The Journal of Immunology

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J Immunol 2004; 172:2834-2844; ;
doi: 10.4049/jimmunol.172.5.2834
http://www.jimmunol.org/content/172/5/2834

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

CD4 T Cell-Dependent CD8 T Cell Maturation[1]

Aaruni Khanolkar, Michael J. Fuller, and Allan J. Zajac[2]

We have investigated the contribution of CD4 T cells to the optimal priming of functionally robust memory CD8 T cell subsets. Intranasal infection of CD4 T cell-deficient ($CD4^{-/-}$) mice with lymphocytic choriomeningitis virus resulted in the elaboration of virus-specific CD8 T cell responses that cleared the infection. However, by comparison with normal mice, the virus-specific CD8 T cells in $CD4^{-/-}$ mice were quantitatively and qualitatively different. In normal mice, lymphocytic choriomeningitis virus-specific memory CD8 T cells are $CD44^{high}$, many are $CD122^{high}$, and a majority of these cells regain expression of CD62L overtime. These cells produce IFN-γ and TNF-α, and a subset also produces IL-2. In the absence of CD4 T cell help, a distinct subset of memory CD8 T cells develops that remains $CD62L^{low}$ up to 1 year after infection and exhibits a $CD44^{int}CD122^{low}$ phenotype. These cells are qualitatively different from their counterparts in normal hosts, as their capacity to produce TNF-α and IL-2 is diminished. In addition, although CD4-independent CD8 T cells can contain the infection following secondary viral challenge, their ability to expand is impaired. These findings suggest that CD4 T cell responses not only contribute to the optimal priming of CD8 T cells in chronically infected hosts, but are also critical for the phenotypic and functional maturation of CD8 T cell responses to Ags that are more rapidly cleared. Moreover, these data imply that the development of $CD62L^{high}$ central memory CD8 T cells is arrested in the absence of CD4 T cell help. *The Journal of Immunology,* **2004, 172: 2834–2844.**

CD8 T cells contribute to the clearance of many viral and bacterial infections as well as the control of the outgrowth of tumorigenic cells (1, 2). Naive CD8 T cells are maintained in the periphery in a relatively quiescent state, but respond vigorously as they encounter presented Ags. These activation events trigger the proliferation and differentiation of the responding T cells, resulting in the acquisition of key functional properties, including the capacity to produce effector cytokines and kill Ag-sensitized target cells (3–6). Although recognition of cognate Ag is critical for induction of primary CD8 T cell responses, other parameters, such as the cell type presenting the Ag, the engagement of costimulatory molecules, and cooperation with other cell types, including CD4 T cells, influence this process.

In recent years, the application of MHC class I tetramer technology as well as the use of single cell assays for cytokine production have enabled the surface phenotype and functions of Ag-specific CD8 T cells to be tracked during the course of various primary immune responses (7, 8). Typically, multiepitope-specific responses are elicited by infectious pathogens, and the T cells that constitute this initial response differ in their anatomical location, effector capabilities, and fate (5, 9–12). The elaboration and function of this overall pool of Ag-specific T cells is critical for the removal of virus-infected cells. This concept is well illustrated by chronic or protracted infections during which the failure to develop or sustain appropriate CD8 T cell responses is associated with impaired viral control (13).

Ideally, primary CD8 T cell responses result in the development of stably maintained populations of memory cells. The cardinal trait of these cells is their capacity to mount accelerated recall responses that rapidly purge the host of infected cells during secondary exposures to pathogens. Memory CD8 T cells are, however, not uniform, but instead are comprised of heterogeneous subsets that can be categorized based on surface marker expression, effector potential, clonal abundance, epitope specificity, and compartmentalization (9, 10, 14, 15). The factors that drive the maturation of Ag-specific CD8 T cells into functionally competent memory cells, as well as which components of the diverse memory pool most effectively confer protective immunity, remain ill defined.

CD8 T cell responses differ in their dependency on CD4 T cell help, as primary responses to certain Ags have been shown to require CD4 T cells, whereas the induction of responses during the acute phase of many viral infections appears CD4 T cell independent. Even though antiviral CD8 T cell responses may be induced in the absence of functional CD4 T cell help, several reports have documented that subsequent secondary immune responses are compromised (16–21). In addition, chronic viral infections are often associated with weak CD4 T cell responses, and under these conditions virus-specific CD8 T cells lose effector activities and succumb to deletion (13, 22, 23). Less is known, however, regarding the role of CD4 T cell responses in orchestrating the transition of primary CD8 T cells into robust memory cells following infections that are rapidly resolved.

Because the goal of vaccination strategies is to elicit effective, long-term immunity, it is necessary to define the parameters that regulate the development, maintenance, and functional quality of memory CD8 T cell responses. In this study, we have addressed how CD8 T cells that are induced in the absence of CD4 T cell help differ from their counterparts that develop in a normal, immunocompetent environment. Our findings show that marked primary CD8 T cell responses can be induced in CD4-deficient ($CD4^{-/-}$) hosts; however, the Ag-specific CD8 T cells that develop under these conditions are functionally aberrant and phenotypically distinct. The extent of this atypical maturation differs depending upon the epitope specificity of the responding cells.

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Received for publication September 30, 2003. Accepted for publication December 30, 2003.

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[1] This work was supported by National Institutes of Health Grant AI49360-03 (to A.J.Z.). M.J.F. was supported in part by Training Grant AI07150.

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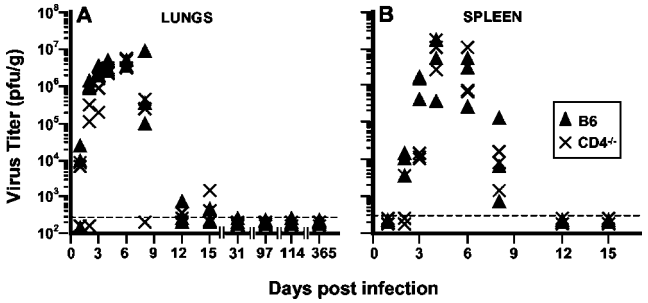

FIGURE 1. Viral clearance is not dependent on CD4 T cell help. Viral titers were determined in the lungs (*A*) and spleens (*B*) at various time points following i.n. infection of B6 (▲) and CD4$^{-/-}$ (×) mice with LCMV-Armstrong. At each time point, three to four mice from each experimental group were analyzed, and results of individual mice are shown. The limit of detection is indicated by the dashed lines.

Nevertheless, the overall population of CD8 T cells that develops following an acute viral infection in the absence of CD4 T cell help is able to confer protection against viral re-exposure.

Materials and Methods

Mice and virus

C57BL/6J (B6) and C57BL/6-Cd4^{tm1Mak} (CD4$^{-/-}$) mice (24), both *H-2^b*, were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were bred and maintained in accredited facilities at the University of Alabama. Male and female mice between 5 and 8 wk of age were used. The Armstrong and clone 13 strains of lymphocytic choriomeningitis virus (LCMV)[3] were kindly provided by R. Ahmed (Emory University, Atlanta, GA). Plaque-purified viral isolates were propagated in BHK-21 cells. For intranasal (i.n.) infections, mice were anesthetized with avertin (2,2,2-tribromoethanol; Aldrich, Milwaukee, WI) and administered 2×10^5 PFU of LCMV-Armstrong in a volume of 20 μl. For rechallenge studies, previously infected mice were injected i.v. with 2×10^6 PFU of the virulent LCMV isolate clone 13. Titers of viral stocks, serum samples, and tissue homogenates were determined by plaque assay using Vero cell monolayers (25).

Cell preparation

Spleens, livers, and lungs were explanted from mice following perfusion with 10 ml of PBS. Single cell suspensions of splenocytes were prepared, as previously described (12). Briefly, spleens were disrupted using wire mesh screens, and erythrocytes were removed by treatment with 0.83% (w/v) NH_4Cl. After washing, cells were finally resuspended in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Livers were disrupted into single cell suspensions, as previously described (26). Tissue debris was removed, and viable lymphocytes were collected by centrifugation over a layer of Histopaque-1083 (Sigma-Aldrich, St. Louis, MO). Lungs were disrupted into single cell suspensions by passage through a 40 μm nylon mesh cell strainer (BD Falcon, Bedford, MA) or wire mesh screens and pooled for each group of mice (27). Pulmonary lymphocytes were then enriched as described above for the livers. Following two washes in supplemented RPMI 1640 containing 1% FCS, cells were finally resuspended as described for the spleens.

