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Preapoptotic Phenotype of Viral Epitope-Specific CD8 T Cells Precludes Memory Development and Is an Intrinsic Property of the Epitope

Xiaoting Z. Wang, Michael A. Brehm, and Raymond M. Welsh

Viral infections result in the activation and proliferation of epitope-specific CD8 T cells within secondary lymphoid organs such as the spleen and lymph nodes, and these T cells migrate into peripheral tissue to combat the infection (1, 2). As the immune response silences after clearance of virus, most of the T cells are removed from the host by apoptotic mechanisms, leaving only a small proportion to form long-lasting memory (3). The mechanisms of apoptosis and the decisions by which some cells are spared to enter the memory state are not well understood. This spontaneous apoptosis at the termination of infection seems unrelated to the preapoptotic signaling molecules Fas, Fas ligand, and TNF-α, which are important for the Ag-driven activation-induced cell death (AICD) and clonal exhaustion of CD8 T cells (4–6). The silencing phase of the T cell response to lymphocytic choriomeningitis virus (LCMV) infection is delayed in perforin−/−, IFN-γ−/−, granzyme B−/−, and CD43−/− mice (6–9), but none of these deficiencies completely eliminated this contraction phase, and prolonged Ag presentation due to delayed clearance of virus may have led to altered kinetics of silencing in some of those deficient mice. It has been proposed that this apoptotic silencing phase of the T cell response may reflect a predetermined, programmed event occurring after T cell proliferation (10), yet some cells are spared this process and become memory cells.

This spontaneous apoptosis after clearance of Ag occurs coincidentally with and perhaps as a consequence of the removal of growth factors such as IL-2 normally synthesized by T cells when their TCRs are triggered by Ag. Of note is that T cells expressing higher levels of Bcl-2, which protects against growth factor removal-induced apoptosis (3, 11), are enriched in the memory pool (12, 13). Alternatively, the T cells may become less responsive to growth and survival factors in their environment. Several cytokines, including IL-2, IL-7, and IL-15, can function as survival and proliferation factors for T cells (14–18). Both naive and memory T cells express high levels of IL-7Ra, but down-regulate it after activation (15). However, a small proportion of CD8 T cell effectors at the resolution of the CD8 T cell response to LCMV infection express relatively high levels of IL-7Ra, and these cells are progenitors to memory cells when transferred into naive mice (19). What controls the expression of IL-7Ra and whether its expression is essential or just correlates with cells that survive in the memory state is not known.

The analysis of apoptotic cells in vivo is difficult because dying cells are rapidly scavenged by macrophages bearing receptors for phosphatidylserine, which is expressed on the surface of apoptotic cells (20). Thus, cells that have fragmented their DNA and demonstrated many of the morphological properties of apoptotic cells are for the most part inapparent. Recent work using annexin V to detect phosphatidylserine on the surface of cells has revealed very high percentages (e.g., >50%) of Ag-specific CD8 T cells in the spleen and lymph nodes reacting with that marker throughout the acute infection and into memory (21). The presumption is that these cells have committed to an apoptotic pathway, but they are best referred to as being preapoptotic, as some studies have questioned whether cells reacting with annexin V are irreversibly destined to die (22, 23). Our analyses of these preapoptotic CD8 T cells during acute infections with a strain of LCMV (Armstrong)
with lymphoid organ tropism showed several remarkable features about preapoptotic virus-specific T cells (21). First, a much higher frequency of the LCMV Ag-specific CD8 T cells from lymphoid organs (spleen and lymph nodes) were preapoptotic than were cells isolated from peripheral tissues such as the peritoneal cavity, fat pad, and lung. Second, these spleen and lymph node T cells had elevated expression of Fas and Fas ligand and were more susceptible to AICD. Third, there was a suggestion, more thoroughly documented in this report, that higher proportions of T cells susceptible to AICD. Third, there was a suggestion, more thoroughly documented in this report, that higher proportions of T cells specifically to certain epitopes may be in a preapoptotic state than those specific to other epitopes. Fourth, relatively high frequencies of preapoptotic spleen and lymph node T cells could be detected throughout the acute infection and into the resting memory state.

