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Antiviral CD4 and CD8 T-cell memory: differences in the size of the response and activation requirements

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Following acute lymphocytic choriomeningitis virus (LCMV) infection, there is a potent antiviral CD8 T-cell response that eliminates the infection. This initial CD8 T-cell response is followed by a period of memory during which elevated numbers of virus-specific CD8 T cells remain in the mouse. CD4 T cells are also activated after LCMV infection, but relatively less is known about the magnitude and duration of the CD4 response. In this study, we used intracellular staining for interferon-γ to measure both CD4 and CD8 responses in the same mice at the single cell level. After LCMV infection, there was an increase in the number of activated CD4 T cells and an associated increase in the number of virus-specific CD4 T cells. At the peak of this expansion phase, the frequency of virus-specific CD4 T cells was 1 in 20 ($0.5–1.0 \times 10^6$ per spleen). Like the CD8 response, long-term CD4 memory could be found up to a year after the infection with frequencies of approximately 1 in 260 ($0.5–1.5 \times 10^5$ per spleen). However, the magnitude of virus-specific CD8 T cells was greater than virus-specific CD4 T cells during all phases of the immune response (expansion, death, and memory). At day 8, there were 20- to 35-fold more virus-specific CD8 T cells than CD4 T cells. This initial difference in cell number lasted into the memory phase as there remained a ten- to 20-fold difference in the CD8 and CD4 responses. These results highlight the importance of the expansion phase in determining the size of the memory T-cell pool. In addition to the difference in the magnitude, the activation requirements of CD8 and CD4 T-cell responses were different: CD8 T responses were not affected by blockade of CD40–CD40 ligand interaction whereas CD4 responses were reduced 90%. So while there is long-term memory in both the CD8 and CD4 compartments, the rules regulating the activation of CD8 and CD4 T cells and the overall magnitude of the responses are different.

Keywords: CD8 T-cell memory; CD4 T-cell memory; CD40–CD40 ligand interaction; T-cell activation; viral immunity

1. INTRODUCTION

T cells play an essential role in the control of viral infections (Ahmed & Gray 1996). Potent CD8 T-cell responses are induced during infections of humans with human immunodeficiency virus (HIV), Epstein–Barr virus, and cytomegalovirus and in mice following infection with lymphocytic choriomeningitis virus (LCMV), and vaccinia virus, vesicular stomatitis virus and murine γ-herpesvirus (Asano & Ahmed 1996; Bi *et al.* 1995; Borrow *et al.* 1997; Butz & Bevan 1998; Callan *et al.* 1996; Cousens *et al.* 1995; Doherty *et al.* 1997; Koup *et al.* 1994; Lau *et al.* 1994; Murali-Krishna *et al.* 1998, 1999; Oldstone 1996; Pantaleo *et al.* 1994, 1997; Selin *et al.* 1996; Slifka *et al.* 1997; Sourdive *et al.* 1998; Steven *et al.* 1997; Sunil-Chandra *et al.* 1994; Whitmire *et al.* 1996; Willis *et al.* 1996). These virus-specific CD8 T cells control viral infections by killing infected cells and also by secreting interferon-γ (IFN-γ) and other cytokines (Butz & Bevan 1998; Murali-Krishna *et al.* 1998; Sourdive *et al.* 1998; Walsh *et al.* 1994). CD4 T cells contribute to viral clearance by producing interleukin-2 (IL-2), which facilitates CD8 T-cell activation and expansion, and by secreting IFN-γ and tumour necrosis factor to activate macrophages and inhibit viral replication. In addition to secreting cytokines, CD4 T-helper cells mediate help to humoral responses by direct cell-to-cell interaction involving CD40 ligand (CD40L) (expressed on activated CD4 T cells) and CD40 expressed on B cells. Despite their importance, relatively little is known about the magnitude and duration of virus-specific CD4 T-cell responses, or if the rules that govern CD4 activation are the same as those responsible for CD8 activation.

