indicated time points, blood samples were collected and resulting sera were evaluated for binding in a GP-specific ELISA. A, Ab titer as a function of time p.i.; each point represents the median titer of groups of seven to eight mice. B, Titration curves of individual sera harvested on day 28 p.i. Each line represents one mouse. Serum from a naive mouse was included for control.

FIGURE 4. Costimulatory molecules B7.1/B7.2 are pivotal for normal clonal expansion of LCMV-specific T lymphocytes. Splenocytes from CD4+ or CD8+ TCR transgenic mice (CD45.1+/CD45.2+ or CD45.1−, respectively) were labeled with CFSE and adoptively transferred into groups of WT and B7.1/B7.2−/− recipients (both CD45.2+). On day 0, the mice were infected with 200 PFU of LCMV Traub, and on the indicated days p.i. splenocytes were harvested and the donor cells were enumerated. A, Numbers of donor lymphocytes were determined by gating for CD4 and CD45.1 (donor CD4+ T cells) or CD8 and CD45.1 (donor CD8+ T cells). B and C, Histograms show the dilution of CFSE on donor TCR transgenic lymphocytes, as determined on day 4 p.i. Numbers above the lines refer to the MFI of staining for CFSE, as expressed by the expanded donor populations of CD8+/CFSE+ T cells (averages ± SD; n = 3 mice/group). C, Splenocytes from CD4+ TCR transgenic mice (CD45.1−/CD45.2+) were adoptively transferred into groups of WT and B7.1/B7.2−/− recipients (both CD45.2+) on day −1. On day 0, the mice were infected with 200 PFU of LCMV Traub, and on days 4 and 5 p.i. splenocytes were harvested. Histograms depict binding of annexin V (top panel) and expression of CD25 (bottom panel) on donor CD4+ T cells gated using CD4 and CD45.1; results are representative of three to four mice per group. Histograms have been normalized to the same area under the curve.
annexin V to CD4+ T cells in B7.1/B7.2−/− compared with WT recipients (Fig. 4C, top panel). In fact, the intensity of annexin V staining of the donor CD4+ T cells was lower in the former recipients, which may reflect the reduced state of activation of these cells (cf. the similarity with the staining of resting CD4+ T cells in uninfected mice). Consistent with this interpretation, we found reduced expression of CD25 on the donor CD4+ T cells in B7.1/B7.2−/− recipients at day 4 p.i. when expression of this activation marker was maximal on donor CD4+ T cells activated in infected WT recipients (Fig. 4C, bottom panel). Taken together, these results strongly indicate that the dominant reason for the markedly impaired expansion of CD4+ T cells recognizing their Ag in the absence of B7 costimulation is a failure of these cells to effectively initiate cell division.

Depending on the context of Ag presentation, B7.1/B7.2 costimulation may be absolutely crucial for the development of an antiviral CD8+ T cell response

Based on studies of CD28−/− mice, it has been claimed that the importance of costimulation may vary with the pathogen, and that viruses with a wide tropism and a systemic spread are less dependent on CD28−/B7 interaction for induction of an efficient T cell response (16). To investigate if this conclusion would also hold in B7.1/B7.2−/− mice, we infected WT and B7.1/B7.2−/− mice with a replication-deficient adenoviral vector expressing the NP of LCMV. The mice were infected in a footpad, and at the peak of the immune response in WT mice (day 11 p.i.) splenic NP-specific (directed toward the NP396–404 peptide) IFN-γ+TNF-α+CD8+ T cells were determined (Fig. 5). As expected, WT mice responded well to this infection, whereas B7.1/B7.2−/− mice contained hardly any epitope-specific cytokine+CD8+ T cells. Considering that systemic LCMV infection of B7.1/B7.2−/− mice resulted in a marked NP396–404-specific CD8 T cell response, these results support the dogma that CD28−B7 interaction for induction of an efficient T cell response, these results support the dogma that CD28–B7 interaction is more important following infection with viruses characterized by localized infection.

