

# Fully Functional Memory CD8 T Cells in the Absence of CD4 T Cells<sup>1</sup>

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The role of CD4 T cells in providing help to CD8 T cells in primary and secondary responses to infection remains controversial. Using recombinant strains of virus and bacteria expressing the same Ag, we determined the requirement for CD4 T cells in endogenous CD8 T cell responses to infection with vesicular stomatitis virus and *Listeria monocytogenes* (LM). Depletion of CD4 T cells had no effect on the frequency of primary or secondary vesicular stomatitis virus-specific CD8 T cells in either lymphoid or nonlymphoid tissues. In contrast, the primary LM-specific CD8 T cell response was CD4 T cell dependent. Surprisingly, the LM-specific CD8 T cell recall response was also CD4 T cell dependent, which correlated with a requirement for CD40/CD40L interactions. However, concomitant inhibition of CD40L and CD4 T cell removal revealed that these pathways may be operating independently. Importantly, despite the absence of CD4 T cells during the recall response or throughout the entire response, CD8 memory T cells were functional effectors and proliferated equivalently to their "helped" counterparts. These data call into question the contention that CD4 T cells condition memory CD8 T cells during the primary response and indicate that the principal role of CD4 T cells in generating CD8 memory cells after infection is augmentation of proliferation or survival through costimulatory signals. *The Journal of Immunology*, 2004, 173: 969–975.

CD8 T cells are important effectors of the adaptive immune system and are essential for protection against infection by many viruses and bacteria. The primary and secondary CD8 T cell responses to infection are characterized by robust expansion of the Ag-specific pool, followed by apoptotic loss of the majority of effectors and eventual development of a stable memory population. It is well established that recognition of peptide-MHC class I by the TCR on naive CD8 T cells in conjunction with costimulatory signals results in activation, expansion, and differentiation into effector CTLs. However, the precise factors that regulate these events are still unclear. In certain cases, CD4 T cells are required for the generation and maintenance of cytolytic CD8 T cells (1). For example, CD8 T cell responses to minor H Ags, soluble proteins, tumor Ags, and peptide-pulsed dendritic cells (DC)<sup>3</sup> require CD4 T cells for induction of optimal primary CTL responses (2–4). In the case of responses to infectious agents, it is believed that the requirement for APC conditioning by CD4 T cells before CD8 T cell priming can be overcome by direct infection of APCs or perhaps by inflammatory mediators induced by infection (5, 6). However, some primary

CD8 T cell responses to virus infection are CD4 T cell dependent, such as the response to HSV infection (7) and to influenza virus infection (8), whereas the CD8 T cell response to lymphocytic choriomeningitis virus (LCMV) infection is largely CD4 T cell independent (9). The factors determining the necessity for CD4 T cell help in the primary CD8 T cell response to one infectious agent vs another are not known. One mechanism by which CD4 T cells provide help to CD8 T cells is via activation of Ag-bearing, CD40-expressing DCs through the interaction of CD40L expressed by CD4 T cells (10–12). CD4 T cell help may also be provided by direct interactions through CD40 expressed by CD8 T cells and CD40L expressed by CD4 T cells (13), although this mechanism has been called into question (14).

Although one effect of CD4 T cells on CD8 T cells is the induction of increased cellular expansion and perhaps survival (15), CD4 T cells may also influence the functional abilities of responding CD8 T cells. For example, in the absence of CD4 T cells, the priming and expansion of mycobacteria-specific CD8 T cells are normal, yet the development of cytotoxic CD8 effector cells is diminished (16). The secondary response of memory CD8 T cells can also be CD4 T cell dependent, such as in the case of anti-H-Y responses (17) and the response to influenza virus infection (8). However, recent studies suggest that CD4 T cells also influence the functional capacity of memory CD8 T cells. In one report, CD4 T cells are needed for the primary and secondary expansion of H-Y-specific CD8 T cells (18), as previously shown (17). However, in the absence of CD4 T cell help during priming, the CD8 T cells are lethargic, in that they proliferate poorly in response to Ag. Nevertheless, such cells are functional effectors, and the proliferative defect is reversible by the addition of CD4 T cells (18). In another study, priming of adenovirus- or LCMV-specific CD8 T cells in CD4-depleted or I- $\beta$ <sup>-/-</sup> mice, respectively, results in memory CD8 T cells that are able to produce IFN- $\gamma$ , but are unable to expand *in vitro* in the absence of CD4 T cells (9). In contrast, when CD4<sup>-/-</sup> mice are infected with recombinant *Listeria monocytogenes* (rLM) expressing LCMV-gp33, a reduced population of

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; LM, *Listeria monocytogenes*; LP, lamina propria; VSV, vesicular stomatitis virus.

CD8 memory T cells is produced that is able to mount a robust recall response to LCMV challenge (19). However, when CD4<sup>-/-</sup> mice are primed by LCMV infection and secondarily challenged with rLM-gp33 (20) or when I-A $\beta$ <sup>-/-</sup> mice are primed and challenged with rLM-OVA (21), the memory CD8 T cells exhibit defective proliferation, even when transferred to hosts with a normal CD4 T cell compartment (20). The reasons for the disparate results obtained in these studies are unclear, but could be linked to the combinations of pathogens used and the types of CD4 T cell-deficient mice analyzed. For example, a recent study provides evidence that in CD4<sup>-/-</sup> mice, the CD8 population contains a large fraction of MHC class II-restricted cells, which could be responsible for the weaker CD8 T cell response observed, suggesting that CD4<sup>-/-</sup> mice are a poor model to study the requirement for T cell help in CD8 T cell responses (22).