In this report, we have examined the magnitude and duration of virus-specific CD4 and CD8 T-cell responses during acute LCMV infection. We show that long-term memory is seen for both CD4 and CD8 T-cell responses. However, the magnitude of the CD4 response was at all times lower than the CD8 response in the same mice. Furthermore, activation of CD4 T cells required CD40–CD40L interaction whereas CD8 T cells were unaffected by blockade of this interaction. These results indicate that

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not only are the activation requirements different between CD4 and CD8 T cells, but the mechanisms regulating the sizes of the responses are different.

2. MATERIAL AND METHODS

(a) *Mice and virus*

C57BL/6 (H-2^b) mice were purchased from Jackson Laboratory, Bar Harbor, ME, USA. Generation of the CD40L-deficient mice has been described previously (Renshaw *et al.* 1994; Xu *et al.* 1994). The mice used in these studies were from a colony established at Emory University, Atlanta, GA, USA. The Armstrong CA 1371 strain of LCMV was used in these studies and mice were infected intraperitoneally with 2×10^5 plaque-forming units (PFU) of LCMV Armstrong (Ahmed *et al.* 1984). The levels of virus in serum and tissues were quantified by a plaque assay on Vero cells (Ahmed *et al.* 1984).

(b) *Flow cytometry and intracellular staining for IFN-γ*

Spleen cells were surface stained with antibodies which recognize CD8 (clone 53–6.7), CD4 (clone RM4–5) and CD44 (clone IM7) using 1 μg of antibody per 10^6 cells. These antibodies were purchased from Pharmingen, La Jolla, CA, USA. Intracellular staining for IFN-γ was used to quantify virus-specific T cells and has been described previously (Murali-Krishna *et al.* 1998; Whitmire *et al.* 1998). Spleen cells (10^6 cells per well in 96-well flat-bottomed plates) were cultured *in vitro* with or without peptide stimulation in the presence of brefeldin A (Golgistop^{TM}, Pharmingen) for 5 h, followed by surface and intracellular stain, as described previously (Murali-Krishna *et al.* 1998). For intracellular IFN-γ stain we used fluorescein isothiocyanate-conjugated monoclonal rat anti-mouse IFN-γ (clone XMG1.2, Pharmingen). For more details regarding the quantification of CD8 T-cell responses, see Murali-Krishna *et al.* (1998), and for a description of assays used to quantify CD4 T-cell responses, see Whitmire *et al.* (1998).

3. RESULTS

(a) *Expansion of CD4 and CD8 T cells*

Adult mice were infected with 2×10^5 PFU of the Armstrong strain of LCMV and T-cell responses in the spleen were analysed by flow cytometry. Consistent with previous reports (Ahmed *et al.* 1984, 1988; Byrne *et al.* 1984; Fung-Leung *et al.* 1991; Kagi *et al.* 1994; Lau *et al.* 1994; Moskophidis *et al.* 1987; Walsh *et al.* 1994), high numbers of cytotoxic T lymphocytes (CTL) were generated and infection was controlled by day 8 after infection as indicated by a plaque assay of serum and several other tissues including spleen, liver, lung and kidney (data not shown). At this time, there was a shift in the percentage of T cells which expressed the activation marker CD44. In uninfected mice, the ratio of CD44^{hi}:CD44^{lo} CD8 T cells was 0.5, and this changed to 11 at day 8 after infection. Afterwards, this ratio returned to levels almost the same or slightly higher than those found in normal, uninfected mice. Similarly, the ratio of CD44^{hi}:CD44^{lo} CD4 T cells was 0.5 at day 0, and this changed to a ratio of 3 by day 8. This ratio changed to 1.9 by day 15 as the immune response to LCMV was subsiding and approached levels seen in uninfected mice by day 30 at approximately 0.9.