Synthetic peptides

The following chemically synthesized peptide epitopes were used to prepare MHC class I tetramers and also to stimulate cytokine production in vitro (28): FQPQNGAFI (nucleoprotein (np)396–404), SGVENPGGYCL (gp276–286), YTVKYPNI (np205–212), KAVYNFATM (gp33–41), and AVYNFATM (gp34–41). Note that to prevent the formation of cystine dimers and improve peptide binding, the naturally occurring C residue at position 41 of the viral glycoprotein is substituted with M. All peptides were obtained from B. Evavold (Emory University).

[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; CD44^{int}, CD44 intermediate; CTO, CellTracker Orange; i.n., intranasal; np, nucleoprotein; Tcm, central memory T cell.

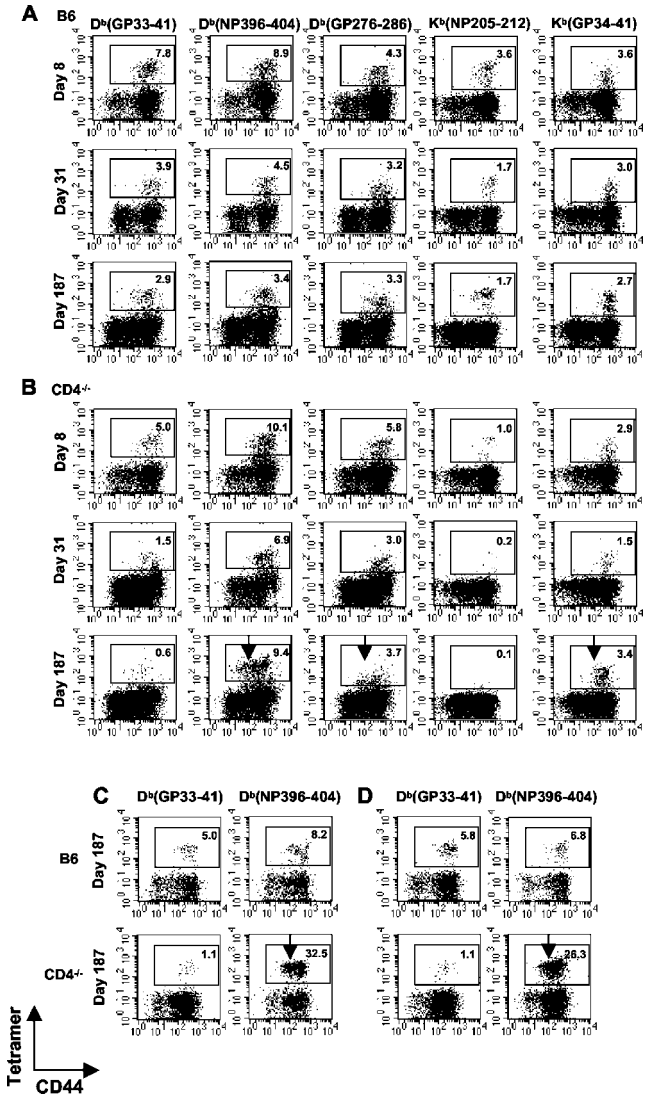

FIGURE 2. Emergence of CD44^{int}CD8 T cells following acute LCMV infection of CD4$^{-/-}$ mice. Virus-specific CD8 T cells were visualized in the spleens (*A* and *B*), livers (*C*), and lungs (*D*) of B6 and CD4$^{-/-}$ mice, as indicated. At the various time points following infection, cells were costained with MHC class I tetramers complexed with the indicated LCMV peptide epitopes together with anti-CD8 and anti-CD44 mAbs. All plots show gated CD8 T cells, and the values given represent the percentages of CD8 T cells that stain positively with the specified tetramers. The arrows highlight CD44^{int}CD8 T cells in the particular panels in which the phenotype is evident. For the spleens and the livers, representative data from two to eight mice analyzed at each time point are shown. The lung data depict gated CD8 T cells from pooled samples taken from two mice in each group.

Preparation of MHC class I tetramers

MHC class I tetramers were prepared, as previously described (12). Briefly, β_2-microglobulin as well as recombinant H-2D^b and H-2K^b H chains fused to a BirA substrate peptide were produced in *Escherichia coli* BL21 (DE3). Monomeric MHC-peptide complexes were refolded with defined peptide epitopes and subsequently enzymatically biotinylated using BirA. Tetramers were formed by the stepwise addition of allophycocyanin-conjugated streptavidin (Molecular Probes, Eugene, OR).

MHC class I tetramer staining

Suspensions of spleen, liver, and lung cells were pretreated with anti-CD16/CD32 mAb (clone 2.4G2). Costaining was then performed using anti-CD44 FITC (clone IM7) and anti-CD122 PE (clone TM-β1) or anti-CD62L FITC (clone MEL-14) mAbs (BD PharMingen, San Diego, CA) together with allophycocyanin-conjugated MHC class I tetramers and anti-

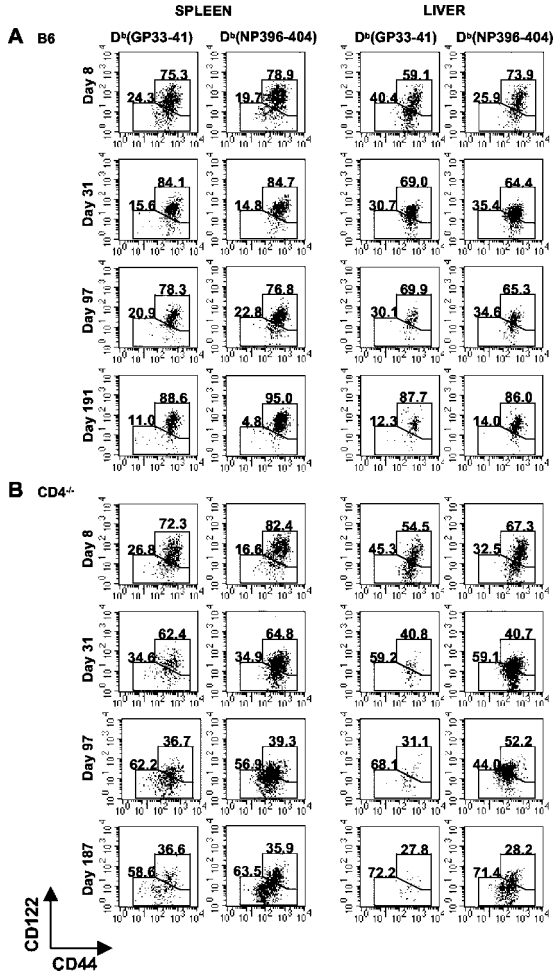

FIGURE 3. CD44^{int} CD8 T cells express low levels of CD122. At various time points following infection, the expression of CD44 and CD122 on MHC class I tetramer$^+$ D^b(gp33–41)- and D^b(np396–404)-specific CD8 T cells was examined in the spleens and livers of B6 (*A*) and CD4$^{-/-}$ (*B*) mice. Each flow cytometric plot shows gated tetramer$^+$ CD8 T cells, and the values given represent the percentage of epitope-specific CD8 T cells that are either CD44^{high}CD122^{high} (*upper region*) or CD44^{int}CD122^{low} (*lower region*). Representative data are shown from two to eight mice analyzed at each time point.

CD8 mAbs. The anti-CD8 PerCP clone 53-6.7 (BD PharMingen) was used in conjunction with H-2D^b tetramers. For costains with H-2K^b tetramers, the anti-CD8 PE mAb clone CT-CD8a (Caltag Laboratories, Burlingame, CA) was used. Staining procedures were performed at 4°C in PBS containing 2% (w/v) BSA and 0.2% (w/v) NaN_3. After incubation with Abs, samples were washed and then fixed in PBS containing 2% (w/v) paraformaldehyde. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using the computer program CellQuest.