Virus-specific CD8+ T cells in LCMV Traub-infected B7.1/ B7.2−/− mice undergo marked changes in the epitope hierarchy as a function of time

Further analysis of the antiviral CD8+ T cell response in chronically infected B7.1/B7.2−/− mice revealed distinct changes in the epitope hierarchy over time, a phenomenon previously observed in the context of persistent LCMV infection, in which case both exhaustion/deletion and expansion of the involved T cell populations may be observed (23, 33). Thus, probably due to prolonged antigenic stimulation in B7.1/B7.2−/− mice, the populations of CD8+ T cells directed toward the immunodominant epitopes GP33–41 and NP396–404 were significantly reduced at 6 mo p.i. compared with the parallel populations in WT mice (Fig. 6). A similar situation was observed for NP396–404-specific CD8+ T cells at 8 mo p.i., whereas the GP33–41-specific response seems to have rebounded to its normal level at this time point despite continued virus persistence. By 10 mo p.i. both CD8+ T cell populations directed toward the immunodominant epitopes matched in size the same populations in WT mice. In contrast, numbers of CD8+ T cells directed against the subdominant epitopes GP276–286 and GP118–125 were markedly increased in B7.1/B7.2−/− mice at this time point compared with the prevalence of cells with the same specificity in WT mice. Thus, in B7.1/B7.2−/− mice, which have difficulty in controlling infection with LCMV Traub virus, dynamic changes in the epitope hierarchies were observed over time. In contrast and in accordance with our previous observations (17), CD28−/− mice matched WT mice quite closely, both with regard to numbers of virus-specific CD8+IFN-γ+ T cells (Fig. 6) and in being able to control the viral infection (results not shown).

Memory CD8+ T cells from LCMV Traub-infected B7.1/B7.2−/− mice are phenotypically shifted toward a more terminally differentiated cell type

To further characterize the CD8 T cells present in B7.1/B7.2−/− mice, we analyzed splenic virus-specific CD8+ T cells for the expression of several cell surface markers (CD127 [IL-7R], CD27, KLRG-1, and CD43) known to be relevant in the characterization of memory cells. “Good” memory cells thus consist of CD8+ T cells that have high expression of CD127 and CD27 (markers of long-term survival) and low expression of KLRG-1 (an inhibitory receptor and a marker of replicative senescence) and CD43 (a molecule involved in cell adhesion and apoptosis and a marker of recent activation) (34).

As expected based on the evidence for ongoing Ag stimulation in B7.1/B7.2−/− mice, our characterization of epitope-specific IFN-γ+CD8+ T cells at 8 and 10 mo p.i. revealed that about half of the virus-specific CD8+ T cells in these mice expressed the hallmarks of a terminally differentiated cell type (Fig. 7, with representative plots depicted in Fig. 8). Thus, only ~40–60% of epitope-specific CD8+IFN-γ+ T cells in B7.1/B7.2−/− mice were CD127high, CD27high, or KLRG-1low, whereas these cell types dominated the LCMV-specific response in WT mice. Interestingly, when examining the expression of CD43, most LCMV-specific CD8+ T cells in B7.1/B7.2−/− mice did not differ in phenotype from the same cells in WT mice, except in the case of CD8+ T cells directed toward the subdominant GP276–286 epitope, ~20% of which showed an effector-like phenotype (i.e., CD43high). This observation is interesting and is in accord with a failure to see increased BrdU incorporation of LCMV-specific CD8+ T cells in B7.1/B7.2−/− mice compared with WT mice (data not shown). The phenotypic differences we observed are probably the result of a long-standing low-grade effect on the immune system that is too subtle to be detected in assays more aimed at providing a snapshot of recent events. Also, a special susceptibility of cells primed in the absence of costimulation to develop a terminally differentiated phenotype may play a role.

Notably, analysis of LCMV Traub-infected CD28−/− mice infected 10 mo, but not 8 mo, earlier also revealed the presence of...
of an increased subset (relative to WT mice) of LCMV-specific memory CD8+ T cells with the phenotype of terminally differentiated cells despite the lack of readily detectable virus in their organs; however, these cells made up a substantially smaller proportion of the virus-specific CD8+ T cells (∼10–40%) in comparison with the situation in matched B7.1/B7.2−/− mice, and the difference to WT mice was not statistically significant for all markers.