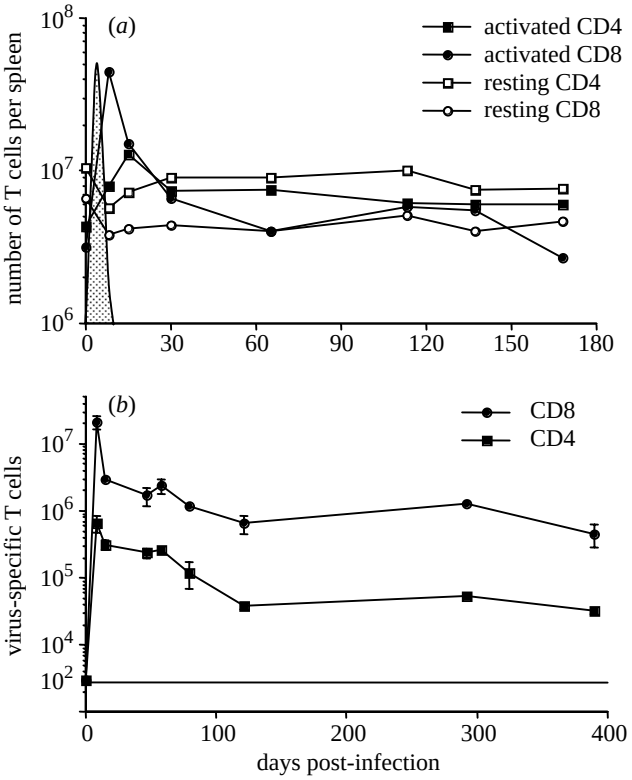

Figure 1. T-cell responses in the spleen after infection. Mice (six to eight weeks old) were infected with LCMV at day 0 and CD8 and CD4 T-cell responses were followed over time. (*a*) The total number of activated and resting T cells was determined by flow cytometry analysis and spleen cell number counts. 'Activated' cells were CD44^{hi} and 'resting' T cells were CD44^{lo}. The expansion of activated T cells peaked at day 8 (for CD8) or days 8–15 (for CD4). In contrast, resting T cells were fairly stable at all times after infection. The data shown are the average from six to 15 mice for each time-point. The dotted curve represents the level of virus which peaks at days 3–4 after infection and is eliminated by day 8. For clarity, error bars have been removed so that activated and resting CD8 and CD4 T-cell responses could be shown together. (*b*) Virus-specific CD8 and CD4 response after infection as determined by intracellular staining for IFN-γ. Virus-specific CD8 T cells (specific to NP396–404, GP33–41, GP276–286, NP205–212) expanded much more than CD4 T cells (specific to GP61–80) during the first week of infection. This was followed by a contraction phase (weeks 2–4) and then a period of memory (after one month) which lasted more than a year for both CD8 and CD4 T cells. Note that the magnitude of the CD8 response was at all times greater than that of the CD4 response. Data were taken from more than four experiments and include 4–15 mice for each time-point. The error bars show standard deviation.

There was also an increase in the total number of activated T cells (figure 1*a*). The number of activated, CD44^{hi} CD4 T cells increased from 4×10^6 per spleen at day 0 to 8×10^6 per spleen at day 8 and 13×10^6 by day 15. In contrast, the number of CD44^{lo} CD4 T cells changed little after infection. There was a slight decrease to approximately 6×10^6 at day 8, but cell numbers returned to approximately 9×10^6 by day 30. For comparison, figure 1*a* also shows activated CD8 T-cell numbers. The number of CD44^{hi} CD8 T cells increased from 3×10^6 per uninfected spleen to 45×10^6 per spleen at day 8 after