Intracellular cytokine staining

Intracellular cytokine staining was performed, as previously described (12). Briefly, cell suspensions from the spleen and livers were either left untreated or stimulated with LCMV-derived peptide epitopes, at a concentration of 1 μg/ml, for 5 h at 37°C. The intracellular accumulation of cytokines was facilitated by the addition of brefeldin A (Golgiplug; BD PharMingen). Surface and intracellular staining were then performed using the mAbs anti-CD8 PE (clone 53-6.7), anti-IL-2 FITC (clone JES6-5H4), anti-IFN-γ FITC or allophycocyanin (clone XMG1.2), and anti-TNF-α allophycocyanin (clone MP6-XT22). In certain experiments, the intracellular cytokine staining procedure was modified to enable the surface exposure of CD107a and CD107b to be monitored (29). For these assays, anti-CD107a FITC (clone 1D4B) and anti-CD107b FITC (clone ABL-93) mAbs or isotype control rat anti-mouse IgG2a,κ FITC (clone R35-95) mAbs were added to cultures at the time of peptide stimulation and intracellular cytokine accumulation facilitated by the addition of monensin (Golgistop; BD PharMingen). After incubation for 5 h, cells were washed in PBS containing 2% (w/v) BSA and 0.2% (w/v) NaN_3 before proceeding with further surface and intracellular staining. Conjugated mAbs were purchased from either BD PharMingen or eBioscience (San Diego, CA). Samples were acquired and analyzed as described for MHC class I tetramer staining.

In vivo CTL assays

Splenocytes from naive B6 mice were prepared as described above and used as target cells for in vivo CTL assays (30, 31). Target cells (10^7 cells/ml) were labeled with either 2 or 0.2 μM CFSE for 5 min at 25°C in PBS. CFSE labeling was then quenched by the addition of FCS to a final concentration of 20% (v/v). Splenocytes labeled with 2 μM CFSE were then sensitized for lysis by pulsing with gp33–41 peptide (1 μg/ml) for 1 h at 37°C. Splenocytes labeled with 0.2 μM CFSE were not peptide pulsed, and used as a control population of target cells. To determine np396–404-specific killing, separate populations of splenocytes suspended in RPMI 1640 medium supplemented with 10% FCS (5×10^6 cells/ml) were labeled with either 5 or 0.5 μM CellTracker Orange (CTO; Molecular Probes) for 1 h at 37°C. Cells labeled with 5 μM CTO were concurrently pulsed with the LCMV np396–404 peptide (1 μg/ml). After labeling and peptide pulsing, all populations of cells were washed and resuspended in HBSS without calcium and magnesium. All four populations of target cells were then mixed together such that recipient mice received 10^7 of each population in a single i.v. injection. Target cells were administered to naive B6 and CD4$^{-/-}$ mice, and to groups of mice between days 250 and 256 post-LCMV infection. Recipient mice were sacrificed 4 h following cell transfer, and single cell suspensions of spleens and livers were prepared as described above. Flow cytometry was used to determine the recoveries of peptide-sensitized and nonpeptide-treated dye-labeled target cells. Detection of CFSE- and CTO-labeled cells was used to determine gp33–41- and np396–404-specific killing, respectively. Percent specific lysis was determined using the following formulas (30): ratio of recovery of nonpeptide-treated control splenocytes to peptide-sensitized splenocytes = (percentage of CFSE^{low} or CTO^{low} cells)/(percentage of CFSE^{high} or CTO^{high} cells).

Percent specific lysis = $100 \times (1 - (\text{ratio of cells recovered from naive mice/ratio of cells recovered from infected mice}))$.

Results

Acute LCMV infection of CD4$^{-/-}$ mice

Resolution of acute infection with the natural mouse pathogen LCMV is mediated by CD8 T cells (28). To determine the consequences of CD4 T cell deficiency on the kinetics of LCMV clearance, B6 and CD4$^{-/-}$ mice were infected by i.n. inoculation with the Armstrong strain of LCMV. Analysis of the viral titers in the lungs and spleens of infected mice demonstrated that both B6 and CD4$^{-/-}$ mice resolved LCMV-Armstrong infection with similar kinetics (Fig. 1). Similar trends were observed in the liver and salivary glands (data not shown). Plaque assays were also used to determine the presence of replicating virus in serum, livers, spleens, and kidneys in both B6 and CD4$^{-/-}$ mice, at various time points between 100 and 200 days postinfection, and were consistently found to be below the limit of detection (data not shown). In addition, lung tissue homogenates prepared from mice 1 mo after infection as well as serum samples obtained from i.n. infected CD4$^{-/-}$ mice up to 1 year after infection failed to cause lethal disease following intracranial inoculation of naive B6 mice (data not shown) (32). Taken together, these data are in agreement with previously published findings that CD4$^{-/-}$ mice control infection with LCMV-Armstrong and imply that the primary antiviral CD8 T cell response is sufficient to control the infection even in the absence of CD4 T cell help (16, 33, 34).

Evolution and maintenance of CD44 intermediate (CD44^{int}) virus-specific CD8 T cells in CD4$^{-/-}$ hosts

MHC class I tetramer staining was used to confirm the induction of LCMV-specific CD8 T cells and determine whether the elaboration and maintenance of these responses were negatively impacted by the lack of CD4 T cell help. By 8 days following i.n. LCMV