Memory CD8+ T cells from LCMV Traub-infected B7.1/B7.2−/− mice are impaired in their capacity to respond to Ag stimulation

In addition to surface phenotyping, a pertinent indicator of a more terminally differentiated/exhausted CD8+ T cell state is an altered capacity for cytokine synthesis. With increased terminal differentiation the cells are subject to a hierarchical loss of function from subtle dysfunction to actual deletion (23, 33). Thus, initially the cells lose the capacity for production of IL-2, and gradually reduced synthesis of IFN-γ follows; in the final stage, complete deletion may be observed. Although CD8+ T cells from B7.1/B7.2−/− mice initially possess the same capacity for peptide-induced synthesis of IFN-γ as do their WT counterparts, based on evaluation of the MFI of virus-specific CD8+ IFN-γ+ T cells harvested on day 10 p.i. and up to ∼2–3 mo p.i., significantly reduced staining for IFN-γ was observed with cells harvested 4 mo p.i.; a representative analysis carried out at 10 d and 10 mo p.i. is presented in Fig. 9A. Moreover, analysis for the ability of virus-specific CD8+IFN-γ+ T cells to coproduce IL-2 revealed that no IL-2-producing cells could be demonstrated in B7.1/B7.2−/− mice infected 8 mo earlier (Fig. 9B); this is in contrast to the situation in WT mice in which a distinct IL-2-producing population can clearly be demonstrated. CD8+ T cells from CD28−/− mice also showed reduced expression of IFN-γ, but to a lesser extent than in the case of B7.1/B7.2−/− mice, and, importantly, in CD28−/− mice a small population of IL-2-expressing CD8+ T cells could be detected, representing another observation that sets CD28−/− mice apart from B7.1/B7.2−/− mice.
In addition to evaluating Ag-induced cytokine expression, we also studied the capacity of the virus-specific CD8+ T cells to immediately kill Ag-expressing cells in vivo. Earlier studies have indicated that cytotoxicity is the last effector function to disappear from terminally differentiated/exhausted CD8+ T cells during chronic infection (35). Thus, LCMV peptide-labeled target cells were injected into WT and matched knockout mice, which had been infected i.v. with 200 FFU of LCMV Traub 6 mo earlier, and 2 h later the survival of the injected cells in the recipient spleen was determined. This analysis disclosed similar killing of GP33-41-labeled targets in all three mouse strains, whereas the killing of NP396-404-labeled targets was markedly reduced, and the killing of GP276-286-labeled targets almost doubled in B7.1/B7.2−/− mice compared with WT or CD28−/− mice (Fig. 9C). These results nicely correlate...
CD8+ T cells, whereas the expansion of GP 33–41-specific CD8+ T cells (Fig. 10). In splenocytes were stimulated and stained as indicated in Fig. 8. Gated CD8+ T cells were infected i.v. with 200 PFU of LCMV Traub, and 10 mo later their numbers were determined. We detected convincing expansion of WT memory CD8+ T cells, result- ing in the generation of a population of secondary effectors with a predominance of NP 396–404-specific CD8+ T cells (Fig. 10). In contrast to WT cells, memory cells from B7.1/B7.2–/– cells did not undergo detectable expansion, and memory cells from CD28–/– mice revealed a 10-fold reduced expansion of NP 396–404-specific CD8+ T cells, whereas the expansion of GP33–41-specific CD8+ T cells could not be clearly differentiated from that of WT cells with the same specificity.