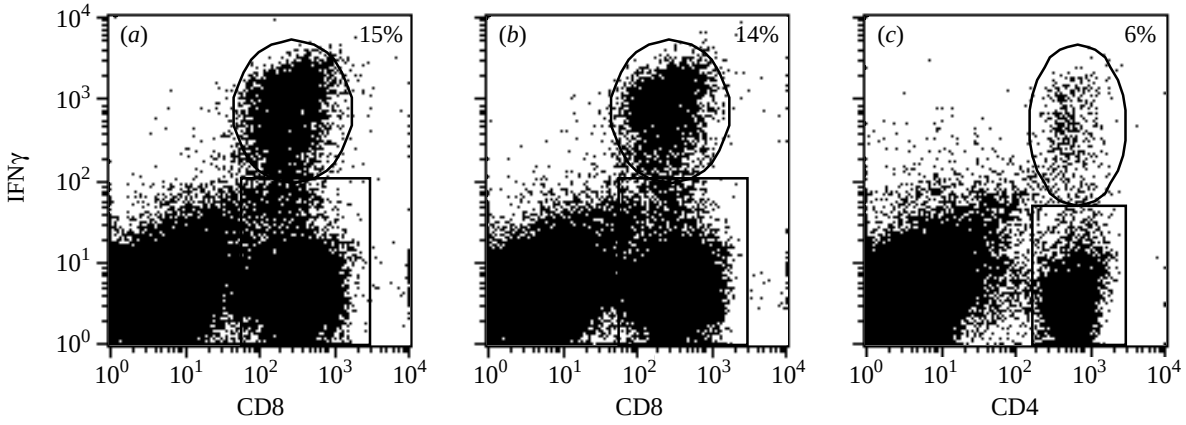

Figure 2. IFN-γ production by virus-specific CD8 (*a*, *b*) and CD4 (*c*) T cells. Spleen cells from day 8 infected mice were stimulated *in vitro* for 5 h with either MHC class-I-restricted epitopes (NP396–404 (*a*), GP33–41 (*b*)) or with MHC class-II-restricted epitope GP61–80 (*c*). Shown is a representative example of CD8 and CD4 responses. Note that the CD8 response was greater than the CD4 response. Numbers shown in the dot plots are the percentage of CD8 or CD4 T cells which were IFN-γ positive. Without stimulation, ⩽0.1% of T cells were IFN-γ positive.