We have now re-examined the requirement for CD4 T cells to provide help during reactivation of memory CD8 T cells *in vivo*. Using a system in which the response to infection with a viral or bacterial strain expressing the same Ag can be monitored, our results indicate that CD4 T cells and CD40L are needed for memory cell reactivation in response to LM, but not to vesicular stomatitis virus (VSV) infection. Interestingly, the requirement for CD4 T cell help was not a property intrinsic to the CD8 memory T cells, but was linked to the infectious agent. Moreover, the major effect of CD4 T cells was amplification of the secondary CD8 T cell response, not functional imprinting of memory cell function. These results are discussed in light of the current paradigms of memory T cell development and function.

## Materials and Methods

### Mice

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

### Infections and detection of Ag-specific CD8 T cells

Recombinant LM-secreting soluble OVA (rLM-OVA) was produced as previously described using a truncated OVA cDNA (23, 24). Mice were infected with  $\sim 1 \times 10^4$  CFU *i.v.* for primary infections, with  $\sim 1 \times 10^3$  CFU *i.v.* for initial priming for recall experiments, and with  $\sim 1 \times 10^4$  CFU for secondary infections. For primary virus infections,  $1 \times 10^6$  PFU of VSV-Indiana or recombinant VSV-OVA (25) was administered *i.v.* Secondary *i.v.* infections were performed using  $1 \times 10^6$  PFU of VSV-OVA. At the indicated times after infection, lymphocytes were isolated, and either OVA-specific or VSV nucleoprotein-specific CD8<sup>+</sup> T cells were detected using an H-2K<sup>b</sup> tetramer containing either the OVA-derived peptide SIINFEKL or the N protein-derived peptide RGYVYQGL (26).

### Depletion and blocking reagents

*In vivo* depletion of CD4<sup>+</sup> T cells was accomplished by treatment with the anti-CD4 mAb, GK1.5. Animals were infected on day 0 and were given 200  $\mu$ l of ascites fluid *i.p.* or 200  $\mu$ g of purified mAb 7 and 6 days before and 1 and 5 days after infection. For long term depletion, after the initial regimen, mice were administered 300  $\mu$ g of purified GK1.5 mAb every 7–10 days. Purified rat IgG was used as a control treatment in all cases. The efficacy of GK1.5 mAb treatment was checked before infection and at the completion of the experiment by flow cytometry using an mAb that does not cross-compete with GK1.5 for binding (RM4-5; BD Pharmingen, San Diego, CA) and consistently showed <0.1% CD4<sup>+</sup> T cells in all tissues analyzed from depleted mice.

For costimulation inhibition studies, 200  $\mu$ g of MR1 (anti-CD40L mAb) or hamster Ig as a control (27) (provided Dr. R. Noelle, Dartmouth Medical School, Hanover, NH) was injected *i.p.* 2 days before infection and every other day until the mice were euthanized. Similar treatments were performed with 200  $\mu$ g of CTLA4-Ig or a CTLA4-Ig mutant as a control (28) (provided by P. Morton, G. D. Searle, St. Louis, MO.). The mutant does not bind B7-1 or B7-2, but retains FcR binding.

### Adoptive transfer

Spleen cells were isolated from C57BL/6-Ly5.1 mice treated with the anti-CD4 mAb, GK1.5, or control IgG starting before primary infection with

$10^3$  LM-OVA and continuing for 10–14 wk. CD8 T cells were enriched by depletion of GK1.5-coated CD4 T cells and B cells with magnetic beads (DynaL Biotech, Oslo, Norway) coupled to anti-mouse Ig and anti-rat Ig. The percentage of tetramer-positive cells was determined, and  $5 \times 10^4$  tetramer-positive cells were transferred to each C57BL/6-Ly5.2 mouse. The total cell number transferred was equalized between groups by the addition of enriched CD8 T cells from naive mice. One day later, transferred mice were infected *i.v.* with  $10^3$  CFU of LM-OVA. Donor cells were detected by reactivity with anti-Ly5.1 mAb, anti-CD8 mAb, and H-2K<sup>b</sup>-SIINFEKL tetramer.

### Isolation and immunofluorescence analysis of lymphocyte populations

Single-cell suspensions were prepared from lymph nodes and spleens using cell strainers. LP, liver, and lung lymphocytes were isolated as previously described (29). Lymphocytes were resuspended in PBS/0.2% BSA/0.1% NaN<sub>3</sub> (PBS/BSA/NaN<sub>3</sub>) at a concentration of  $1 \times 10^6$  to  $1 \times 10^7$  cells/ml, followed by incubation at room temperature for 1 h with tetramer-allophycocyanin plus the appropriate dilution of PerCP-conjugated anti-CD8 mAb (clone 53.6.7; BD Pharmingen). Cells were washed with PBS/BSA/NaN<sub>3</sub>, stained with FITC-conjugated anti-CD11a mAb and PE-conjugated anti-CD4 mAb (clone RM4-5; BD Pharmingen), incubated at 4°C for 20 min, washed, and fixed in 3% paraformaldehyde in PBS. Relative fluorescence intensities were then measured with a FACSCalibur (BD Biosciences, San Jose, CA). Data were analyzed using WinMDI software (J. Trotter, BD Biosciences).