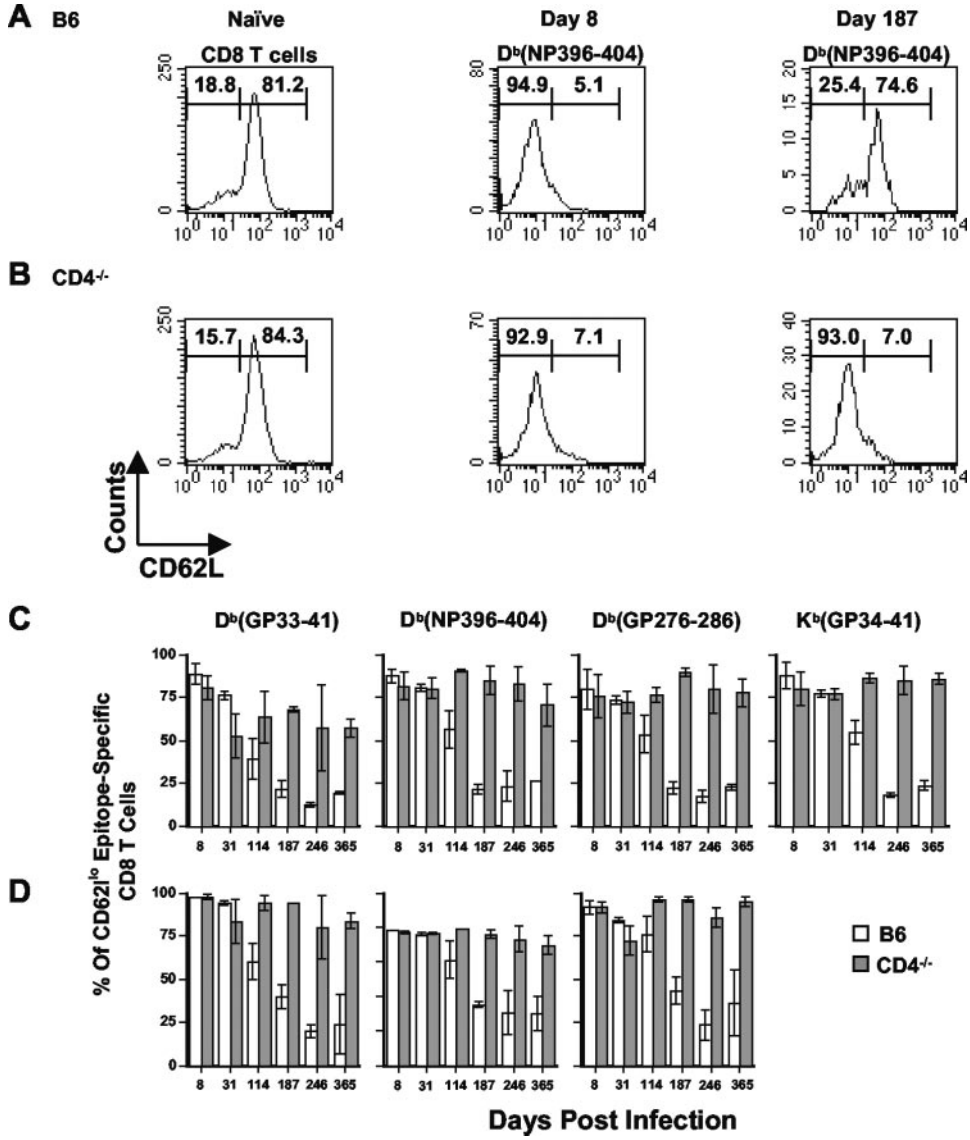

FIGURE 4. Maintenance of CD62L^{low}CD8 T cells in the absence of CD4 T cell help. *Upper panels*, Show representative CD62L staining profiles of naive or LCMV-specific CD8 T cells isolated from spleens of either naive or infected B6 (*A*) or CD4$^{-/-}$ (*B*) mice. Values in each histogram give the percentage of either gated naive or D^b(np396–404)-specific CD8 T cells that are either CD62L^{low} (*left region*) or CD62L^{high} (*right region*). *C* and *D*, Show the mean percentages ± SD of epitope-specific CD8 T cells that are CD62L^{low} in B6 (□) or CD4$^{-/-}$ mice (▨). At various time points following infection, four separate specificities of CD8 T cells were analyzed in the spleen (*C*), and three specificities were examined in the liver (*D*). Between two and eight mice from each group were analyzed at each time point.

infection, a multiepitope-specific CD8 T cell response was readily detectable in the spleens of both B6 and CD4$^{-/-}$ mice (Fig. 2, *A* and *B*). In CD4$^{-/-}$ mice, alterations in the expected patterns of immunodominance emerged as a diminution in the proportion of D^b(gp33–41)-specific CD8 T cells, and a more marked attenuation in the K^b(np205–212) response became clearly evident by day 187 postinfection. Whereas all specificities of virus-specific CD8 T cells in B6 mice were CD44^{high} at each time point examined, virus-specific CD8 T cells that were maintained in the absence of CD4 T cell help gradually developed a CD44^{int} phenotype.

CD8 T cell responses were examined in the livers and lungs to address whether the distribution and phenotype of virus-specific CD8 T cells in tertiary tissues were similarly affected by the absence of CD4 T cells. In the liver and lungs of CD4$^{-/-}$ mice, D^b(np396–404)-specific CD8 T cells were strikingly more frequent than D^b(gp33–41)-specific CD8 T cells. Consistent with the splenic data, the D^b(np396–404) CD8 T cells that were preferentially maintained in CD4$^{-/-}$ mice also exhibited a CD44^{int} phenotype (Fig. 2, *C* and *D*). Taken together, these findings suggest that phenotypically distinct subsets of memory CD8 T cells develop in the absence of CD4 T cell help.

Down-regulated CD122 expression on CD44^{int}CD8 T cells

In B6 mice, typical Ag-specific memory CD8 T cells are CD44^{high}, and the stable maintenance of these cells in the absence of Ag has been shown to be influenced by IL-15 (35). CD122 is the β-subunit shared by the IL-2 and IL-15 receptors, and normal memory CD8 T cells exhibit a CD122^{high} phenotype (36, 37). Given that the absence of CD4 T cell help is associated with the emergence and maintenance of an atypical population of CD44^{int}CD8 T cells, we next determined whether the expression of CD122 was also altered on this subset of CD8 T cells (Fig. 3). As expected, the majority of D^b(gp33–41)- and D^b(np396–404)-specific responses were CD44^{high}CD122^{high} in the spleens and livers at all the time points following acute LCMV infection of B6 mice (Fig. 3*A*). By contrast, the virus-specific CD44^{int}CD8 T cells that developed in the absence of CD4 T cell help expressed diminished levels of CD122. This was evident by the preponderance of CD44^{int}CD122^{low} cells that emerged over time in the spleens and livers of CD4$^{-/-}$ mice (Fig. 3*B*). Similar trends were observed in the lungs (data not shown). These data are consistent with the hypothesis that CD4 T cells contribute to the programming of normal memory CD8 T

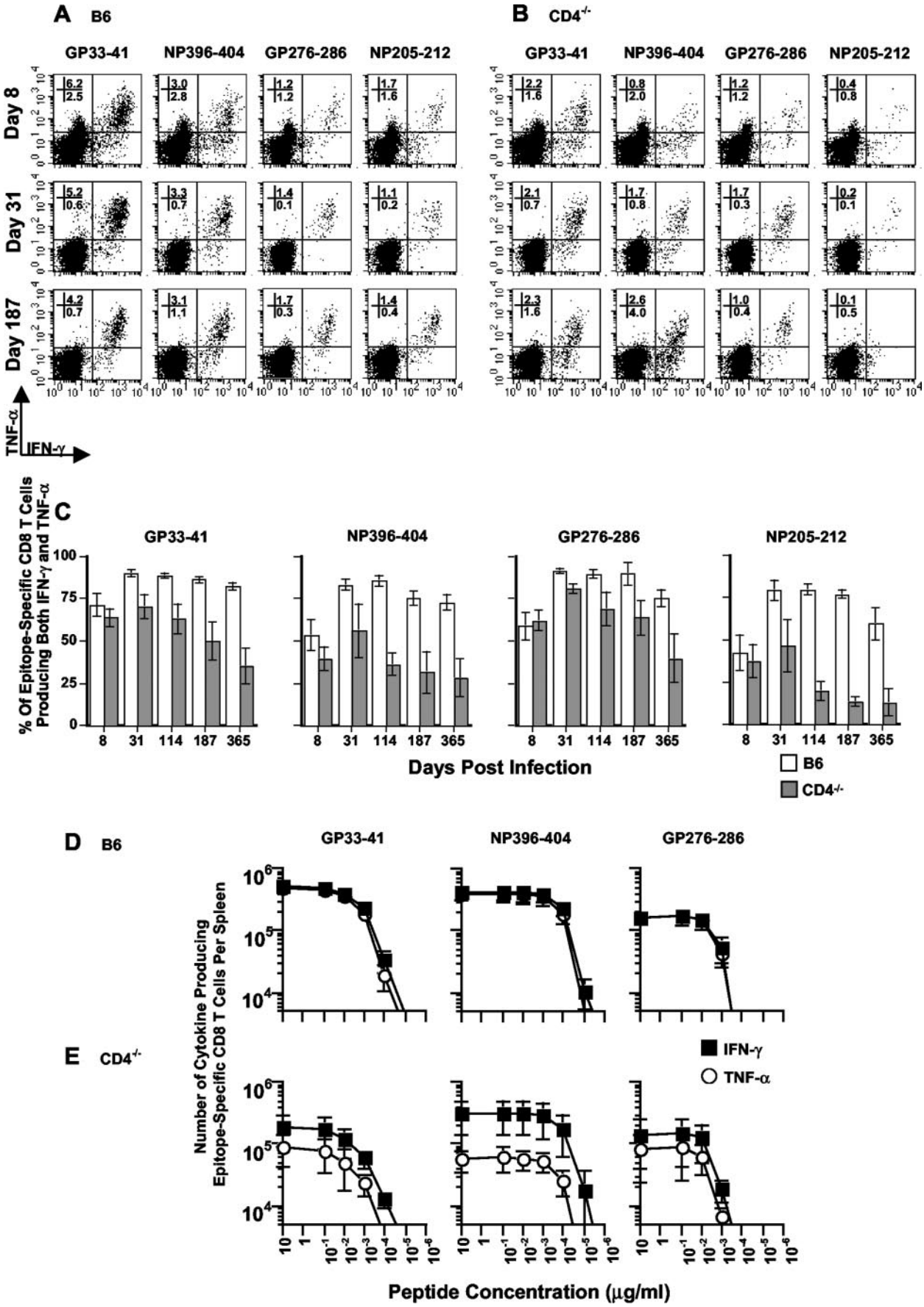

FIGURE 5. Altered functional maturation of virus-specific CD8 T cells in CD4$^{-/-}$ mice. At various time points following LCMV-Armstrong infection of B6 (*A*) and CD4$^{-/-}$ (*B*) mice, the capacity of splenic virus-specific CD8 T cells to produce IFN-γ and TNF-α was evaluated by intracellular cytokine analysis, following a 5-h stimulation with four separate viral peptide epitopes. Each flow cytometric plot shows (*Figure legend continues*)

cells and that phenotypically distinct subsets of Ag-experienced CD8 T cells develop if insufficient CD4 T cell help is provided.

