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Differential Sensitivity of Naive and Memory CD8+ T Cells to Apoptosis in Vivo

Jason M. Grayson, Laurie E. Harrington, J. Gibson Lanier, E. John Wherry, and Rafi Ahmed

Apoptosis is a critical regulator of homeostasis in the immune system. In this study we demonstrate that memory CD8⁺ T cells are more resistant to apoptosis than naive cells. After whole body irradiation of mice, both naive and memory CD8⁺ T cells decreased in number, but the reduction in the number of naive cells was 8-fold greater than that in memory CD8⁺ T cells. In addition to examining radiation-induced apoptosis, we analyzed the expansion and contraction of naive and memory CD8⁺ T cells in vivo following exposure to Ag. We found that memory CD8⁺ T cells not only responded more quickly than naive cells after viral infection, but that secondary effector cells generated from memory cells underwent much less contraction compared with primary effectors generated from naive cells (3- to 5-fold vs 10- to 20-fold decrease). Increased numbers of secondary memory cells were observed in both lymphoid and non-lymphoid tissues. When naive and memory cells were transferred into the same animal, secondary effectors underwent less contraction than primary effector cells. These experiments analyzing apoptosis of primary and secondary effectors in the same animal show unequivocally that decreased downsizing of the secondary response reflects an intrinsic property of the memory T cells and is not simply due to environmental effects. These findings have implications for designing prime/boost vaccine strategies and also for optimizing immunotherapeutic regimens for treatment of chronic infections. The Journal of Immunology, 2002, 169: 3760–3770.

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poptosis plays a critical role in the development and regulation of the immune system. Because immune responses are often characterized by large clonal expansions of lymphocytes, mechanisms must exist to prevent the accumulation of large numbers of cells and to cull the expansion once the antigenic stimulus has been cleared. The immune system returns to homeostasis through apoptosis of the vast majority of lymphocytes that are expanded. Although much has been learned about the sensitivity of lymphocytes of undefined specificity to apoptosis in vitro, very little is known about the relative sensitivity to apoptosis of Ag-specific CD8⁺ memory T cells in vivo.

CD8⁺ T cells are a component of the cellular immune response critical for the clearance of intracellular pathogens and certain tumors. Throughout the development of T cells there are multiple chances for a cell to undergo apoptosis. One of the first opportunities occurs in the thymus, where a developing cell can undergo death by neglect if it lacks a TCR with sufficient affinity for MHC class I complexed with the appropriate self ligand or death through negative selection if the TCR/MHC interaction is too strong (1, 2). Throughout the double-positive (CD8⁺CD4⁺) stages these thymocytes are extremely sensitive to agents such as dexamethasone and irradiation, which induce apoptosis through production of reactive oxygen species and loss of mitochondrial function (3, 4). When single-positive CD8⁺ T cells migrate to the peripheral lymphoid organs, they acquire an increased resistance to apoptosis compared with thymocytes. These cells express moderate levels of the anti-apoptotic protein Bcl-2 (5) and continue to require MHC interaction for survival (6, 7). If a naive T cell encounters its cognate peptide in the appropriate MHC context along with costimulation it will become activated and begin to proliferate. These cells also acquire effector functions such as cytokine production and cytotoxicity. Once Ag is cleared, these cells continue through a brief, Ag-independent replication phase where their numbers continue to increase (8–10). At the completion of this phase, Bcl-2 levels decrease substantially, and effector cells begin to undergo a period of apoptosis in vivo. This period of apoptosis lasts for ~2–3 wk, during which ≥90% of the Ag-specific CD8⁺ T cells undergo apoptosis (11). The surviving memory cells can persist for extended periods and provide long term protective immunity (12). Additionally, these memory CD8⁺ T cells no longer require MHC class I interactions for their survival in vivo (6).

Previously we have documented that Ag-specific memory CD8⁺ T cells contain increased levels of Bcl-2 compared with either naive or effector cells (5). This raised the question of whether memory cells are more resistant to apoptosis than naive cells. Previous studies have examined whether primed CD4 cells were more resistant to Fas-induced death in vitro. These authors found that memory cells underwent less death in vitro (13, 14).

We examined apoptosis of Ag-specific CD8⁺ memory T cells after whole body irradiation and during the contraction of an immune response following acute infection with lymphocytic choriomeningitis virus (LCMV)⁴ or vaccinia virus (VV) expressing the LCMV CD8⁺ T cell epitope gp33–41. We found that after whole body irradiation memory cells survived preferentially compared with naive cells. Rechallenge of immune mice generated a

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4 Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; Bu5F; bromodeoxyuridine; NP, nucleoprotein; VV, vaccinia virus; 7-AAD, 7-aminoactinomycin D.
vigorouos secondary response, and the amount of contraction in a secondary response was only 2- to 5-fold compared with a 10- to 15-fold decrease observed in a primary response. To equalize numbers of Ag-specific cells we adoptively transferred P14 transgenic CD8 naïve or memory T cells (which express a TCR specific for gp33-41) into naïve recipients and challenged with virus. After exposure to Ag we found not only that memory cells underwent faster expansion than naïve cells during the effector phase, but that memory CD8 T cells underwent less death during the contraction phase.