### Intracellular detection of IFN- $\gamma$

Lymphocytes were isolated from the indicated tissues and cultured for 5 h with 1  $\mu$ g/ml GolgiStop (BD Pharmingen) with or without 1  $\mu$ g/ml SIINFEKL peptide. After culture, cells were stained for surface molecules, then fixed, and cell membranes were permeabilized in Cytofix/Cytoperm solution (BD Pharmingen) and stained with anti-IFN- $\gamma$ -FITC (XMG1.2, 5  $\mu$ g/ml; BD Pharmingen) or control rat IgG1-FITC (R3-34, 5  $\mu$ g/ml; BD Pharmingen). Cells were then washed, and fluorescence intensities were measured using a FACSCalibur.

### Measurement of cytolytic activity

Cytolytic activity was measured using [<sup>51</sup>Cr]sodium chromate-labeled EL4 cells, with or without the addition of 10  $\mu$ g/ml OVA-derived peptide SIINFEKL. Serial dilutions of effector cells were incubated in a 96-well, round-bottom microtiter plates with  $2.5 \times 10^3$  target cells for 5 h at 37°C. The percent specific lysis was calculated as:  $100 \times [(cpm \text{ released with effectors}) - (cpm \text{ released alone})] / [(cpm \text{ released by detergent}) - (cpm \text{ released alone})]$ .

### Statistical analysis

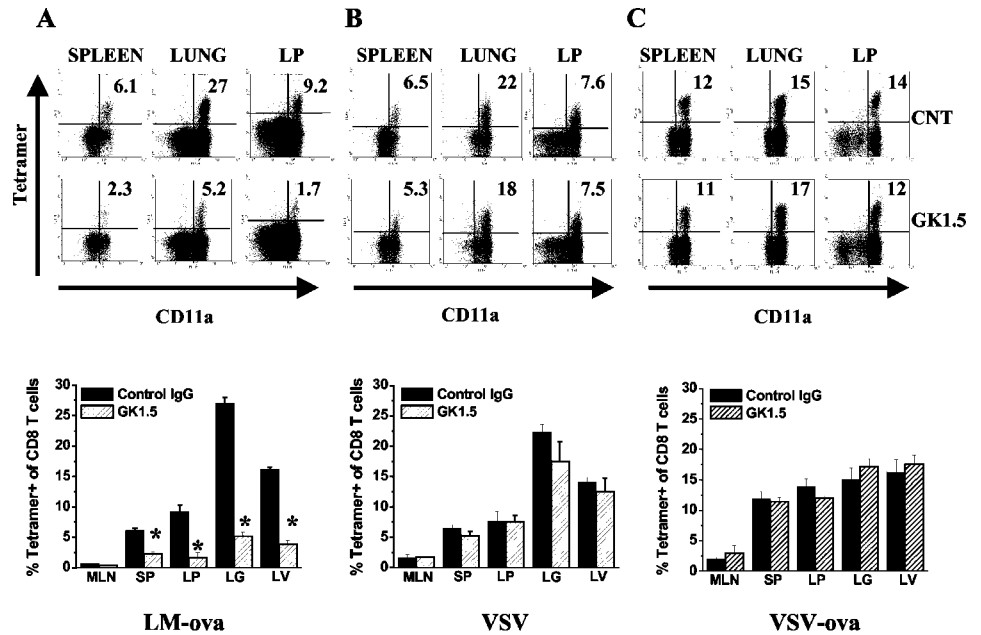
Statistical differences between groups were determined using Student's *t* test. A value of *p* < 0.05 was considered significant.

## Results

### The primary CD8 T cell response to LM infection requires CD4 T cells

Our previous results using MHC I-A $\beta$ <sup>-/-</sup> mice indicated a variable requirement for CD4 T cells in the primary CD8 T cell response to rLM-OVA infection (23), with minimal effect on the splenic response, but a more dramatic diminution of the response in the intestinal lamina propria (LP). However, there is low level expression of chimeric MHC class II in I-A $\beta$ <sup>-/-</sup> mice (30), there are residual CD4 T cells in I-A $\beta$ <sup>-/-</sup> mice (31, 32), and it is also possible that APCs lacking MHC class II may exhibit developmental abnormalities. Therefore, we examined the rLM-OVA-specific CD8 T cell primary response after short term depletion of CD4 T cells by mAb treatment (Fig. 1A). The response in all tissues was greatly decreased in the absence of CD4 T cells, indicating that CD4 T cell help was essential to mount the primary CD8 T cell response to OVA expressed by LM. Similar results were obtained when the response to the listeriolysin *O*-derived peptide (LLO<sub>91–99</sub>) presented by H-2K<sup>d</sup>, was measured after infection of intact or CD4 T cell-depleted (BALB/c $\times$ C57BL/6J)F<sub>1</sub> mice (data not shown). In contrast, the CD8 T cell response to

**FIGURE 1.** CD4 T cells are required for the primary CD8 response to rLM-OVA, but not to VSV infection. Mice were treated on days  $-6$ ,  $-5$ ,  $0$ , and  $6$  with control IgG or the anti-CD4 mAb GK1.5. Mice were infected with either LM-OVA (A), VSV (B), or VSV-OVA (C) at the peak of each response (days 9, 7, and 7, respectively), lymphocytes were isolated from mesenteric LN (MLN), spleen (SP), intestinal LP, lung (LG), and liver (LV) and analyzed for the expression of CD8, CD11a, and reactivity with either OVA-tetramer or N-tetramer. \*,  $p < 0.05$ . Data are representative of three experiments with at least three mice per group.