Virus-specific CD8 T cells remain CD62L^{low} in the absence of CD4 T cells

Expression of the adhesion molecule CD62L has been used to discriminate between memory CD8 T cell subsets (9, 15). Changes in the expression of CD62L on LCMV-specific CD8 T cells were tracked over time to further elucidate how CD4 T cells influence memory CD8 T cell development. As expected, CD8 T cells isolated from the spleens of both naive B6 and CD4$^{-/-}$ mice were CD62L^{high} (Fig. 4, *A* and *B*, *left panels*). In concordance with the previously reported dynamics of CD62L expression (15, 38), Ag-specific effector CD8 T cells isolated from spleens and livers, at 8 days following LCMV-Armstrong infection, were CD62L^{low} (Fig. 4, *A* and *B*, *middle panels*). In immunocompetent B6 mice, this CD62L^{low} phenotype reverted over time as Ag-specific CD62L^{high} cells gradually re-emerged. Of significant interest was the prolonged maintenance of the CD62L^{low} phenotype on a majority of LCMV-specific CD8 T cells in the spleens (Fig. 4, *B* and *C*), livers (Fig. 4*D*), and lungs (data not shown) of CD4$^{-/-}$ mice. This failure to revert to a CD62L^{high} phenotype in the absence of CD4 T cells resulted in a marked divergence between epitope-specific CD8 T cells in normal and CD4$^{-/-}$ mice that became clearly apparent at later time points.

Altered cytokine production by CD8 T cells in CD4$^{-/-}$ mice

Intracellular cytokine staining was used to investigate whether the development of phenotypically distinct subsets of CD8 T cells in the absence of CD4 T cell help was associated with an altered capacity to produce effector cytokines (Fig. 5, *A* and *B*). By day 8 postinfection, the overall pattern of IFN-γ and TNF-α production by virus-specific CD8 T cells in the spleens of B6 and CD4$^{-/-}$ mice was fairly similar. In both groups of mice, CD8 T cells had developed that either coproduced IFN-γ and TNF-α or produced only IFN-γ following transient stimulation with viral peptide epitopes. As the CD8 T cells progressed through the effector phase, their functional quality improved in B6 mice as a greater proportion of the responding cells exhibited the ability to produce both cytokines (Fig. 5, *A* and *C*) (12, 39, 40). This functional maturation was less pronounced in the absence of CD4 T cell help as the relative proportion of virus-specific CD8 T cells that coproduced IFN-γ and TNF-α was lower than that observed in B6 mice (Fig. 5, *A–C*). Following day 31 postinfection, reductions in the functional quality of virus-specific CD8 T cells became further apparent as, in CD4$^{-/-}$ mice, the proportion of CD8 T cells that were capable of coproducing IFN-γ and TNF-α decreased over time. Epitope-dependent differences in cytokine production also emerged in CD4$^{-/-}$ mice as the functional capacity of np-specific responses was more severely impacted than glycoprotein-specific responses (Fig. 5, *A–C*). Similar trends were observed in the livers (Fig. 6*B*, and data not shown).

Cytokine production was measured following exposure to graded doses of antigenic peptides to address whether the altered functional capacity of CD8 T cells in CD4$^{-/-}$ mice reflected different sensitivities to peptide stimulation. Although, by comparison with B6 mice, a greater proportion of CD8 T cells from CD4$^{-/-}$ mice produced only IFN-γ, similar overall dose-response curves were observed (Fig. 5, *D* and *E*). These data imply that both populations of cells have similar sensitivities to antigenic stimulation, but differ in their intrinsic ability to elaborate downstream effector functions.

Kinetic analysis of antiviral CD8 T cell responses further highlighted the development of functionally distinct memory CD8 T cells in the absence of CD4 T cell help (Fig. 6). As illustrated in Figs. 5 and 6, this altered functional capacity manifested as an inability to produce TNF-α and was most marked for D^b(np396–404)-specific responses, whereas D^b(gp276–286)-specific CD8 T cells appeared to be less affected. Enumeration of IL-2-producing LCMV-specific CD8 T cells in the spleens further supported the hypothesis that phenotypically distinct subsets of CD8 T cells develop if insufficient CD4 T cell help is provided. In CD4$^{-/-}$ mice, markedly reduced numbers of virus-specific CD8 T cells capable of producing IL-2 became apparent (Fig. 6*A*). Similar qualitative differences in TNF-α and IL-2 production were also observed in pooled virus-specific CD8 T cells isolated from the lungs of CD4$^{-/-}$ mice at late time points (data not shown). Taken together, these data show that the absence of CD4 T cell help during acute LCMV infection results in the evolution and preferential maintenance of phenotypically distinct (CD62L^{low}CD44^{int}CD122^{low}) subsets of memory CD8 T cells and is associated with a diminished capacity to produce IL-2 and TNF-α.

In i.n. infected CD4$^{-/-}$ mice, the contraction phase that usually ensues following the peak of the T cell response was not pronounced. This reduced down-regulation may reflect differences in the immunization strategy, as a preliminary comparative analysis of responses following i.n. and i.p. infections showed that peak CD8 T cell responses following i.p. infection of CD4$^{-/-}$ mice were greater in magnitude, but were followed by a more distinct and pronounced death phase (data not shown).

Protective and proliferative properties of CD8 T cells in CD4$^{-/-}$ mice

Because the functional robustness of virus-specific CD8 T cells is reduced in CD4$^{-/-}$ mice, we investigated whether these cells could mount anamnestic responses and confer immunological protection following viral rechallenge. Groups of naive B6 and CD4$^{-/-}$ mice as well as mice that had experienced acute LCMV infection greater than 16 wk previously were inoculated with the virulent LCMV isolate clone 13. As expected, significant viral titers were detected in the serum, spleens, and livers of B6 and CD4$^{-/-}$ mice during the course of primary LCMV-clone 13 infection (Fig. 7*A*) (23, 34, 41). By contrast, immune B6 mice rapidly controlled the rechallenge inoculum, and viral titers were below the limits of detection by 8 days following secondary infection. Similarly, CD4$^{-/-}$ mice that had previously undergone acute LCMV infection were also able to contain rechallenge with LCMV-clone 13. By 8 days postinfection, seven of the nine

gated CD8 T cells. The values in the *upper right* and *lower right quadrants* represent the percentage of CD8 T cells that coproduce both IFN-γ and TNF-α or only produce IFN-γ, respectively. At each time point, two to eight mice per group were analyzed, and representative data are shown. The bar graph (*C*) shows the percentage of cytokine-producing epitope-specific CD8 T cells in the spleens of B6 (□) and CD4$^{-/-}$ (▨) mice that coproduce both IFN-γ and TNF-α at various time points following infection. Four different epitope-specific responses were evaluated, and mean values ± SD are plotted. *D* and *E*, Epitope-specific IFN-γ- and TNF-α-producing CD8 T cells were enumerated by intracellular cytokine analysis following stimulation with varying concentrations of three separate viral peptide epitopes. Splenocytes were prepared and analyzed from B6 (*D*) and CD4$^{-/-}$ (*E*) mice at 97 days following LCMV-Armstrong infection. The mean number of CD8 T cells that produced IFN-γ (■) or TNF-α (○) in response to a given dose of peptide is plotted. Error bars represent SD.