Materials and Methods

Mice
Six- to 8-wk-old C57BL/6, B6.PL-Thy1.1/Cy, and BALB/c mice were purchased from National Cancer Institute (Frederick, MD). P14 TCR transgenic (H-2b and Thy1.2) mice have been described previously (15). Thy1.1 P14 transgenic mice were generated by crossing P14 mice onto a B6.PL-Thy1.1/Cy (H-2b and Thy1.1) background.

Viruses
For LCMV infection, mice were infected i.p. with 2 × 10^6 PFU of the Armstrong strain or i.v. with 2 × 10^6 PFU of the clone 13 strain. LCMV was prepared and quantitated as described previously (16). For vaccinia infections mice were infected i.p. with 5 × 10^6 PFU of VV-gp33, a recombinant VV that expresses the D^b-restricted LCMV CD8 T cell epitope gp33-41. This virus and its preparation have been described previously (17).

Preparation of MHC class I tetramers
The construction and purification of L^d NP118-126, D^gp33-41, D^NP3936-404, and D^gp276-286 have been described previously (11).

Surface and intracellular staining
All Abs were purchased from BD PharMingen (San Diego, CA). Surface staining was performed as described previously (11). For Bc1-2 staining, cells were first surface stained with anti-CD8α and D^gp33-41, and then intracellular staining was performed with anti-Bc1-2 as described previously (5). Bromodeoxyuridine (BrdU) staining was performed using the BrdU Kit from BD PharMingen according to the manufacturer’s instructions. For analysis of direct ex vivo apoptosis, splenocytes were isolated, surface stained as described above, briefly incubated with annexin V and 7-amino-actinomycin D (7-AAD) at room temperature in the dark, and removed immediately. Samples were obtained on a FACSCalibur instrument and analyzed using CellQuest software (BD Biosciences, San Diego, CA).

Irradiation
LCMV immune mice were irradiated in a Gammacell irradiator (Atomic Energy of Canada, Ottawa, Canada) to receive 200, 400, or 600 rad whole body gamma irradiation.

Cell isolation
Lymphocytes were isolated from the spleen, lymph nodes, bone marrow, and PBL as described previously (5, 11, 18). For isolation of non-lymphoid tissues, mice were euthanized, the abdomen was opened, the hepatic vein was cut, and 5 ml ice-cold PBS was injected directly into the hepatic artery to perfuse the liver. The gall bladder was removed, and the entire liver was excised. The liver tissue was homogenized using a wire screen and was incubated in 0.25 mg/ml collagenase B (Roche, Mannheim, Germany) and 1 U/ml DNase (Sigma, St. Louis, MO) at 37°C for 45 min. Digested liver was centrifuged, and the pellet was resuspended in 5–10 ml 4% Percoll (Sigma). This solution was underlaid with 56% Percoll and spun at 2000 rpm for 20 min at 20°C. The intrahepatic lymphocyte populations were harvested from the interface, and RBC were lysed using 0.83% ammonium chloride, washed, and counted. Lung lymphocytes were isolated in a similar manner. At the peak of the response on day 7, 1.50–2 × 10^7 lymphocytes were isolated from the liver, of which ~80% were CD8^- T cells; from lung 5–10 × 10^6 cells were isolated, of which 80% were CD8^- T cells. By day 32 postinfection we could isolate ~2 × 10^6 cells from the liver (25–44% CD8^- T cells) and ~4–7 × 10^5 cells from the lungs (~25% CD8^- T cells).

Generation of memory CD8^- T cells
The P14 Tg memory cells used in experiments with VV-gp33 (Fig. 3) were generated as follows. Naïve P14 TCR transgenic cells (10^4) were injected i.v. into naive C57BL/6 hosts. Four hours later these mice were infected with 2 × 10^6 PFU LCMV-Armstrong. Under these conditions we have shown that 98% of the gp33-specific CD8^- T cells are derived from transgenic precursors (19). After 60 days these mice were sacrificed, and the number of transgenic memory cells was determined by staining with α-CD8α and D^gp33-41. Splenocytes were transferred i.v. from these LCMV-immune mice into naive C57BL/6 mice to ensure that the recipient mice received 10^6 P14 memory CD8^- T cells. These animals are referred to as memory transfer. Naïve transfer was performed by injecting 10^6 naïve P14 transgenic cells i.v. into naive C57BL/6 mice. These groups of mice were then infected with 5 × 10^6 PFU VV-gp33 i.p. and analyzed at the indicated time points.