VSV infection, regardless of whether the N- or OVA-specific response was measured, was CD4 T cell independent (Fig. 1, B and C, respectively).

#### CD4 T cells are essential for secondary expansion of Listeria-specific CD8 T cells

As the primary CD8 T cell response to LM infection was CD4 T cell dependent, we determined whether a similar requirement existed for the recall response to secondary infection. To test this, the recall CD8 T cell response was analyzed in C57BL/6J mice primed with  $10^3$  CFU of LM-OVA, rested for at least 7 mo, and then depleted of CD4 T cells before and during secondary challenge with  $10^4$  CFU of LM-OVA. Control mice mounted a robust recall response in the blood, which peaked on day 6 after reinfection (Fig. 2A). However, although CD4 T cell-depleted mice mounted a recall response, Ag-specific T cell numbers in the blood were markedly reduced throughout the response, and by  $\sim 1$  mo after infection were decreased 80% compared with controls (Fig. 2A). At the peak of the recall response, in the absence of CD4 T cells, substantially fewer OVA-specific CD8<sup>+</sup> T cells were present in tissues, such as the spleen and LP, compared with control mice (70 and 79% reductions, respectively; Fig. 2B). The responses in the lung and liver were less affected by the absence of CD4 T cells, suggesting potential tissue-specific effects of CD4 T cell help. Interestingly, when CD4 T cells were continuously depleted after the recall, the effect became more evident when the generation of secondary CD8 memory cells was examined. Thus, 50 days after recall, the percentages of OVA-specific CD8 T cells in the spleen, LP, lung, and liver were all reduced (85, 90, 85, and 81%, respectively; Fig. 2, C and D). This difference was also evident when comparing the total number of tetramer-positive cells (Fig. 2E).

We also examined the requirement for CD4 T cells in promoting a secondary CD8 T cell response at various times after primary infection. Surprisingly, the CD4 dependence of the CD8 recall response was accentuated as the time after initial infection increased (Fig. 2F). Thus, the peak recall response was inhibited by  $\sim 50\%$  at 3 mo after infection and by  $\sim 60\%$  at 5 mo, and at 7 mo after infection,  $>80\%$  inhibition was noted when CD4 T cells were depleted only during the recall response. Although we do not yet know the basis for this finding, it does not appear to be due to

major changes in memory precursor frequencies, which were not significantly different from 3–7 mo after primary infection (data not shown).

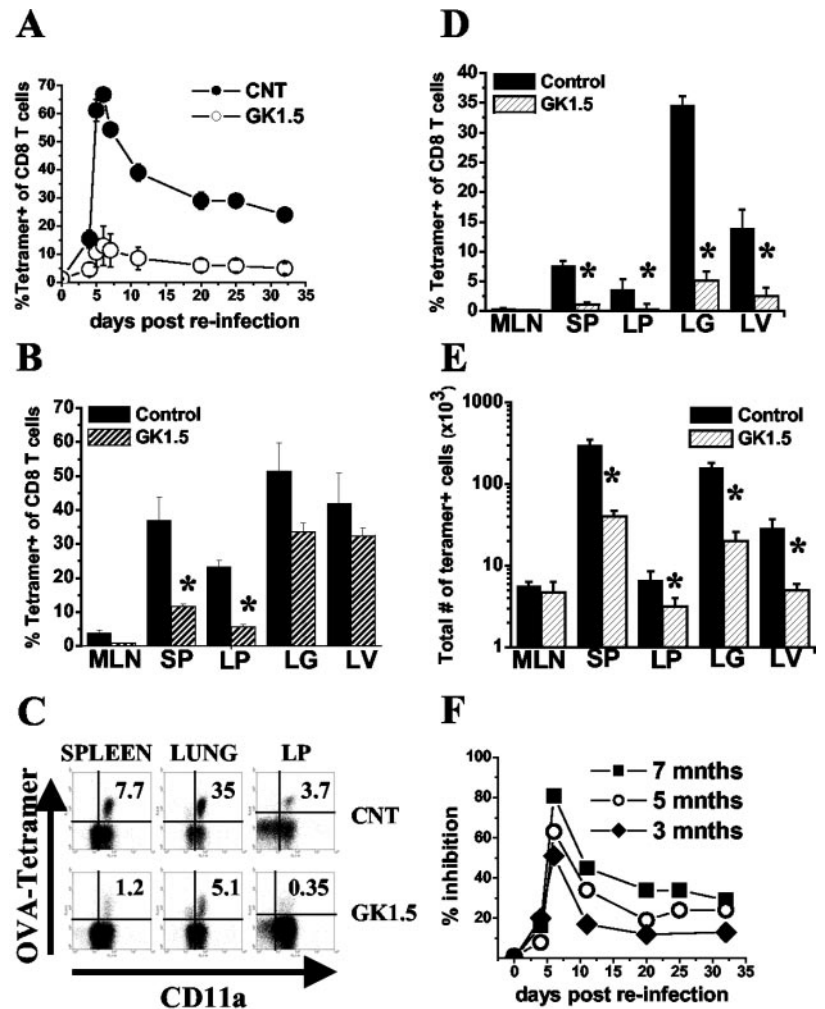
#### The requirement for CD4 T cell help is not a property intrinsic to CD8 memory T cells