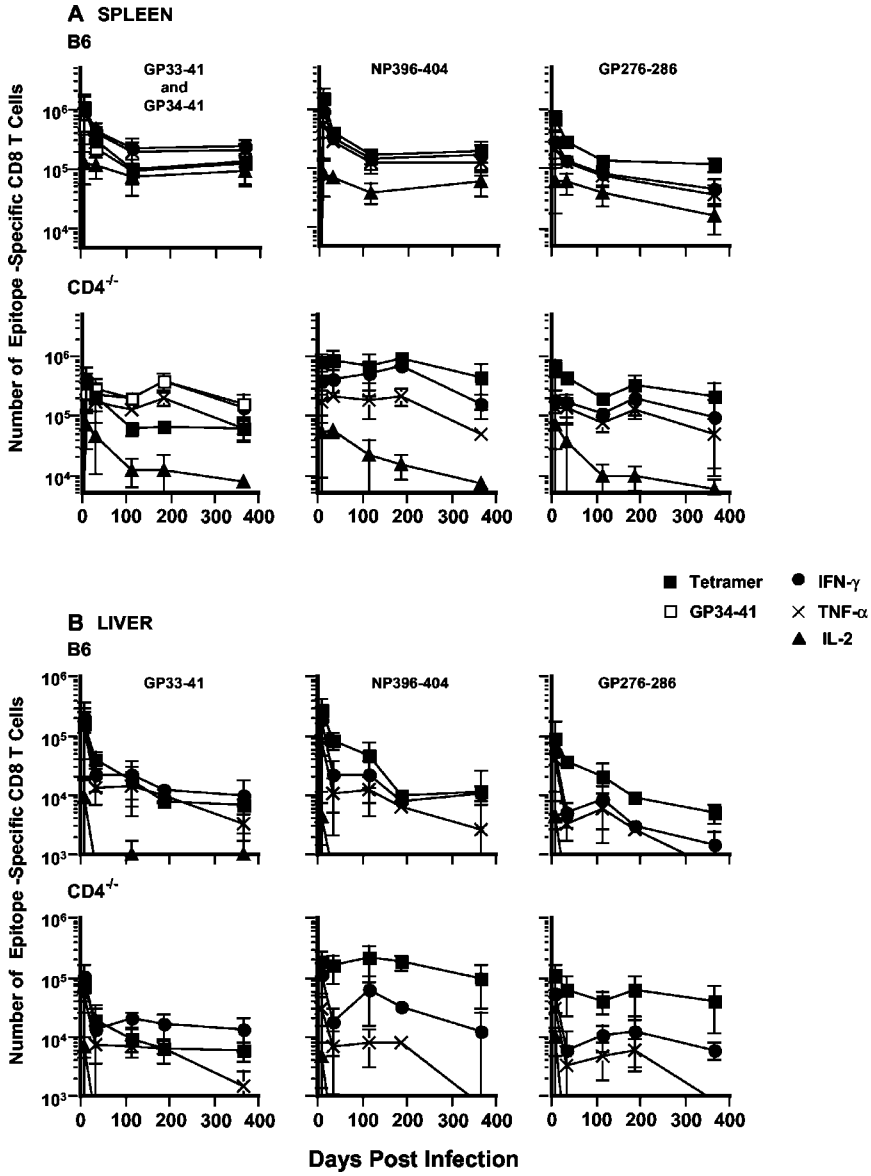

FIGURE 6. Quantitative and qualitative alterations of virus-specific CD8 T cells in CD4$^{-/-}$ mice. LCMV-specific CD8 T cell responses were enumerated in the spleens (*A*) and livers (*B*) of B6 and CD4$^{-/-}$ mice at various times following i.n. infection with LCMV-Armstrong. The presence of virus-specific CD8 T cells was visualized using MHC class I tetramers (■, □). IFN-γ (●), TNF-α (×), and IL-2 (▲)-producing cells were analyzed by intracellular cytokine staining following a 5- to 6-h stimulation with peptide epitopes. Note that the gp33–41 peptide encompasses overlapping D^b- and K^b-restricted epitopes and that *A* (*left panels*) shows both D^b(gp33–41) tetramer$^+$ cells (■) as well as K^b(gp34–41) tetramer$^+$ cells (□). Mean values are shown for two to eight mice at each time point, and error bars represent SD.

CD4$^{-/-}$ mice tested had no detectable levels of LCMV in the serum, spleen, or liver. In two of the nine rechallenged CD4$^{-/-}$ mice, virus was detectable; however, the titers were markedly lower than those observed in CD4$^{-/-}$ mice undergoing primary LCMV-clone 13 infection. Thus, all of the CD4$^{-/-}$ mice checked showed a substantial degree of viral control following secondary LCMV-clone 13 infection.

The rapid control of secondary LCMV infections suggested that all groups of mice were able to elicit accelerated recall responses. As previously shown, a multiepitope-specific CD8 T cell response was elaborated in both B6 and CD4$^{-/-}$ mice following primary acute LCMV infection (Figs. 2–6). By 8 days following rechallenge with LCMV-clone 13, pronounced secondary responses were apparent in both B6 and CD4$^{-/-}$ mice (Fig. 7, *B* and *C*). Strikingly, the immune response to the D^b(np396–404) epitope became overwhelmingly dominant in both groups of rechallenged mice. D^b(gp276–286)-specific CD8 T cells emerged as the next most prevalent population, followed by K^b(gp34–41)-specific T cells. This altered pattern of immunodominance was apparent in both the spleens and livers. Further inspection of the data showed that by 8 days following rechallenge, the majority of virus-specific cells were CD44^{high}CD122^{high} in both B6 and CD4$^{-/-}$ mice. In the spleens, the proportion of CD8 T cells that were CD44^{high}CD122^{high} was greater than that observed in the livers (data not shown).

Although both B6 and CD4$^{-/-}$ mice were protected against viral rechallenge, and the overall profile of secondary immune responses was similar in both groups, the proliferative potential of virus-specific CD8 T cells in CD4$^{-/-}$ mice appeared lower than their counterparts in normal mice. The fold increase in the numbers of virus-specific CD8 T cells in CD4$^{-/-}$ mice undergoing secondary LCMV infections was consistently lower than that observed in B6 mice (Fig. 7, *D* and *E*). These data suggest that the altered phenotypic maturation and selective functional deficits that emerge in CD4$^{-/-}$ mice are also associated with a reduction in the capacity of these CD8 T cells to expand following re-exposure to viral Ags.

Cytotoxic activity of memory CD8 T cells in CD4$^{-/-}$ mice

In addition to the production of antiviral cytokines, including IFN-γ and TNF-α, a prominent mechanism by which CD8 T cells control viruses is by directly killing infected cells (1, 2). Fig. 7*A* shows that both B6 and CD4$^{-/-}$ mice that have previously undergone acute LCMV infection can control secondary viral challenge.