The P14 Tg memory cells used in experiments with LCMV (Figs. 4–6 and 9) were generated as follows. Naïve P14 TCR transgenic cells (10^4) were injected i.v. into naive C57BL/6 hosts. Four hours later, these mice were infected with 5 × 10^6 VV-gp33 i.p. After 60 days these mice were sacrificed, and the number of transgenic memory cells was determined by staining with α-CD8α and D^gp33-41. Splenocytes were transferred i.v. from these VV-gp33-immune mice into naive C57BL/6 mice to ensure that the recipient mice received 10^6 P14 memory CD8^- T cells. These animals are referred to as memory transfer. Naïve transfer was performed by injecting 10^6 naïve P14 transgenic cells i.v. into naive C57BL/6 mice. These groups of mice were then infected with 2 × 10^6 PFU LCMV-Armstrong i.p. and used at the indicated time points for primary infection. Secondary infection was set up as described for memory transfer in the preceding paragraph. To generate secondary memory cells for use in tertiary infections, enough splenocytes were transferred i.v. from memory transfer mice that had been infected with VV-gp33 to ensure that the recipient mice received 10^6 secondary memory cells. There were no detectable Ag-specific CD8^- T cells for other LCMV epitopes besides gp33.

Isolation and transfer of naïve and memory P14 Tg cells into the same animal
Naïve cells were isolated from naive P14 Thy1.2^- mice and enumerated by staining with α-CD8α and D^gp33-41. Enough splenocytes were transferred i.v. to give the desired numbers of naïve gp33-specific CD8^- T cells. To generate Thy1.1^- P14 memory cells, 10^6 naïve Thy1.1^- P14 CD8^- T cells were transferred i.v. into naïve C57 BL/6 mice and infected with 2 × 10^6 PFU LCMV-Armstrong i.p. After 90 days these animals were sacrificed, and memory cells were isolated by FACS sorting with CD8α and Thy1.1 Abs. The isolated population was 98% pure. These cells were mixed with naïve P14 Thy1.2^- splenocytes and injected i.v. into naïve C57BL/6 recipients. These mice were infected with 2 × 10^6 PFU LCMV-Armstrong i.p. and analyzed at the indicated time points.

Characterization of naïve and memory P14 Tg CD8^- T cells
The naïve P14 cells were CD44low, CD122low, and LFA-1low and did not produce IFN-γ in a 4- to 6-h assay after stimulation with Ag. Memory P14 cells were CD44high, CD122high, LFA-1high, CD43low (effector cells are CD43high), CD25low, and CD69low and rapidly (2–4 h) produced IFN-γ after stimulation with peptide. Neither naïve nor memory P14 cells exhibited direct ex vivo cytotoxicity against virus-infected targets.

Results
Memory cells are resistant to irradiation-induced apoptosis
To determine whether memory cells were more resistant to apoptosis than naïve cells we subjected LCMV-immune mice to whole body irradiation and examined the survival of naïve and memory cells in the same animal. After 18 h apoptosis was complete, and we analyzed survival at this time point to minimize the effects of homeostatic proliferation of the few remaining cells. Fig. 1A shows that adult BALB/c mice contained ~4 × 10^6 naïve (LFA-1low) CD8^- T cells and ~1.5 × 10^5 memory (LFA-1high) cells in
the spleen. After whole body irradiation both cell populations decreased, but naive cells declined to $10^5$ cells (40-fold drop), while activated/memory cells only decreased to $3 \times 10^5$ (5-fold drop; Fig. 1B). In a dose-dependent manner memory CD8$^+$ T cells became enriched in the spleen as they increased from 20% of the total CD8$^+$ population in untreated mice to 76% after 600 rad. LCMV-immune mice contained 10% splenic CD8$^+$ T cells specific for the LCMV CTL epitope NP118–126. After irradiation (Fig. 1C), the total number of Ag-specific memory cells decreased from $6 \times 10^6$ to $1.6 \times 10^6$ (4- to 5-fold drop; Fig. 1D), but these cells also became enriched as they increased in a dose-responsive manner from 15% at 200 rad to 46% of the total CD8$^+$ population after 600 rad. Similar trends were observed in the lymph nodes, bone marrow, and PBL (data not shown).

Contraction during a secondary infection is less than that during a primary infection

We next examined the expansion and contraction of LCMV-specific CD8$^+$ T cells during a secondary infection. In this experiment and all subsequent ones in this paper we will use the following nomenclature to describe the various cell populations. Effector cells generated from naive cells will be referred to as primary effectors and the surviving cells from the pool of these primary effector cells as memory cells (naive→primary effector→primary memory). Ag-specific CD8$^+$ T cells generated upon secondary infection will be referred to as secondary effector and secondary memory cells (primary memory→secondary effectors→secondary memory), and those generated after a tertiary infection will be referred to as tertiary effectors and tertiary memory cells (secondary memory→secondary effectors→tertiary memory).