In the case of memory B7.1/B7.2–/– cells, the results fit well with our phenotypic and functional findings presented above, which indicate that the virus-specific CD8+ T cells in B7.1/B7.2–/– mice to a large extent represent exhausted (reduced expression of cyto- kines), terminally differentiated, and senescent cells. However, the markedly reduced response of memory cells from CD28–/– mice could not be explained in this manner and required further analysis of the underlying mechanism. Because CD28 is expressed on the T cell surface, no CD28–B7 costimulation would be avail- able during the recall response of CD28–/– cells, even in a WT environment. In this case, therefore, but not in the case of cells from B7.1/B7.2–/– mice, the response following adoptive transfer into normal mice would reflect not only the functionality of the cells prior to restimulation, but it also includes a test for the role of costimulation during the recall response. Consequently, the re- duced response of CD28–/– T cells might therefore primarily reflect absence of costimulation during the recall response itself. To test this possibility, we performed another adoptive transfer exper- iment in which LCMV-primed T cells from WT mice were trans- ferred into either naive WT recipients or naive B7.1/B7.2–/– mice. By using this approach, we found that the recall response was reduced if CD28–B7 interaction was prevented during the recall (Fig. 11), but only about half as much as if the donor cells were deficient in expression of CD28 (3.6- vs 7.3-fold). Thus, while absence of costimulation during restimulation alone clearly reduced the recall response, the reduction with primed cells from CD28–/– mice was more pronounced, suggesting that CD28–B7 costimulation is important also during priming and/or maintenance of the memory cells.

Notably, all of the above results were obtained studying cells from LCMV Traub-infected mice, and given the ability of this virus strain to persist at a low level even in WT mice, it could not be excluded that all of the above results primarily reflected the require- ments for costimulation in the context of chronic Ag stimulation. Therefore, to address this question we repeated selected experiments using mice infected with LCMV Armstrong instead of LCMV Traub.

Similar to the situation in LCMV Traub-infected mice, we found virtually no impairment of the acute primary CD8+ T cell response in LCMV Armstrong-infected B7.1/B7.2–/– mice as compared with WT mice (Fig. 12), whereas the primary CD4+ T cell re- sponse was almost completely missing in the former mice (data not shown).

Regarding the analysis of virus-specific CD8+ T cells present during the memory phase of the Armstrong infection (6–8 mo p.i.), we found that the CD8+ T cell response against all epitopes tended to be increased in B7.1/B7.2–/– mice (Fig. 12A and data not shown). It is possible that this increase reflects the absence of a negative feedback signal via CTLA-4 during the acute response in B7.1/B7.2–/– mice since a similar trend was observed already on day 8 p.i. (cf. Fig. 12A). More importantly, similar to the virus-specific CD8+ T cells from Traub-infected B7.1/B7.2–/– mice, we found evidence for a phenotypic shift toward a more terminally differentiated cell type in these mice as compared with matched WT mice (Fig. 13B, 13C), and the capacity of their cells for IFN-γ synthesis was also significantly reduced (Fig. 13D). However, particularly with regard to KLRG-1, the changes tended to be less pronounced in Armstrong-infected mice compared with Traub-infected animals.

Finally, we evaluated the ability of cells from Armstrong- infected mice to raise a recall response following adoptive transfer to naive WT recipients. As previously observed following infection with LCMV Traub, the recall response of Armstrong-primed cells from B7.1/B7.2–/– mice was significantly reduced compared with that of matched WT cells (Fig. 14.). Notably, this difference in the capacity for secondary CD8+ T cell expansion was observed de- spite the fact that we could not detect infectious virus at late time points in the organs of any Armstrong-infected animals irrespec- tive of their genotype (data not shown). Thus, virus persistence was not required to induce the observed difference in the
functionality of virus-specific CD8 T cells from B7.1/B7.2−/− mice versus WT mice.

Discussion
In the present report we clearly show that LCMV is not able to induce a normally sustained CD8+ T cell immune response in the absence of B7.1/B7.2, and that most of the sustained CD8+ T cells behave like terminally differentiated cells with reduced functionality and a limited capacity for supporting a recall response. This was highly surprising given that earlier studies have indicated that CD8+ T cell memory is unimpaired in CD28-deficient mice infected with similar doses of LCMV (12, 13, 17). However, our subsequent, direct comparison of CD28- and B7.1/B7.2-deficient mice confirmed that the difference was real. Consequently, these results indicate that the absence of B7.1/B7.2 has a more profound impact on the LCMV-specific immune response than does the lack of CD28, and the results may therefore suggest that there are additional stimulatory receptors for B7.1/B7.2 besides CD28. A few other studies have pointed toward the same conclusion (19–21), but so far no such receptor has been unequivocally identified.