As recent reports suggest that CD4 T cells may imprint functional properties on memory cell precursors (20, 21), we determined whether the requirement for CD4 T cells in the recall response was engendered during initial priming. To this end, mice were either primed by LM-OVA or VSV-OVA infection and secondarily infected with the heterologous pathogen (Fig. 3). CD4 T cells were depleted only during the recall response. Mice initially infected with LM-OVA mounted a CD4 T cell-independent secondary CD8 T cell response to VSV-OVA infection (Fig. 3A). In stark contrast, CD8 memory T cells generated via VSV-OVA infection exhibited a profound requirement for CD4 T cells when responding to LM-OVA infection (Fig. 3B). Fifty days later, with continuous CD4 T cell depletion, the memory cell levels in the tissues reflected the effects observed in blood (Fig. 3, C–F), suggesting that CD4 T cells did not play a significant role in memory cell maintenance at least until day 50. These results indicated that memory cells induced after LM-OVA infection were not inherently CD4 T cell dependent, but that the dependence of the CD8 T cell response on help was a property linked to infection with LM-OVA.

#### Functional CD8 memory cell development is CD4 T cell independent

Our results to date indicated that CD4 T cells controlled the magnitude of the responses of naive and memory CD8 T cells to LM infection. However, it remained possible that during priming, CD4 T cells also afforded functionality to memory CD8 T cells, as recently reported (20, 21). To test this possibility, CD4 T cells were depleted continuously throughout the LM-OVA primary and recall responses or only during the recall response to LM-OVA infection. Sixty days after primary infection, in mice in which CD4 T cells had been depleted from the start, memory cell levels were  $\sim 40\%$  those in control mice (0.2 and 0.45% of blood CD8 T cells, respectively). After secondary infection, the recall response was inhibited to a similar extent ( $\sim 60\%$  of control) whether CD4 T cells

**FIGURE 2.** The recall CD8 T cell response to LM-OVA infection requires CD4 T cells. Mice were infected i.v. with  $10^3$  CFU of LM-OVA and, after 7 mo, treated with control IgG or the anti-CD4 mAb GK1.5 from 6 and 5 days before re-infection with  $10^4$  CFU of LM-OVA and every 6 days thereafter until day 50 (A–E). At various time points, the percentage of Ag-specific CD8 T cells in the blood was assessed by flow cytometry (A). Six (B) and 50 (C–E) days after recall infection, cells were isolated from the various tissues, and the mean percentage  $\pm$  SEM (C and D) or the total number  $\pm$  SEM (E) of OVA-specific CD8 T cells was assessed by flow cytometry. F, Mice rested for 3, 5, or 7 mo after primary infection with  $10^3$  CFU of LM-OVA, were depleted of CD4 T cells from 6 and 5 days before secondary infection with  $10^4$  CFU of LM-OVA and every 6 days thereafter. The percent inhibition was calculated by comparing the total percentage of tetramer-positive cells in the blood in control and anti-CD4 mAb-treated mice at the indicated times after challenge infection. \*,  $p < 0.05$ . Data are representative of three experiments with three mice per group.



were depleted throughout the response or only during the recall (Fig. 4A). A similar effect was observed in the tissues (data not shown). As memory cell numbers were fewer in the continuously CD4 T cell-depleted mice, this result indicated that memory CD8 cells generated in the absence of CD4 T cells responded normally to rechallenge. Similar results were obtained using CD4-intact or depleted VSV-infected mice (data not shown). As the number of CD8 memory cells is inversely proportional to the magnitude of the recall response to LM infection (23, 33), the possibility remained that on a per cell basis, “helped” and “unhelped” memory cells were not responding equivalently. Thus, we directly compared each memory population by transferring equal numbers of tetramer-positive cells to normal hosts, followed by infection with LM-OVA. The kinetics of the response were followed beginning on day 3 after infection (Fig. 4B). No difference was detected in the recall response of memory CD8 T cells generated in the presence or the absence of CD4 T cells. In another experiment, tetramer-positive cells were quantitated in the tissues at the peak of the response, and no differences were observed between the response of CD8 memory cells spawned in the presence or the absence of CD4 T cells (data not shown).

#### Effector memory cell function does not require CD4 T cells

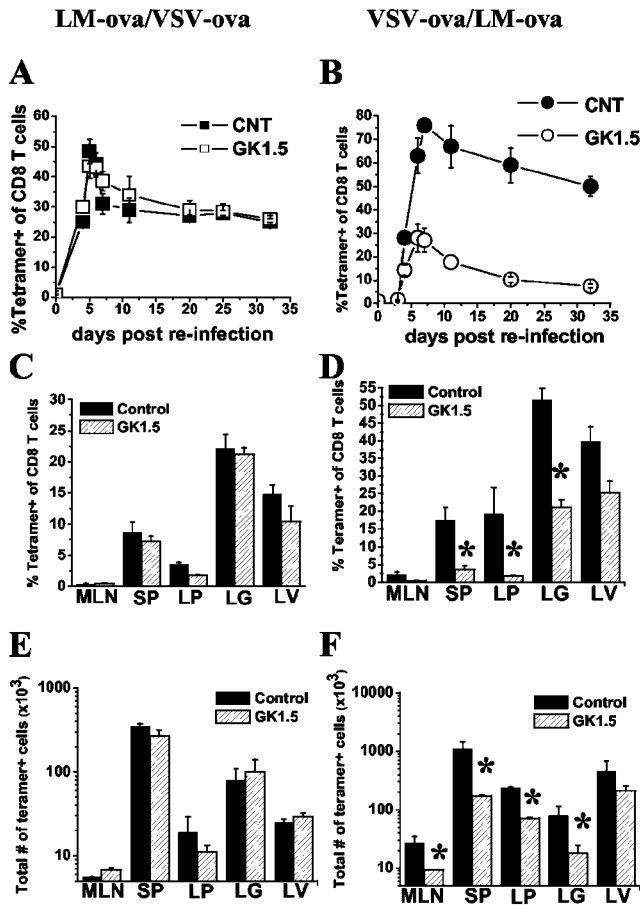
As CD4 T cells augmented memory CD8 T cell proliferation or survival, it was of interest to examine other functional properties of memory CD8 T cells. To this end, the ability to produce IFN- $\gamma$  and the ex vivo lytic activity of lung effector memory cells generated