Groups of naive and LCMV-immune BALB/c mice were challenged with virus, and the expansion and contraction of the CD8$^+$ T cell responses during the primary and secondary infections was monitored with MHC class I tetramers. As shown in Fig. 2 (A and B) contraction of the primary response was much greater (15-fold) than that of the secondary response (2- to 3-fold). At the peak of the primary response (day 8) there were $10^7$ NP118-specific CD8$^+$ T cells, and 90% of these primary effector cells died by day 30 to give a memory pool of $7 \times 10^5$ cells (15-fold drop). In striking contrast the secondary effectors only decreased from $10^7$ at the peak (day 5) to $4 \times 10^6$ on day 36 (2.5-fold drop). Since the LCMV-specific CD8$^+$ T cell response in BALB/c mice is heavily directed toward a single immunodominant epitope (NP118–126), we also examined secondary responses in C57BL/6 mice that elaborate sizable responses to three epitopes, gp33–41, NP396–404, and gp276–286. Fig. 2C shows that for all three CD8$^+$ epitopes there was a 10- to 15-fold reduction during the primary response and only a 3-fold reduction during the secondary response.

FIGURE 1. Memory CD8$^+$ T cells are more resistant to radiation-induced apoptosis than are naive cells. BALB/c mice were infected with LCMV and 110 days postinfection were subjected to whole body gamma irradiation with 200, 400, or 600 rad. Animals were sacrificed, splenocytes were prepared, and CD8$^+$ LFA-1$^{low}$ or LFA-1$^{high}$ cells were identified and enumerated (A and B). Additionally, CD8$^+$ L$^d$NP118–126$^+$ (C) Ag-specific CD8$^+$ memory T cells were enumerated (C and D). The number in the upper right corner of each dot plot indicates the percentage the gated population constitutes of total CD8$^+$ T cells. The average and SD of each group are plotted in the graph. Four to six mice were analyzed at each time point. The number in the graph indicates the fold reduction in the number of naive or memory CD8$^+$ T cells.
FIGURE 2. Secondary effector cells undergo less contraction than primary effector cells. BALB/c mice were infected with LCMV and were analyzed 8 and 60 days postinfection. After 60 days immune animals were rechallenged with LCMV and examined 4, 5, 7, 12, and 36 days post-secondary infection. Splenocytes were surface-stained with anti-CD8α and LdNP118–126, and total numbers of tetramer-positive cells in the spleen were quantitated and plotted (A) as the average of four to six mice with the SD. A representative FACS analysis of primary and secondary CD8+ T cell responses after infection is shown in B. C57BL/6 mice were infected in a similar manner, and enumeration was made for three Dβ-restricted epitopes: gp33–41, NP396–404, and gp276–286 (C).

FIGURE 3. Responses of naive and memory cells after immunization with recombinant VV expressing gp33 epitope. Naive or memory transgenic P14 T cells (10^4) were transferred i.v. into naive C57BL/6 mice, and these mice were challenged with VV-gp33 and examined 5, 8, 12, and 30 days postinfection. Splenocytes were isolated and stained with anti-CD8α and Dβgp33–41 (A). The value in the dot plot indicates the percentage of CD8+ T cells that are gp33–41 specific. Ag-specific cells were quantitated in the spleen, and the average and SD were plotted (B). Four mice were analyzed at each time point. C. The fold reduction between the peak of the effector response and the memory set-point for primary (naive P14 cells) and secondary (memory P14 cells) effectors.
Memory cells proliferate more rapidly than naive cells and secondary and tertiary effectors undergo less death than primary effectors

Upon secondary infection we observed significantly less death of effector cells than after primary infection. However, there is a vast difference in the precursor frequency of Ag-specific CD8+ T cells between naive and immune mice. Naive mice have been estimated to contain 100–500 precursors for any given epitope (19, 20). In LCMV-immune mice there are between 10^5–10^7 memory cells for the epitopes we analyzed. To reach 10^7 effector cells in immune mice requires a much fewer number of divisions compared with the primary response. It is possible that the differences in the amount of death we observed may simply be due to differences in the number of divisions that the T cells undergo during the expansion phase. To better control for this we used an adoptive transfer system where we transferred similar numbers of naive or memory transgenic cells specific for the LCMV D^b-restricted epitope gp33–41 into naive recipients. Thus, in these experiments the starting point is the same for memory and naive CD8+ T cells. These mice were then challenged with either LCMV or VV expressing the gp33–41 epitope.