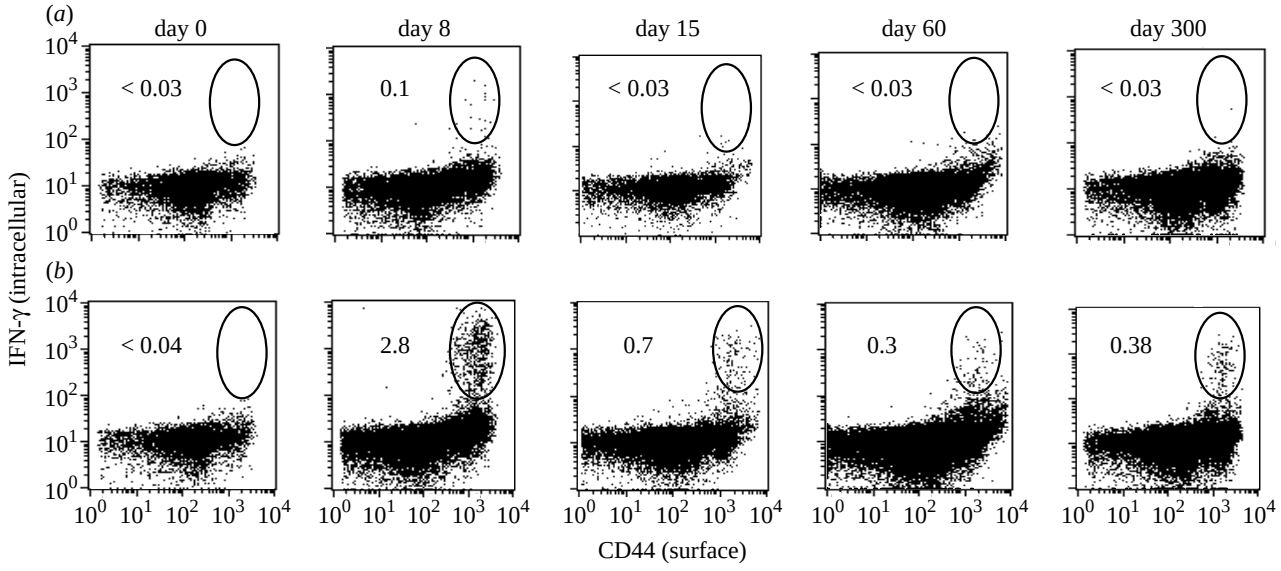

Figure 3. Longevity of LCMV-specific CD4 T cells. Intracellular IFN-γ in CD4 T cells responding to GP61–80 and NP309–328 at days 0, 8, 15, 60 and 300 after infection. (*a*) No peptide and (*b*) peptide stimulation. Spleen cells were stimulated for 5 h with a mixture of both peptides and then surface stained for CD4 and CD44 and stained for intracellular IFN-γ. Flow cytometry dot plots gated on CD4 T cells show expression of IFN-γ (*y*-axis) versus that of CD44 (*x*-axis). The numbers shown are the percentage of CD4 cells which are IFN-γ positive. Following the expansion of LCMV-specific CD4 T cells at day 8 (IFN-γ positive), there was a decrease which can be seen at day 15. The frequency of memory cells established by day 60 remained unchanged even at day 300. At day 8, some CD4 T cells made IFN-γ even without stimulation. These probably represent activated cells which were IFN-γ positive *in vivo* and retained the cytokine throughout the staining process. Note that all CD4 T cells making IFN-γ in response to these peptides were $CD44^{hi}$. Taken from Whitmire *et al.* (1998).

infection, fivefold more than the number of activated CD4 T cells. These cells also decreased in number to homeostatic levels by days 30–40. In contrast, the number of $CD44^{lo}$ CD8 T cells remained fairly stable at all times after infection.

Expansion of activated T cells was associated with an increase in the number of virus-specific T cells (figures 1*b* and 2). The antiviral CD8 response peaked at day 8 with $2–3 \times 10^7$ cells per spleen. This was followed by a contraction period (days 8–30) where 95% of these cells died. Afterwards, a relatively stable number of virus-specific CD8 T cells (approximately 1×10^6) persisted for the life of the mouse. The antiviral CD4 response showed a similar pattern (i.e. three distinct phases: expansion, death, and memory). At day 8, $5–9 \times 10^5$ GP61–80-specific cells could be found. This was followed by a contraction period in which approximately 90% of the CD4 T cells died, and then a period of memory. During the memory period, approximately 1 in 260 CD4 or $0.5–2 \times 10^5$ GP61–80-specific CD4 T cells remained. Interestingly, the virus-specific CD8 response was always stronger than the CD4 response. This trend started at