in the presence or the absence of CD4 T cells was measured (Fig. 5, A and B, respectively). Analysis was performed 50 days after secondary infection with continuous CD4 T cell depletion. After in vitro stimulation with peptide, lung-derived LM-specific memory CD8<sup>+</sup> T cells generated in the absence of a concurrent CD4 T cell response were able to produce IFN- $\gamma$  at either the peak of the recall response (data not shown) or 50 days after recall (Fig. 5B).

When the direct ex vivo lytic activity of lung memory CD8 T cells was compared on a per cell basis, no difference in lytic activity was detected between cells isolated from CD4-intact vs CD4-depleted mice (Fig. 5C). Due to the low number of Ag-specific CD8 T cells in the spleens of CD4-depleted mice E:T cell ratios were too low to obtain informative data. Lung CD8 memory T cells from mice recalled with VSV-OVA and depleted of CD4 T cells also exhibited no significant difference in CTL activity compared with memory cells from control mice (Fig. 5D). Thus, whether the response was CD4 dependent (LM) or CD4 independent (VSV), CD8 memory cells were able to respond proliferatively and functionally despite the absence of CD4 T cells during priming or recall.

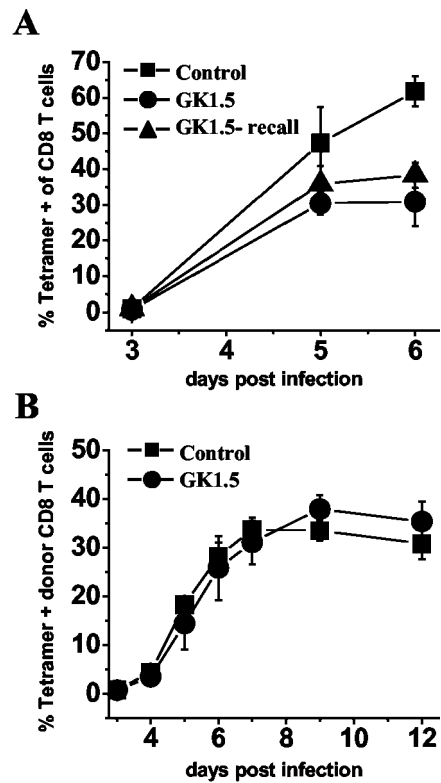
#### CD40L is required for the reactivation of Ag-specific memory LM CD8 T cells

Although memory cells have been suggested to be less dependent on costimulation than naive cells for activation (34–36), we examined whether there was a linkage between the requirement for CD4 T cells during recall and a role for costimulation. To this end,



**FIGURE 3.** The dependence of memory CD8 T cells on help is linked to the infectious agent. Seven months after primary infection with  $10^3$  CFU of LM-OVA (A, C, and E) or 4 mo after infection with  $10^6$  PFU of VSV-OVA (B, D, and F), mice were treated with control IgG or the anti-CD4 mAb GK1.5 from 6 and 5 days before secondary infection with VSV-OVA i.v. (A, C, and E) or LM-OVA i.v. (B, D, and F), and treatment was continued every 6 days thereafter. At various time points the percentage of Ag-specific CD8 T cells in the blood was assessed by flow cytometry (A and B). C–F, Fifty days after recall infection (with continuous anti-CD4 treatment), cells were isolated from the various tissues, and the percentage (C and D) or total cell number (E and F) of Ag-specific CD8 T cells was assessed by flow cytometry.

secondary infection of mice primed 6 mo previously with LM-OVA was conducted in the presence of anti-CD40L mAb to block CD40/CD40L interactions. This treatment was performed in CD4 T cell-intact or -depleted mice to test potential interactive effects of CD4 T cells and CD40L. Tetramer-positive CD8 T cells were quantitated in various tissues 6 days after reinfection (Fig. 6A). CD40L blockade resulted in 75% inhibition of the splenic CD8 T cell response, which was a level of inhibition similar to that obtained with removal of CD4 T cells (62% inhibition). Interestingly, when CD40L/CD40 interactions were blocked in the absence of CD4 T cells, inhibition was increased to 94%. The enhanced inhibition observed with concomitant CD40L block and CD4 T cell removal was also evident in tissues such as the lung and liver, where the response was inhibited 82 and 80%, respectively, compared with treatments with anti-CD40L or anti-CD4 alone, which resulted in modest levels of inhibition (39 and 31% inhibition in the lung; 48 and 33% inhibition in the liver, respectively). These levels of inhibition were similar when the total number of tetramer-positive cells in each organ was determined (Fig. 6B). We also examined the role of B7 costimulation in the recall response