Fig. 3 (A and B) shows analysis of primary and secondary CD8 responses in the spleen after infection with recombinant VV expressing gp33. Early in the response, on day 5 postinfection, secondary effector cells had expanded to almost 10-fold greater numbers than primary effector cells. At the peak of the response, on day 8, secondary effector cells were present in greater numbers, and this difference was maintained as the response contracted. When the decrease in cell numbers in both populations was compared, we observed a 5-fold contraction of the primary effectors compared with a 2.5-fold contraction of the secondary effectors (Fig. 3C).

In addition to examining how naive and memory cells respond in the context of a recombinant vaccine (i.e., VV expressing gp33 epitope) we also analyzed the responses following infection with the native virus. To address this we transferred naive and memory Tg cells into naive C57BL/6 hosts and then challenged them with LCMV. Five days postinfection there were 2 × 10^6 secondary effector and 4 × 10^5 primary effector cells in the spleen (Fig. 4, A and B). The number of secondary effectors reached a plateau on day 6, edged slightly higher on day 7, and began to contract by day 8. This contrasted with the primary effector pool, which plateaued on day 7, remained constant on day 8, and then began to contract. After viral clearance the new memory pool was established, and the contraction of the secondary response was significantly less than the primary response. Comparison of the death phase revealed that transferred naive cells underwent ~18-fold reduction from the peak on day 8, while transferred memory cells underwent ~6-fold reduction from the peak on day 7. In addition to examining cell numbers in the spleen we examined other lymphoid and non-lymphoid tissues. Fig. 5 shows that lymphoid tissues such as the bone marrow (A) and lymph nodes (B) contain similar numbers of primary and secondary effector cells on days 6–8 postinfection, but by the establishment of memory there were more secondary memory cells. Examination of cell numbers in two non-lymphoid tissues the lung (Fig. 5C) and liver (Fig. 5D) revealed similar trends.

In both the LCMV and vaccinia systems we observed differences in the contraction of the immune response after stimulation of naive and memory CD8+ T cells. One potential explanation for

FIGURE 4. Responses of naive and memory cells after infection with LCMV. Naive or memory transgenic P14 T cells (10^4) were transferred i.v. into naive C57BL/6 mice, and these mice were challenged with LCMV and examined 5, 6, 7, 8, 15, and 32 days postinfection. Splenocytes were isolated and stained with anti-CD8α and Db gp33–41 (A). Ag-specific cells were quantitated, and the average and SD are shown in B. Three to six mice were analyzed at each time point. C, The fold reduction between the peak of the effector response and the memory set-point for primary (naive P14 cells) and secondary (memory P14 cells) effectors.
this phenomenon is that the cytokine milieu during the secondary infection is inherently different, and all cells, even primary effectors, would undergo less contraction in this environment. Although the majority of the LCMV response in the adoptive transfer model is directed against donor gp33–41-specific cells, there is an easily measurable host NP396–404 response. In both naive and memory cell transfers the endogenous NP396-specific T cell response will represent primary effector cells and would rule out nonautonomous factors controlling cell fate decisions. When the NP396–404 response was examined (Fig. 6) after transfer of either naive or memory gp33-specific cells, a similar peak of NP396/H11001-specific T cells was observed on day 8, and similar numbers were also observed after completion of the death phase on day 32. Thus, the contraction of the primary effectors is similar in both groups of mice (i.e., transgenic naive or memory cell recipients).