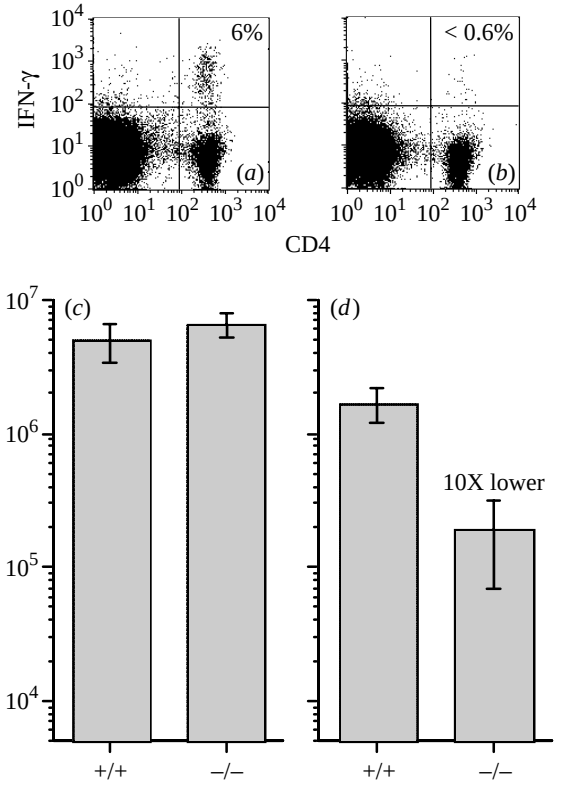

Figure 4. Differential requirement of CD40–CD40L for virus-specific CD8 and CD4 T cells. T-cell responses in day 8 infected +/+ (*a*) and CD40L-deficient (*b*) mice were analysed by intracellular staining for IFN-γ. Flow cytometry dot plots showing levels of intracellular IFN-γ (*y*-axis) and surface CD4 (*x*-axis) after stimulation with GP61–80 are shown in (*a*, *b*). The numbers in the upper quadrant are the percentage of CD4 T cells which were IFN-γ positive. Note that the frequency of virus-specific CD4 responses was lower in the CD40L-deficient mice. The total number of LCMV-specific CD8 (*c*) and CD4 (*d*) responses in the same mice. Spleen cells were stimulated with GP33–41 for the CD8 response and with GP61–80 for the CD4 response. Note that the CD8 response was not affected by the absence of CD40–CD40L interaction whereas the CD4 response was reduced 90%. Data are the average of four mice with standard deviations.

day 8, with the CD8 response 20- to 35-fold greater than the CD4 response, and continued into the memory phase with the CD8 response ten- to 20-fold greater than the CD4 response. However, these lower levels of CD4 memory were stable, and they lasted for more than one year after infection. Immune mice analysed during this time were able to mount anamnestic CD4 responses as indicated by a second expansion of virus-specific CD4 T cells after reinfection with LCMV. Furthermore, both Th1 (IL-2 secreting) and Th2 (IL-4 secreting) memory T cells could be detected during this period (data not shown).

Figure 3 shows an example of antiviral CD4 responses measured over time. It depicts CD4 T-cells surface-stained for CD44 and stained for intracellular IFN-γ. No virus-specific CD4 T-cell responses were detected in uninfected mice, but by day 8, 2.8% (total of 7.8×10^5 per spleen) of CD4 T cells made IFN-γ upon restimulation. Since all of these cells were in the $CD44^{hi}$ subset this corresponded to a frequency of 1 in 18 activated CD4 T cells. There was a decrease in the percentage of CD4 T cells which made IFN-γ at day 15 to 0.7% (1 in 143), and a drop in number to 5.9×10^4 specific cells per spleen. Afterwards, CD4 memory was stable as the percentage of virus-specific CD4 T cells changed very little from day 60 (0.3%) to day 300 (0.4%). The frequency of memory cells per activated CD4 was 1 in 47 at day 300, which corresponded to 4.1×10^4 CD4 T cells per spleen.

Since the antiviral CD4 and CD8 T-cell responses differed greatly in magnitude, it was of interest to examine if these subsets also had distinct activation requirements. Since CD40–CD40L interaction has been found to be important for CD4 responses in other systems (Campbell *et al.* 1996; Grewal *et al.* 1995; Kamanaka *et al.* 1996; Soong *et al.* 1996), we examined how LCMV-specific CD4 and CD8 T-cell responses would be affected by the absence of this interaction. Therefore, CD40L-deficient mice were infected and antiviral T-cell responses were quantified at day 8 after infection. CD40L-deficient mice generate normal levels of LCMV-specific CTL and clear the infection by day 8 (Borrow *et al.* 1996; Oxenius *et al.* 1996; Whitmire *et al.* 1996). High percentages (8–20%) of CD8 T cells were specific to NP396–404 in both +/+ and CD40L-deficient mice. In contrast, +/+ and CD40L-deficient mice differed in their CD4 responses. Figure 4*a* shows that 6% of the CD4 T cells were specific to GP61–80 in +/+ mice, whereas < 0.6% were specific in the CD40L-deficient mice. Figure 4*b* shows the average number of antiviral T cells found in +/+ and CD40L-deficient mice. While the number of GP33–41-specific CD8 T cells was similar in +/+ and CD40L-deficient mice, the number of GP61–80-specific CD4 T cells was reduced approximately 90% in the absence of CD40–CD40L interaction in these same mice. Also, when the numbers of IL-2- or IL-4-secreting CD4 T cells were quantified by enzyme-linked immunospot assay (ELISPOT) or when cytokines made by purified CD4 T cells were measured using enzyme-linked immunosorbent assay (ELISA), the responses were weaker in the CD40L-deficient mice (data not shown). Taken together, these results show that CD40–CD40L co-stimulation is dispensable for antiviral CD8 T-cell responses but plays a major role in the generation of virus-specific CD4 T-cell responses.