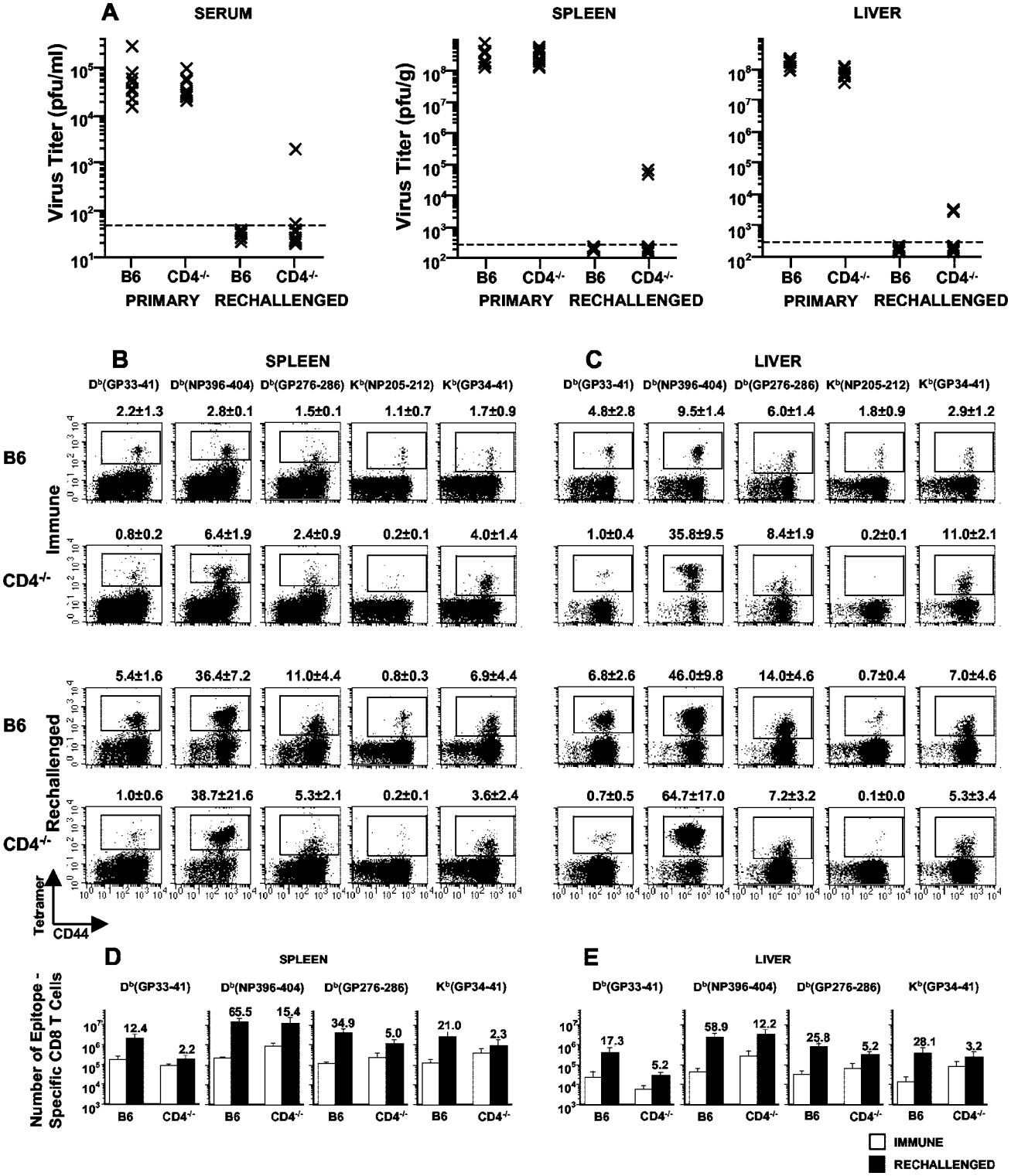

FIGURE 7. Epitope-dependent recall responses in the spleens and livers of B6 and CD4$^{-/-}$ mice. *A*, Viral titers were determined in the serum, spleen, and liver at 8 days following primary LCMV-clone 13 infection of B6 ($n = 8$) and CD4$^{-/-}$ ($n = 9$) mice. Separate groups of B6 ($n = 6$) and CD4$^{-/-}$ ($n = 9$) mice that had undergone acute LCMV infection >115 days previously were rechallenged with LCMV-clone 13, and viral titers were also assayed 8 days later. Results of individual mice are shown, and the limit of detection is indicated by the dashed line. Virus-specific CD8 T cells in the spleens (*B*) and livers (*C*) of immune and rechallenged mice were visualized by MHC class I tetramer staining. Each flow cytometry plot shows gated CD8 T cells, and the values given indicate the mean percentage ± SD of CD8 T cells that bind the specified tetramer. The bar graphs show the absolute number ± SD of epitope-specific CD8 T cells in the spleens (*D*) and livers (*E*) of immune (□) and rechallenged (■) B6 and CD4$^{-/-}$ mice. The values given above each shaded column indicate the fold increase in the mean numbers of epitope-specific CD8 T cells in rechallenged mice over those measured in immune mice analyzed in the same experiment.

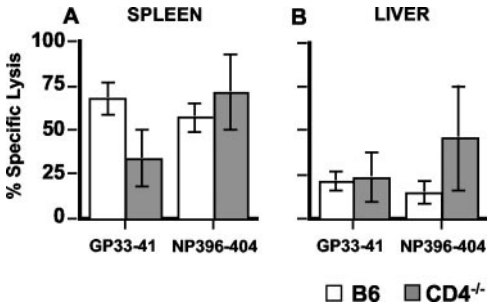

FIGURE 8. In vivo cytotoxicity of helpless CD8 T cells. CTL activity was evaluated in the spleens (*A*) and livers (*B*) of B6 (□; $n = 8$) and CD4$^{-/-}$ (▨; $n = 6$) mice between days 250 and 256 postinfection. CFSE- or CTO-labeled target cells were pulsed with gp33–41 and np396–404 peptide epitopes, respectively, and infused into recipient mice together with control populations of cells not treated with antigenic peptides. After 4 h, the recovery of target cells from naive or previously infected mice was determined by flow cytometry. The values plotted represent mean percentage of specific lysis. Composite data from two independent experiments are shown, and error bars represent SD.

In vivo CTL assays were performed to examine how effectively CD8 T cells that develop in the absence of CD4 T cell help remove target cells presenting viral epitopes (30, 31). The gp33–41- and np396–404-specific killing activity was evaluated 4 h following target cell transfer into B6 or CD4$^{-/-}$ mice that had been infected with LCMV-Armstrong at least 250 days previously (Fig. 8). In both groups of mice, cytotoxic activity was detectable in the spleens and livers. The cytotoxic competency of virus-specific CD8 T cells in CD4$^{-/-}$ mice was further investigated in in vitro assays to determine the degranulation-associated surface exposure of CD107a and CD107b following peptide stimulation. These intracellular proteins are incorporated in the membrane of cytotoxic granules, but can be detected on the surface of cells that have recently released their granule contents (29). Degranulation potential was determined in two separate experiments performed using splenocytes prepared between days 230 and 235 postinfection (data not shown). Consistent with the in vivo CTL results, surface exposure of CD107a and CD107b was detectable on virus-specific CD8 T cells from both B6 and CD4$^{-/-}$ mice. Notably, cells that produced IFN-γ as assessed by intracellular cytokine staining also acquired surface CD107 expression.

Taken together, the results presented in this report show that virus-specific CD8 T cells in CD4$^{-/-}$ hosts are phenotypically distinct and not necessarily as functionally robust as their counterparts in normal hosts. Nevertheless, a sufficient number of these cells are maintained that can act together to bring secondary infections under control.

Discussion

In this study, we have investigated how the absence of CD4 T cell help impacts the optimal priming of CD8 T cell responses following acute LCMV infection. A central finding of this study is that insufficient CD4 T cell help detrimentally affects CD8 T cell responses even to infections that are rapidly resolved. The atypical populations of CD8 T cells that emerge under these conditions display a distinct CD44^{int}CD122^{low}CD62L^{low} phenotype as well as exhibit a selective loss in the capacity to produce effector cytokines, including TNF-α and IL-2. The extent of this altered maturation is at least somewhat influenced by the epitope specificity of the responding cells, as certain specificities show a more marked impairment of cytokine production and altered surface phenotypes. The decreased functional quality of virus-specific CD8 T cells is also associated with reduced expansion following viral rechallenge; nevertheless, the overall secondary response adequately contains the infection. Thus, taken together, these data demonstrate CD4 T cell help is critical for the phenotypic and functional differentiation of peripheral CD8 T cells.

In immunocompetent hosts, the primary immune response leads to the establishment of heterogenous subsets of memory CD8 T cells, and a key finding of this study is that distinct populations of these cells persist in the absence of CD4 T cell help. Normally, following the effector phase of the CD8 T cell response, the virus-specific T cells that survive functionally mature as their capacity to produce TNF-α and IL-2 improves (12, 39, 40, 42). These populations are stably maintained and gradually regain expression of CD62L as well as retain a CD44^{high}CD122^{high} phenotype. This maturation of the memory CD8 T cell pool may reflect either an intrinsic developmental change within the Ag-specific T cell or result from the preferred maintenance of CD62L^{high}CD44^{high}CD122^{high} T cells. Phenotypically distinct subsets of memory T cells have been described, termed central (Tcm) and effector memory cells, based on differential expression of surface markers, including CD62L and CCR7, altered functional activities, and disparate homing properties (9, 10, 15, 43). Thus, in normal hosts, the pool of antiviral CD8 T cells undergoes a transition as the functional quality improves and CD62L^{high} cells (Tcm) emerge.