Although we observed similar contraction of NP396-specific CD8+ T cells in both primary and secondary infections, these cells are of a different specificity than gp33. To address the fate of naive and memory cells of the same specificity, we transferred naive and memory P14 cells into the same mouse. These two populations (i.e., naive and memory P14 cells) can be distinguished from each other by staining with anti-Thy1.1 Abs, as the memory cells were generated from Thy1.1/H11001 P14 mice. Naive and memory cells were mixed at various ratios (naive cells/memory cells), transferred into naive recipients, and then infected with LCMV. A longitudinal analysis of PBL was performed by retroorbitally bleeding the recipient mice on days 5, 7, 15, and 33 postinfection. P14 cells were identified by staining with /H9251-CD8/H9251 and D b gp33–41. Tetramer-positive cells were further characterized to determine whether they were Thy1.1 positive (derived from transferred memory P14 cells). The results of these experiments are shown in Fig. 7. The data from the control groups (naive or memory cell transfer only) are shown in Fig. 7A, and the experimental groups (both naive and memory cells transferred into the same mouse) are shown in Fig. 7B. The striking finding is that regardless of the ratio of naive/memory P14 cells on day 0, memory cells always contracted less than naive cells following viral infection. Thus, as a result of this decreased downsizing of the secondary effectors, Thy1.1 P14 cells became the predominant Ag-specific cells in all groups of mice. This is summarized in Fig. 7B, and raw data from a representative mouse are shown in Fig. 7C. These results showing decreased contraction of secondary effectors compared with primary effectors of the same antigenic specificity (P14 Tg cells) and in the same animal and prove unequivocally that this important difference in the response of naive and memory CD8 T cells is due to intrinsic
FIGURE 7. Longitudinal analysis of the expansion and contraction of naive and memory P14 cells in the same mouse. A total of $10^4$ naive (Thy1.2$^+$) and memory (Thy1.1$^+$) transgenic CD8$^+$ T cells were transferred separately (A) or at the indicated ratios (B) into naive mice and infected with LCMV. PBL were isolated from the same mouse on days 5, 7, 15, and 33. Cells were stained with anti-CD8$^+$, anti-Thy1.1, and D b gp33–41. Using Thy1.1 Abs the contribution of transferred memory cells could be determined. At each time point the percentage of CD8$^+$ T cells that was either D b gp33–41$^+$Thy1.2$^+$ (naive) or D b gp33–41$^+$Thy1.1$^+$ (memory) was determined. The average and SD are plotted. Three or four mice were included in each group. E, Percentage of gp33-specific cells derived from naive P14 cells; F, percentage of Ag-specific cells derived from memory P14 cells. Note that in all groups of mice the contraction of secondary effectors is less than that of primary effectors, such that regardless of the ratio of naive/memory cells transferred, there were always more Ag-specific CD8$^+$ T cells derived from memory P14 cells on day 33 postinfection. An example of the raw data from the 2.5:1 (naive/memory) cell ratio is shown in C. In the dot plot on the left of the figure the percentage of CD8$^+$ T cells that are D b gp33–41$^+$ is indicated in the left corner of each plot. The dot plot in the right column is gated on tetramer-positive cells and shows the percentage of Ag-specific cells derived from naive (Thy1.2) or memory (Thy1.1) P14 cells. Note that Thy1.1 gp33$^+$ cells became the predominant population by day 15 after infection.
differences in the (i.e., cell autonomous) and is not the result of environmental effects.

We next asked whether the decreased contraction of secondary effectors was due to increased proliferation during the contraction phase or if it truly represented less apoptosis of the activated cells. To determine which factor played a greater role we examined the proliferation of effector cells as the response contracted. BrdU was added to the drinking water of the animals from days 8–15 of infection to determine whether cells had synthesized DNA. Examination of the rates of incorporation revealed that 7.2% of primary effector cells had synthesized DNA compared with 2.7% of secondary effector cells (Fig. 8A). This shows that the decreased downsizing of secondary effectors cannot be explained by increased proliferation of these cells during the death phase. In fact, if anything their proliferation was slightly less than that of primary effectors during the contraction phase. In addition to measuring proliferation we determined ex vivo apoptosis of primary and secondary effector cells on days 7 and 15 postinfection (Fig. 8B). High levels of annexin V binding are indicative of cells in the early stages of apoptosis, while cells that bind both annexin V and 7-AAD are in the later stages of apoptosis or are dead. Directly ex vivo many more primary effector cells (56%) on day 7 were in the early stages of apoptosis compared with secondary effector cells (28%). By day 15, 36% of primary effector cells were in the early stages of apoptosis compared with 16% of secondary effector cells. Taken together, these results (Fig. 8) show that decreased downsizing of secondary effectors is most likely due to decreased apoptosis.

Because cells after a secondary infection appeared to undergo less death we challenged secondary immune animals and compared the death after primary, secondary, and tertiary infections. Fig. 9A shows a longitudinal analysis of the PBL of a representative animal as it passes through a primary, secondary, or tertiary response. All animals started with equal numbers of P14 TCR transgenic cells. Similar to secondary effector cells, tertiary effectors proliferated to a greater extent than primary effector cells 5 days postinfection. These cells also peaked at a level similar to that of the primary effector cells on day 8 postinfection. By the time the memory phase was established, both secondary and tertiary infections had generated greater numbers of cells than the primary infection (Fig. 9C). Because of the critical role of Bcl-2 in controlling cell death of CD8 cells we examined its expression in primary, secondary, and tertiary effector cells (Fig. 9). Fig. 9D shows that after T cell activation (day 8 postinfection) Bcl-2 levels decreased in all three populations compared with naive cells, but secondary and tertiary effectors contained slightly higher levels of Bcl-2 than primary effectors.

**Discussion**

In this study we show that Ag-specific CD8\(^{+}\) memory T cells are more resistant to apoptosis than naive cells. When memory cells were exposed to a strong physical stimulus such as gamma irradiation, their survival was greater than that of naive phenotype cells. Re-exposure of memory cells to Ag through viral infection resulted in a more rapid expansion and diminished contraction compared with those of naive cells. Finally, secondary memory cells behaved in a similar fashion to primary memory cells when they were re-exposed to Ag.