4. DISCUSSION

This report shows that virus-specific CD4 T-cell responses, like the CD8 T-cell response, has three phases following viral infection: (i) the activation and expansion phase that occurs during the first week of infection; (ii) a death phase that ensues during the second week of infection; and (iii) a memory phase that commences after one month and lasts for at least 300 days after infection.

The increase in the number of virus-specific CD4 T cells seen during the first week after infection was most probably due to expansion of clones of cells rather than recruitment of cells to the spleen from other sites. All of the virus-specific CD4 T cells that could make IFN-γ were $CD44^{hi}$ (figure 3), and only $CD44^{hi}$ cells divided during this period. T-cell division was determined by feeding

mice bromodeoxyuridine (BrdU) in their drinking water during the first week of the T-cell response. These experiments showed that 84% of activated CD4 T cells incorporated BrdU (Whitmire *et al.* 1998). At day 8 after infection, intracellular IFN-γ staining (figures 1–3) indicated the frequency of LCMV GP61–80-specific cells was 1 in 35 to 1 in 20 CD4 T cells. Furthermore, epitope analysis at day 8 indicated that $5–10 \times 10^5$ cells per spleen were specific for GP61–80 and 1×10^5 were specific for another I-A^b-restricted LCMV peptide, NP309–328 (Whitmire *et al.* 1998).

Previous studies have shown that most (50–70%) of the activated CD8 T cells expanding after infection are specific for LCMV (Butz & Bevan 1998; Murali-Krishna *et al.* 1998). The data shown in this report indicate that the expansion of LCMV-specific CD8 T cells is 35-fold greater than that of LCMV-specific CD4 T cells. As can be seen in figure 1, even if all of the activated CD4 T cells expanding after infection were specific for LCMV, there would still be an approximately fivefold smaller burst size for the T-helper compartment. Why is the antiviral CD8 response greater than the CD4 response? The CD8 co-receptor could deliver a stronger proliferative signal than the CD4 co-receptor to T cells. Another possibility is that during the initial expansion phase there is more antigen for CD8 T cells to see since most infected cells express major histocompatibility complex (MHC) class I. In contrast, fewer cells can express MHC class II, so CD4 T cells will see less antigen and therefore will not expand in number as much as CD8 T cells.

The death phase occurred during weeks 2–4 after infection. Annexin-V staining indicated that there were threefold more apoptotic $CD4^+$ T cells during this period than in naive mice. The number of virus-specific IFN-γ-secreting CD4 cells dropped approximately 90% during this period, mirroring what happens to the CD8 T-cell response (Ahmed & Gray 1996; Asano & Ahmed 1996; Lau *et al.* 1994; Murali-Krishna *et al.* 1998). There was a similar drop in the number of NP309–328-specific CD4 T cells, and CD4 T cells specific to GP61–80 or NP309–328 dropped approximately 15-fold between days 8 and 150. Interestingly, the contraction phase appeared to be quicker for CD8 T cells than for CD4 T cells. It could be that proliferation, with the associated changes in Bcl2, BclX, telomerase activity, and other factors affecting cell cycling and cell survival, predisposes cells to apoptose faster.