The most marked divergence between CD8 T cells in normal and CD4$^{-/-}$ hosts manifests following the acute phase of the antiviral response. The persistence of a CD62L^{low} phenotype as well as the decreased expression of CD44 and CD122 suggest that the development of CD62L^{high} Tcm cells is arrested following the acute phase of the response. Phenotypic alterations in CD8 T cells have also been shown to occur during the Ag-independent proliferation of naive T cells in lymphopenic hosts (44, 45). Nevertheless, CD8 T cells that expand without the full complement of usual accessory signals do not necessarily proceed to attain all of the attributes typically associated with functionally robust CD8 T cells. In the case of acute viral infections, CD4 T cells appear to promote the progression of antiviral CD8 T cell maturation and favor the development of effective memory cell subsets.

Following the peak of primary CD8 T cell responses, a programmed death phase is usually, but not always, observed in the spleen (1–6, 46, 47). Notably, this contraction is attenuated during the course of LCMV infection of CD4$^{-/-}$ mice initiated via the i.n. route. This most likely reflects a unique aspect of i.n. LCMV infection, as an expected down-regulation of CD8 T cell responses does occur in CD4$^{-/-}$ mice following i.p. LCMV infection. Thus, CD4 T cells are not strictly required for the diminution of primary CD8 T cell responses, but may influence this under certain circumstances. It has been postulated that the extent of CD8 T cell proliferation may determine the subsequent degree of cell death (47). Thus, CD4 T cells may promote CD8 T cell expansion by the production of IL-2 or other factors, and this may then be reflected by a more pronounced contraction.

The persistence of CD44^{high}CD122^{high} memory cells has been shown to be positively regulated by IL-15, but inhibited by IL-2 (35–37). In the absence of CD4 T cell help, however, the CD8 T cells that are maintained express reduced levels of CD122, a key component of the IL-15R and IL-2R. Thus, the survival of CD44^{int}CD122^{low}CD62L^{low} CD8 T cells in CD4$^{-/-}$ hosts may be regulated by other factors. Notably, it is unlikely that the proliferation of these cells is held in check by IL-2, as the persisting CD8 T cells fail to express this cytokine, are CD122^{low}, and CD4 T cells are also absent. Therefore, CD4 T cells appear to license the transition of effector CD8 T cells into mature CD62L^{high} Tcm cells. This transition stalls in the absence of CD4 T cell help, and

the subsets of memory cells that subsequently persist are phenotypically divergent and are likely to be maintained by distinct survival signals.

Aberrant populations of CD8 T cells have been shown to emerge in chronically infected hosts (13). Widely disseminated high grade LCMV infection is associated with the progressive loss of CD8 T cell effector activities and the rapid deletion of D^b(np396–404)-specific CD8 T cells (12, 22, 23, 41, 46). The pattern of CD8 T cell responses is clearly different in acutely infected $CD4^{-/-}$ mice, as a significant proportion of virus-specific CD8 T cells retains the capacity to produce both IFN-γ and TNF-α, and D^b(np396–404)-specific T cells are maintained. Nevertheless, the possibility that following i.n. LCMV infection of $CD4^{-/-}$ a low level of viral Ag persists, which is undetectable by conventional assays, cannot be ruled out. If, however, viral Ag does remain present, our findings would suggest that the absence of CD4 T cell help as well as low levels of persistent Ag are detrimental for the development and maintenance of robust memory CD8 T cells.

A hallmark of immunological memory is the capacity to mount accelerated recall responses. By using MHC class I tetramers, striking differences in the recruitment of epitope-specific CD8 T cells have been revealed following secondary viral challenge, which may have been missed if only monoclonal TCR transgenic T cells were monitored. In immunocompetent B6 mice, secondary CD8 T cell responses were characterized by an overwhelming expansion of D^b(np396–404)-specific cells in both the spleens and livers. D^b(gp276–286)-specific CD8 T cells comprised the next most dominant secondary response, but D^b(gp33–41)- and K^b(np205–212)-specific responses were less prominent (Fig. 7). Several factors are likely to contribute to this biased secondary recruitment of epitope-specific CD8 T cells from the poly-specific pool of memory cells. These include the frequencies of epitope-specific memory cells, the kinetics of viral protein synthesis and subsequent presentation, the abundance of presented Ag, and also the type of cells that initially display the Ags for immunosurveillance by memory CD8 T cells (48). In vitro studies have documented that Ag presentation by fibroblasts favors the outgrowth of D^b(gp276–286)-specific responses, whereas dendritic cell lines preferentially give rise to D^b(np396–404)- and gp33–41-specific cells (49). Alterations in immunodominance during secondary influenza virus infections have also been in part attributed to the differential presentation of Ags by dendritic and nondendritic cells. Primary influenza virus infection of B6 mice elicits both flu np366–374- and flu PA224–233-specific responses; however, during viral rechallenge, there is preferential recruitment of flu np366–374-specific CD8 T cells (50, 51). Overall, multiple factors are likely to govern the mobilization of secondary immune responses. An intriguing possibility is that the magnitude of recall responses also reflects the intrinsic capacity of memory epitope-specific CD8 T cells to react to viral rechallenge in vivo. In this scenario, the responsiveness of individual populations of epitope-specific CD8 T cells would be differentially programmed during the primary infection and depend upon the viral epitope recognized. This encoding of T cells during primary infections could in turn be influenced by the activation state and type of cells that most effectively present the cognate Ag.

$CD4^{-/-}$ mice exhibit a significant degree of immunological protection following rechallenge with the virulent LCMV isolate clone 13. Since antiviral Ab responses are curtailed because of the lack of helper activity, the accelerated viral clearance observed in immune $CD4^{-/-}$ mice is solely conferred by the CD8 T cell response (16, 34). Even though immune $CD4^{-/-}$ hosts control secondary viral challenge, the fold increase in the numbers of virus-specific CD8 T cells is markedly reduced. This is consistent with several reports of impaired secondary responses by CD8 T cells that have been primed in the absence of CD4 T cells (16–21). The virus-specific CD8 T cells detectable in $CD4^{-/-}$ mice following secondary viral challenge phenotypically more closely resemble their counterparts in normal hosts, as the majority are $CD44^{high}CD122^{high}$. This suggests that pre-existing $CD44^{high}CD122^{high}$ T cells may preferentially proliferate during recall responses; however, the possibility that $CD44^{int}CD122^{low}$ cells convert to $CD44^{high}CD122^{high}$ cells cannot be ruled out. Despite the reduced overall secondary response, a considerable number of D^b(np396–404)-specific CD8 T cells do expand, and this specificity of antiviral T cells most likely contributes substantially to the containment of the secondary infection. Thus, the phenotypically divergent pools of virus-specific CD8 T cells that arise in $CD4^{-/-}$ mice are maintained at adequate levels to have protective potential even though their functional quality and capacity to expand are reduced.

Insufficient CD4 T cell help impacts the formation of optimal memory CD8 T cell responses (16–21). Consequently, CD8 T cell-mediated immunity to pathogens is likely to be best conferred by a broad multiepitope-specific pool of cells that has been primed and is maintained in the presence of CD4 T cells. Vaccines focused on only eliciting CD8 T cell responses are likely to be less useful, as, although primary responses may be generated, the long-term efficacy of the memory cells that subsequently develop may be compromised. This may be overcome if sufficiently high numbers of virus-specific cells of appropriate specificity can be induced. Thus, protective immunity is impacted by both the overall abundance and functional quality of antiviral T cells.

This study has shown that phenotypically and functionally distinct subsets of memory CD8 T cells arise in the absence of CD4 T cells, and that helper functions are necessary for the evolution of $CD62L^{high}$ Tcm. Further investigation will be required to define how CD4 T cells mediate the transition of effector CD8 T cells into protective memory subsets that can persist in the absence of Ag. Obvious candidates for consideration include the production of soluble mediators such as cytokines, including IL-2, as well as the conditioning of accessory cells, including dendritic cells.

Acknowledgments

We thank Kyoko Kojima for excellent technical assistance, and Anne Tebo, Michael Betts, Robin Stephens, and Laurie Harrington for helpful advice and discussions. We also thank Rafi Ahmed for supplying viral seed stocks, and John Altman, and the University of Alabama Molecular Biology Core and Fermentation Facility, for advice and assistance with MHC-tetramer production.

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