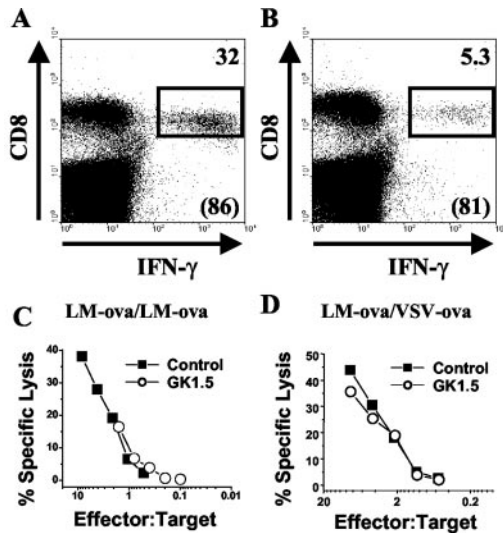


**FIGURE 4.** CD8 T cell priming in the absence of CD4 T cells generates functional memory. A, Mice were treated with either control IgG (■) or the anti-CD4 mAb GK1.5 on days -7, -6, and 0 before the primary infection and continuously throughout the recall response (●) or were only treated for the secondary response on days -3, -2, and 0 before the secondary infection (Δ). Mice were infected with  $10^3$  CFU of LM-OVA for primary infections, rested for 2 mo, and then infected with  $10^4$  CFU of LM-OVA for secondary infections. B, Equal numbers ( $5 \times 10^4$ ) of memory CD8 T cells from continuously CD4 T cell-depleted (●) or control C57BL/6-Ly5.1 mice (■), primed at least 70 days previously, were adoptively transferred into naive B6 mice (C57BL/6-Ly5.2) and challenged with LM-OVA ( $10^3$  CFU). Ag-specific CD8 T cells were quantitated in the blood at various time points after challenge. Data are representative of two experiments with five mice per group.

by treating mice with CTLA4-Ig during secondary infection. Although CTLA4-Ig treatment inhibited the response by ~40%, this level of inhibition was not enhanced in the absence of CD4 T cells (data not shown).

### Discussion

The results presented in this study demonstrated a differential requirement for CD4 T cells in the response of memory CD8 T cells to secondary challenge. This requirement mirrored a similar necessity in the primary CD8 T cell response to infection. Although the primary and recall CD8 T cell responses to VSV infection were largely helper independent, both responses against LM infection required CD4 T cells. The CD4 T cell requirement extended to CD8 T cell responses in both lymphoid and nonlymphoid tissues after infection with LM. In the case of recall responses, it is generally held that memory T cells are less dependent on costimulation for reactivation (34–36) and are able to respond more rapidly and to much lower levels of Ag than naive T cells (36), although in at least one case human influenza virus-specific memory cells required CD28 costimulation for reactivation *in vitro* (37). Our results indicated that the requirement for CD4 T cells to augment the CD8 T cell response was related to the pathogen inciting the

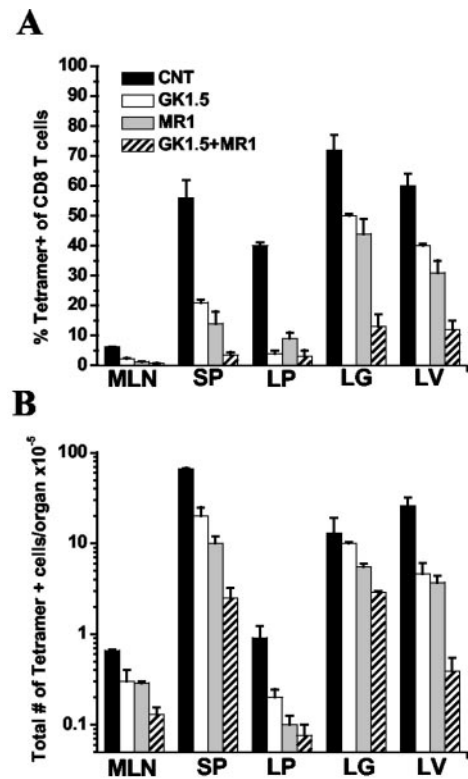


**FIGURE 5.** CD8 T cell effector memory functions are not affected in the absence of CD4 T cells. Fifty days after a secondary challenge with LM-OVA, lung T cells from CD4 T cell-intact (A) or CD4 T cell-depleted (B) mice were analyzed for their capacity to synthesize IFN- $\gamma$ . Numbers in parentheses are the percentage of tetramer-positive CD8 T cells that secrete IFN- $\gamma$ . Fifty days after secondary challenge with either  $10^4$  CFU of LM-OVA (C) or  $10^6$  PFU of VSV-OVA (D), lymphocytes were incubated for 5 h with  $^{51}\text{Cr}$ -labeled untreated EL4 target cells or target cells pulsed with SIINFEKL peptide. The E:T cell ratios shown are corrected for the number of tetramer-positive cells. Data shown are from a pool of two mice (an equivalent pool from two additional mice gave similar results).

secondary infection. Thus, whether memory cells were induced by VSV or LM infection, a secondary response to LM infection required CD4 T cell help, indicating that the necessity for help was not a property intrinsic to the memory CD8 T cells.