Initially, the role of B7.1/B7.2 was studied in mice infected with the LCMV Traub strain. Under these experimental conditions we observed that virus was not completely cleared in most of the infected mice, and this was associated with a perturbed epitope hierarchy. For an epitope such as NP 396–404, most of the functional CD8+ T cells transiently disappeared, whereas for other epitopes virus persistence led to increased numbers of cells with the phenotype of terminally differentiated cells (e.g., KLRG-1 high) and reduced functionality. CD8+ T cells directed toward the GP 33–41 epitope seem to present an intermediate pattern, in that this subset went...
through a very short period with low numbers of functional cells followed by rapid recovery to the same level as in WT mice while maintaining a dysfunctional state as determined by surface phenotype and reduced responsiveness to Ag stimulation. What explains these differences in the behavior of T cells directed toward different epitopes is not clear, but the pattern with more pronounced depression of the response to NP 396–404, as well as a compensatory increase in numbers of CD8+ T cells directed toward the subdominant epitopes, closely recapitulates the pattern observed in other situations where chronic LCMV infection is the result (24, 36). Perhaps the average avidity of the different T cell subpopulations for their cognate epitopes together with the abundance of epitope/MHC complexes expressed on infected cells during chronic infection determine the severity of Ag-induced perturbation (i.e., expansion/dysfunction/anergy/deletion).

Although the above results immediately point to the importance of optimal costimulation as a prerequisite to stable immune surveillance during chronic viral infection, they also raise questions...
regarding the relevance of chronic Ag stimulation in driving this outcome. To resolve this issue we also studied B7.1/B7.2-deficient mice infected with LCMV Armstrong. In these mice we never detected persistent virus infection, and the epitope hierarchy remained unaffected by the absence of costimulation. However, phenotypic and functional analysis revealed that a substantial proportion of the virus-specific CD8+ T cells reached a similar dysfunctional state as found in LCMV Traub-infected knockout mice. Taken together, these observations indicate that although the changes in the epitope hierarchy may simply be a reflection of prolonged Ag stimulation, the reduced functionality is only in part driven by Ag persistence and clearly contains an important element directly related to the absence of costimulation. This interpretation is supported by our findings in LCMV Traub-infected CD28−/− mice, in which the epitope hierarchy is similar to that in WT mice, but phenotypic analysis also points to the presence of more terminally differentiated cells late p.i. Importantly, as with Armstrong-infected B7.1/B7.2−/− mice, we have never seen persistent infection in LCMV Traub-infected CD28−/− mice when these mice were infected with the virus doses used in this study (17).

The evaluation of recall responses in the adoptive setting strongly suggests that although costimulation is essential for secondary expansion of the CD8+ T cells even for optimally primed WT cells, costimulation is also required for initial imprinting of future memory cells during priming and/or to sustain the cells even in the absence of Ag, as demonstrated by the reduced secondary expansion of CD8+ T cells from LCMV Armstrong-infected B7.1/B7.2−/− donors in a WT environment. Note, however, that chronic Ag stimulation (as in Traub-infected B7.1/B7.2-deficient mice) may even further erode the memory potential of T cells provided with no costimulation during neither the primary response nor the maintenance phase. Also note that delivery of normal costimulation during the recall response does not suffice to circumvent the requirement for costimulation at earlier time points.

The observation that costimulation is required during priming strongly suggests that although costimulation is essential for secondary expansion of the CD8+ T cells even for optimally primed WT cells, costimulation is also required for initial imprinting of future memory cells during priming and/or to sustain the cells even in the absence of Ag, as demonstrated by the reduced secondary expansion of CD8+ T cells from LCMV Armstrong-infected B7.1/B7.2−/− donors in a WT environment. Note, however, that chronic Ag stimulation (as in Traub-infected B7.1/B7.2-deficient mice) may even further erode the memory potential of T cells provided with no costimulation during neither the primary response nor the maintenance phase. Also note that delivery of normal costimulation during the recall response does not suffice to circumvent the requirement for costimulation at earlier time points.