Radiation-induced apoptosis is a critical technique used to treat certain malignancies. Sensitivity to radiation-induced death was traditionally assessed in clonogenic survival assays in vitro where the number of colony-forming cells was compared against varying doses of radiation. After irradiation it has been observed that cells with the greatest proliferative capacity, such as lymphocytes, are among the most sensitive to apoptosis. Teleologically this makes sense because of the risk of expanding a population of cells that harbor DNA damage that could eventually lead to malignancy. Why, then, do memory cells undergo less apoptosis than naive

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Greater contraction of primary effectors compared with secondary effectors is due to increased apoptosis of primary effectors and not to increased proliferation of secondary effectors. Proliferation during the contraction phase (days 8–15) was assessed (A) by transferring 10\(^4\) naive or memory transgenic P14 T cells into naive C57 Bl/6 animals infected with LCMV and then giving the mice BrdU in their drinking water from days 8–15 after infection. Splenocytes were isolated on day 15 postinfection and were stained with anti-CD8\(\alpha\), D\(^b\)gp33–41, and anti-BrdU Ab. The plots are gated on CD8\(^{+}\)D\(^b\)gp33–41 cells, and the number on the dot plot indicates the percentage of Ag-specific cells that are BrdU\(^{+}\). Direct ex vivo apoptosis on days 7 and 15 postinfection (B) was assessed by surface staining splenocytes as described above and then performing a brief incubation with annexin V and 7-AAD, followed by immediate acquisition. The value in each quadrant indicates the percentage of CD8\(^{+}\)gp33–41\(^{+}\) cells that fall in the quadrant. Three mice were examined at each time point, and data from a representative mouse are presented.
cells? Studies have shown that memory cells undergo a slow homeostatic proliferation, while naive cells undergo little or no proliferation (6). In theory, memory cells should be more sensitive to irradiation-induced death, as they cycle more and should have more of a chance for DNA damage-sensing mechanisms, such as the tumor suppressor p53, to kill the cells. Although multiple mechanisms may account for the radioresistance, this apparent conundrum could be explained by the increased expression of Bcl-2, as previous studies by Strasser (21) and colleagues have shown that lymphocytes that overexpress Bcl-2 are more resistant to irradiation-induced death in vitro.

Irradiation of Ag-specific CD8^+ memory T cells represents one type of apoptotic stimuli these cells may respond to; the death that occurs after expansion induced by exposure to Ag during reinfection or vaccine boosting represents another. Rechallenge of LCMV-immune mice results in a vigorous amanestic response. The amount of contraction of the secondary response was ~2- to 5-fold compared with the ~10- to 15-fold contraction observed after primary infection. One potential explanation for the difference observed in contraction between the two infections is that the lower number of divisions in secondary infection compared with primary infection causes less death. We used an adoptive transfer system to illustrate that differential death after exposure to Ag is an innate property of memory compared with naive cells and is not solely due to differences in proliferation. In the adoptive transfer experiment, equal cell numbers of naive and memory cells were transferred. We found that memory cells underwent greater expansion than naive cells. These results confirm and extend a similar finding by Veiga-Fernandes et al. (22), who demonstrated that memory cells specific for the HY Ag proliferated faster than naive cells upon exposure to male splenocytes. We extend these findings to demonstrate that memory cells also expand to greater numbers more quickly in viral infections. At the peak of the response the memory cells were either greater (VV) or almost equal (LCMV) compared with the naive cell transfers. Previous studies by Zimmerman et al. (23) have documented similar total expansion of naive and memory cells at the peak of the LCMV response. When the immune response had finished contracting, the cells generated from memory cells had always contracted less. Most of these cells were present not only in several lymphoid tissues (spleen, lymph nodes, and bone marrow), but also in non-lymphoid tissues such as lung and liver (Fig. 5). In addition to observing differential expansion and contraction of naive and memory P14 cells when they were transferred into separate mice, we observed similar trends when these cells were transferred into the same mouse. This finding is important because it allows us to rule out nonautonomous factors such as the cytokine milieu as the main reason for the differential contraction of naive and memory cells and shows that decreased downsizing of secondary effectors is due to intrinsic differences between naive and memory cells.

Survival of secondary P14 Tg effectors was better than that of primary P14 effectors after challenge with either LCMV or VV-gp33. However, the overall contraction was greater after LCMV infection compared with VV-gp33 infection. One possible reason for this difference could be the duration of antigenic stimulation. There is likely to be more Ag after LCMV infection than after VV-gp33 infection, and a longer period of stimulation may result in greater downsizing of the response. Additional experiments will...