Our findings are consistent with the possibility that distinct pathogens differentially modulate DC costimulatory capacity. Perhaps in the case of VSV, DC are directly infected, resulting in APC activation and direct up-regulation of the necessary costimulatory molecules. In contrast, although LM infection incites a robust inflammatory response, CD4 T cells mediate DC activation via a CD40-CD40L interaction. It is also possible that VSV, but not LM, infection induces CD8 T cells to express CD40, which can also drive APC activation. Other reports have examined the role of CD4 T cells in driving a secondary CD8 T cell response to infection. The primary and recall CD8 T cell responses to influenza virus infection require CD4 T cells to bring about normal CD8 T cell expansion (8). In another study, when CD4 $^{-/-}$  mice were infected with rLM expressing LCMV-gp33, a reduced population of CD8 memory T cells was produced that was able to mount a robust recall response to LCMV challenge (19). In this case, the recall response was reduced in proportion to the reduced number of memory cells generated without CD4 T cell help. As the CD8 response to LCMV is CD4 and CD28 independent (9, 38), this result is perhaps not surprising. However, this finding is not in keeping with recent results suggesting that CD8 memory T cells induced in CD4 $^{-/-}$  or I- $\text{A}\beta^{-/-}$  mice by LCMV/LM (20) or LM/LM (21) primary/secondary infections, respectively, mount a poor proliferative recall response. Similarly, priming of adenovirus- or LCMV-specific CD8 T cells in CD4-depleted or I- $\text{A}\beta^{-/-}$  mice, respectively, results in memory CD8 T cells that are able to produce IFN- $\gamma$ , but are unable to expand *in vitro* in the absence of CD4 T cells (9).

In our hands the primary and secondary CD8 T cell responses to LM infection were consistently CD4 T cell dependent, and the



**FIGURE 6.** CD40L is required for optimal reactivation of LM-specific memory CD8 T cells. Mice were initially infected with  $10^5$  CFU of LM-OVA and, after 6–7 mo, were reinfected with  $10^4$  CFU of LM-OVA. Control or CD4 T cell-depleted mice were treated with anti-CD40L on days 0, 2, and 4 starting on the day of reinfection. On day 6 after reinfection, the percentage (A) or total number (B) of OVA-specific CD8 T cells was assessed by flow cytometry. Values are means derived from three or four mice per group  $\pm$  SEM.

memory cells generated in the absence of CD4 T cells were fully functional. This was true in the case of a help-dependent response (LM infection) and a help-independent response (VSV infection). Moreover, LM-specific CD8 memory cells generated and maintained in the absence of CD4 T cells and transferred to normal hosts responded to challenge infection as efficiently as did their “helped” counterparts. The reasons for the discrepancies in the different systems could be related to the infectious dose, the pathogen used, or the timing and kinetics of the responses. With regard to the latter, it is interesting to note that the dependency of CD8 memory T cells on CD4 help increased with increasing time after primary infection. This phenomenon was not related to the precursor frequency of CD8 memory cells, suggesting that the effect was cell autonomous or dependent on other factors, such as APC function. In any case, our results do not support a general concept where CD8 memory T cells are instilled with functional properties by CD4 T cells during the primary response. Indeed, even in cases where the immunogen is weak and the primary CD4 T cell response is minimal, the CD8 memory T cells produced mount a robust recall response (39).

The requirement for CD4 T cell help in the recall response correlated with the requirement for costimulation. Inhibition of CD40-CD40L interactions led to inhibition of the CD8 T cell recall response to LM infection to a magnitude similar to that obtained with CD4 T cell depletion (Fig. 6). Interestingly, the combination of anti-CD40L mAb and CD4 T cell depletion resulted in inhibition substantially greater than that observed with either treatment alone. This result suggests the novel possibility that CD4 T cells

and CD40L augmentation are operating independently. In contrast, although treatment with CTLA4-Ig also inhibited the secondary response in the spleen, albeit to a lesser extent than CD40L blockade, the combination of CTLA4-Ig and CD4 T cell depletion had no additive effect (data not shown). A recent report also showed a requirement for CD28 in the secondary response to LM infection (40). However, CD28<sup>-/-</sup> mice were used, and in our hands blocking the CD28-B7 interactions greatly inhibited the primary response, suggesting that the reduced secondary response seen in CD28<sup>-/-</sup> mice may be a reflection of a reduced primary response. Therefore, CD28 may play an ancillary role in the recall response, although the confounding effects of B7 blockade on CTLA4 function (41) may mask a larger role for CD28-B7 interactions in augmenting the response. Nonetheless, despite the heightened frequency of Ag-specific T cells and their heightened activation status as memory cells, costimulation played a major role in magnifying their capacity to proliferate and/or survive. Importantly, even in nonlymphoid tissues, the recall response was CD4 T cell and costimulation dependent. This finding suggested that CD8 memory T cells interacted with professional APC to spawn a vigorous secondary response. It might be expected that memory CD8 T cells encountering infected parenchymal cells, such as liver hepatocytes or intestinal epithelial cells, would be triggered to secrete lytic molecules and cytokines and to proliferate. Alternatively, cross-presentation of Ag could be mediated by APC within the nonlymphoid tissues. It is also possible that triggering of effector pathways is dissociated from the proliferative program in nonlymphoid tissues. If this were the case, then the secondary proliferative response could perhaps be driven primarily in secondary lymphoid tissues, which results in migration of effector cells to all tissues (26).

Overall, our data support the concept that in response to primary and secondary infection, one of the major roles of CD4 T cells is to maximize the magnitude of the CD8 T cell response, rather than imprint functional properties. Given the substantial magnitude of many CD8 T cell responses, a protective CD8 T cell response may nevertheless be generated without CD4 T cells. However, with regard to achieving effective vaccination, understanding the role of T cell help in producing a functionally and numerically significant population of memory CD8 T cells remains an important goal.

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