The observation that costimulation is required during priming as well as during recall (also explaining the surprisingly impaired secondary expansion of CD28−/− CD8+ T cells despite the absence of persistent infection) is consistent with what has recently been reported based on studies of infections with vaccinia and MHV68.
However, considering that LCMV has always been set apart as a virus that requires little or no costimulation, the present results are important in indicating that costimulation is essential at both stages of infection even when this virus is involved; that is, there seems to be no exceptions to this rule. The importance of Ag persistence in influencing the requirement for costimulation during the maintenance phase is clearly new. In previous studies different viruses have been used to induce acute (vaccinia) and chronic (MHV68) viral infections (18), which makes a comparison difficult if not invalid. In the present study we have used variants of the same virus to induce both courses of infection, and our results strongly suggest that the capacity for raising a recall response is more impaired following persistence of the infection. For example, looking at the capacity for recall, no secondary expansion could be detected with CD8+ T cells from Traub-infected mice, whereas primed cells from Armstrong-infected mice did undergo secondary expansion, albeit less efficiently (~2- to 3-fold) than did matched cells from WT mice.

One issue that was not directly addressed in this study is to what extent the results regarding CD8+ T cell phenotype and functionality reflect a requirement for direct costimulation of the CD8+ T cells themselves versus the role played by the reduced priming of CD4+ T cells, which is also observed in these mice. Analysis of the CD8+ T cell recall, which following infection with LCMV is independent of primed CD4+ T cells (22), as well as our phenotypic analysis clearly demonstrate that the CD8+ T cells in B7.1/B7.2-deficient mice suffer from intrinsic deficiencies; however, to which extent these derive from absence of CD4+ T cell help during CD8+ T cell priming and/or maintenance is not clear. Indeed, this question is not easily resolved, because the normal approach of making mixed bone marrow chimeras where CD28-deficient and -proficient T cells are stimulated and maintained in the same environment may not work, because the phenotype is much less clearly expressed when B7.1/B7.2 is lacking than when the receptor is absent. However, note that the transfer of memory cells from B7.1/B7.2-/- mice to WT recipients for recall does not compensate for the lack of costimulation during priming/maintenance. This appears to be different from the situation regarding helpless CD8+ T cells (37), suggesting that the deficiencies of CD8+ T cells in B7.1/B7.2-/- mice does not merely reflect the absence of CD4+ T cell help. Regarding the impact of the CD4+ T cell deficiency in B7.1/B7.2-/- mice, the associated impairment of Ab production may also play role in the erosion of CD8+ T cell functionality observed in mice with persistent infection. Thus, there are some indications from other studies that lack of antiviral Abs may contribute to a higher viral burden in chronically infected mice (22, 29, 38), which in turn could enhance an intrinsic propensity of CD8+ T cells lacking costimulation to become exhausted. However, in view of the fact that the ability to produce Abs toward the surface GP seems to be equally reduced in Traub-infected B7.1/B7.2-/- and CD8+ CD28-/- mice, we do not consider it very likely that reduced CD4 T cell help to B cells is a major factor in the erosion of CD8 T cell functionality, which is much more severe in B7.1/B7.2-/- mice than in CD28-/- mice.

In summary, unlike what has been concluded earlier based on analysis of CD28-deficient mice (12, 13, 17), our findings in B7.1/B7.2-deficient mice indicate that LCMV similar to other viruses causing localized infection or possessing a lesser capacity for activating the innate immune system also requires costimulation through CD28 and possible other receptors with similar ligands for the induction and maintenance of a normal virus-specific CD8+ T cell response. Without costimulation a high proportion of the generated CD8 T cells eventually acquire the phenotype of terminally differentiated cells and become compromised in their capacity to respond to Ag stimulation. However, virus dose and strain affect the precise requirements for costimulation as well as the long-term consequences. Thus, following infection with a virus that causes chronic infection, further functional erosion may take place compared with that solely ascribed to the absence of costimulation. This virus isolate dependency as well as the fact that the CD8+ T cells directed toward different epitopes may differ in some aspects of their behavior during what appear to be identical situations serve to underscore how careful one has to be when trying to extrapolate from simple animal experiments to outbred humans in a varied environment.

Disclosures
The authors have no financial conflicts of interest.

References