Figure 9. Secondary memory cells also expand more rapidly than naive cells and undergo less contraction. Naive or primary or secondary memory transgenic P14 T cells (10^5) were transferred i.v. into naive C57BL/6 mice, and these mice were challenged with LCMV. PBL were isolated 5, 8, 15, and 32 days postinfection and stained as described in Fig. 3A. The number in the dot plot indicates the percentage of CD8^+ T cells that are D^b gp33–41^+. The percentage of CD8^+ D^bgp33–41^+ in the CD8^+ T cells was plotted over time (B). Each measurement is the average of 5–12 mice. The bars indicate the SD. The numbers of CD8^+ D^b gp33–41^+ cells in the spleen on days 8 and 32 were assessed as described previously (C). Bcl-2 levels were assessed in naive and primary, secondary, and tertiary effector cells on day 8 postinfection (D).
need to be performed to precisely define the reasons that underlie the differences we have observed between LCMV and VV-gp33 infections. These studies are beyond the scope of this work. However, in terms of the present study it is worth emphasizing again that regardless of the virus infection, the contraction of primary effectors was always greater than that of secondary effectors.

What is the mechanistic basis for the difference in the numbers of Ag-specific memory cells observed following primary and secondary infections? From our BrdU labeling experiments we demonstrate that the difference cannot be ascribed to excess proliferation during the contraction phase following viral clearance. Our data point to differences in apoptosis causing differences in cell numbers. This is also supported by our observation that secondary effector cells contained lower levels of annexin V binding directly ex vivo compared with primary effector cells on both days 7 and 15. Although we cannot formally exclude in vitro manipulation influencing cell death, it is important to note that secondary effector cells still contained fewer apoptotic cells than primary effectors. Apoptosis is a complex, multifactorial process that is controlled by many genes. In recent years considerable research has implicated mitochondria as a critical cellular component of apoptosis (24, 25). When cells are subjected to apoptotic stresses, mitochondria undergo changes in membrane permeability. These changes result in the release into the cytoplasm of proteins such as cytochrome c (26, 27), apoptosis-inducing factor (28), and second mitochondria-derived activator of caspase/Diablo (29). Once they enter the cytoplasm, these proteins activate and maintain caspase cascades and cause DNA degradation. Bcl-2 prevents apoptosis by preventing the release of these proteins into the cytosol (30). In our study we show that at the peak of the response secondary and tertiary effector cells contained slightly increased levels of Bcl-2 compared with primary effector cells. While increased Bcl-2 may partially explain increased survival, two lines of evidence suggest that other mechanisms may be involved as well. First, the Bcl-2 family is composed of many genes, some of which induce apoptosis. Increased survival could also be due to decreased levels of pro-apoptotic proteins such as Bad, Bax, Bim, or Bid (31). The second line of evidence is based on observations regarding the levels of reactive oxygen species and how these affects T cell apoptosis. Previous studies by Hildebrandt et al. (32) showed that treatment of superantigen-activated T cells with Mn(III) tetrakis(5,10,15,20-benzoic acid) porphyrin, a compound that catalyzes the destruction of reactive oxygen species, decreases T cell death in vitro. Early in LCMV responses (day 5), primary effector cells contain increased levels of superoxide compared with naive cells (J. Grayson and R. Ahmed, unpublished observations). The production of superoxide may cause intracellular damage culminating in apoptosis. It will be critical to determine whether secondary effector cells contain decreased levels of reactive oxygen species compared with primary effector cells.

In addition to documenting increased resistance to apoptosis of memory cells after irradiation and decreased downsizing of secondary effectors, we found that this property does not become altered following an additional (third) exposure to Ag. This finding has important implications for vaccination protocols. Many vaccines rely on initial priming followed by multiple boosts. It is unclear how much time is required after boosting for effector cells to reacquire the memory cell’s resistance to apoptosis and capacity for rapid proliferation. It is also critical to determine whether increased exposure to Ag selects for a subpopulation of memory cells that becomes more resistant to apoptosis with each boost. Since we only exposed memory cells to Ag twice, we do not know how this property will change with repeated boosting. Using the systems described in this paper, these key questions can be addressed in future studies. Our findings may also be useful in optimizing structured treatment interruption of HIV-infected individuals. This treatment results in increasing HIV load by cessation of anti-retroviral therapy for a brief period. In some patients (33) and in non-human primates (34, 35) this is followed by increases in the number of Ag-specific cells, which decline again after therapy is reinstituted. Although HIV infection is a complex process, with expansion of Ag-specific CD8 T cells being influenced by multiple factors, such as viral load, CD4 numbers, and the time of initial HIV exposure, structured treatment interruption is essentially a regimen involving a cyclical increase and decrease in Ag. General principles derived from studies of memory cells and their response to Ag may be applicable and provide guidelines for optimizing structured treatment interruption or therapeutic vaccination regimens.

References