The CD4 T-cell memory established after one month was stable for six to ten months after infection. All of the virus-specific CD4 T cells that persisted in immune mice were $CD44^{hi}$ (figure 3) and $CD69^{lo}$ (data not shown), indicating that they were memory cells and not recently activated effector cells ($CD69^{hi}$). Mice were also able to mount rapid secondary CD4 T-cell responses upon re-infection with LCMV. Longevity of CD4 T-cell memory was comparable with that of CD8 T cells but was approximately tenfold lower in magnitude. The number of memory CD4 T cells was most probably established by the size of the of the expansion phase, and since there was less expansion of CD4 cells than CD8 cells, the size of the memory pool was set lower.

Since levels of memory appeared to be most affected by the expansion phase, we examined if CD4 and CD8 T cells differ in their activation requirements. Previous studies have shown that CD40–CD40L interaction is not required for CTL generation as measured by CTL killing of virally infected target cells (Borrow *et al.* 1996; Oxenius *et al.* 1996; Whitmire *et al.* 1996). The results reported here indicate that in contrast to the CD8 response, virus-specific CD4 T-cell responses were severely compromised (approximately 90% inhibition) in CD40L-deficient mice. This result shows that the activation requirements of CD4 and CD8 T cells are different; so why are CD4 T cells but not CD8 T cells affected by the absence of CD40–CD40L interaction? It is likely that the amount of antigen that CD8 T cells encounter is sufficient to activate them, whereas CD4 T cells encounter less antigen during the viral infection. One study which examined the number of T-cell receptors (TCR) required to activate T cells found that approximately 8000 TCR molecules had to be engaged in order to produce activation, but if the T cells were also stimulated through CD28, then the number of TCR which had to be engaged was reduced to approximately 1500 per cell (Viola & Lanzavecchia 1996). It is possible that a large number of MHC class I molecules contain viral peptides, so a sufficient number of TCR molecules may be engaged for CD8 T-cell activation. Also, most cells express MHC class I molecules. Fewer cells (namely B cells, macrophages and dendritic cells) express MHC class II, and it is possible that antigen density per cell (i.e. MHC class II molecules presenting viral peptides) is not sufficient to activate CD4 T cells in the absence of CD40–CD40L co-stimulation. Hence, CD4 T cells may not reach the activation threshold without additional co-stimulatory signals. Circumstantial evidence that CD4 T cells may require more CD40L signalling is the fact that CD4 T cells express higher levels of CD40L than CD8 T cells after infection (data not shown). Another possibility is that there may be additional molecules used by CD8 T cells. For example, 4-1BB–4-1BBL interaction has been shown to be important for alloreactive CD8 T cells (Shuford *et al.* 1997), and it may also be important for antiviral CD8 T cells (Tan *et al.* 1999).

Dissecting the mechanisms which are important for generating and maintaining CD8 and CD4 T-cell responses will lead to improved vaccines and strategies for the prevention and treatment of chronic viral infections. Some antiviral $CD8^+$ T-cell responses are critically dependent upon $CD4^+$ T-cell help (Cardin *et al.* 1996; Jennings *et al.* 1991; Matloubian *et al.* 1994; Von Herrath *et al.* 1996). The importance of $CD4^+$ T cells is highlighted by the finding that mice deficient in functional $CD4^+$ T-cell responses (CD4-depleted or $CD4^{-/-}$ mice) are unable to generate large numbers of CTL and cannot control strains of LCMV which replicate quickly (Matloubian *et al.* 1994; Zajac *et al.* 1998). In humans, the decrease in T-helper cell number caused by HIV infection is associated with a loss of virus-specific $CD8^+$ CTL and an increase in viral titre and susceptibility to other infectious agents. Help from the $CD4^+$ set probably plays a pivotal role in driving and maintaining the CTL responses under conditions of protracted viral infection (Matloubian *et al.* 1994; Rosenberg *et al.* 1997; Zajac *et al.* 1998). This is one of the first studies quantifying the initial burst size of the CD4 T-cell response which

demonstrates long-term CD4 memory in an acute viral infection. Future investigations should address what the activation requirements are for both T-cell compartments.

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