In this study, we sought to understand the significance of this preapoptotic state by examining the fate of the preapoptotic cells in vitro and in vivo. We demonstrate that cultivation of preapoptotic CD8 T cells in vitro with IL-2 and/or anti-CD3 can inhibit them from fragmenting their DNA. However, the preapoptotic cells were mostly lost when transferred in vivo, and nearly all memory was derived from the annexin V−CD8 cell population. Secondly, we show that the preapoptotic phenotype differs between epitope-specific CD8 T cell populations, whether or not the Ags are expressed by biologically different LCMV strains or by vaccinia virus (VV) recombinants expressing LCMV proteins. These diverging phenotypes are seen in primary, memory, and recall responses. Finally, we correlate the reduced expression of IL-7Ra with the tissue- and epitope-dependent patterns of preapoptotic T cells.

Materials and Methods

Mice

C57BL/6J, B6.PLThy1c, and 129×1(129SvJ) male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.SJL (Ly5.1) male mice were purchased from Taconic Farms (Germantown, NY). Mice were used at 6–12 wk of age. P14 TCR transgenic mice harboring transgenic T cells specific for the Dβ-restricted LCMV epitope gp33−41 were purchased from The Jackson Laboratory and were backcrossed onto Rag-/-C57BL/6 mice (Thy 1.2+) by Dr. J. Maciaszek (University of Massachusetts Medical School, Worcester, MA).

Virus

LCMV strain Armstrong and its highly disseminating clone 13 variant were propagated in baby hamster kidney (BHK)/21 cells. Mice were inoculated i.p. with 0.1 ml of 5 × 105 PFU. VV recombinants expressing LCMV genes were provided to us by Dr. J. Lindsay Whitton ( Scripps Research Foundation, La Jolla, CA) (24).

Preparation of leukocytes

Mice were anesthetized by an i.p. injection of nembutal (Abbott Laboratories, Abbott Park, IL). Peritoneal exudate cells (PEC) from the peritoneal cavity were collected as previously described (26). For staining, 1 μg of peptide-charged dimer was incubated with 106 leukocytes for 1.5 h at 4°C. The cells were then incubated with biotinylated anti-mouse IgG1 and anti-CD3, and then with PE-streptavidin (BD Pharlmagen, San Diego, CA), all done at 4°C. For tetramer staining, 106 leukocytes were incubated with allophycocyanin-labeled NP396- or gp33-specific tetramers (31, 32) and anti-CD8 (BD Pharlmagen) for 1 h at 4°C. Cells stained with MHC dimers were then stained with FITC-annexin V (BD Pharlmagen), as previously described (21). In our hands annexin V stain of MHC dimmer-bound cells was more reliable than tetramer-bound cells. The cells were analyzed by flow cytometry immediately. In some experiments, anti-CD127 (IL-7Ra) (BD Pharlmagen) was added.

Intracellular IFN-γ assay

Leukocytes were stimulated with 5 μM peptides, 10 U/ml human rIL-2 (BD Pharlmagen), and 0.2 μl of Golgiplug (containing brefeldin A) at 37°C for 5 h. IFN-γ-producing CD8 T cells were detected with the Cytofix-Cytoperm kit Plus (BD Pharlmagen), according to the manufacturer’s protocol as described (26).

Cell sorting and adoptive transfer

Splenocytes from LCMV-infected mice were stained with anti-CD8 and annexin V. Cells were sorted by flow cytometry using a FACStar™ sorter (BD Biosciences, Mountain View, CA). CD8+ annexin V+ or CD8+ annexin V+ Ly5.2− T cells (1−2 × 106) were transferred into Ly5.1 congenic mice, and the donor cells were traced in the PBL by tail-bleed. In the study with transgenic cells, 1 × 106 splenocytes of P14 Thy1.2+ mice were transferred into Thy1.1 hosts, and LCMV was given the next day. Eight days after infection, annexin V+ and annexin V− splenocytes were sorted.

TUNEL staining and propidium iodide (PI) staining

For in vitro studies, 106 sorted cells were cultured in 48-well plates with or without 0.02 μg of human rIL-2 (BD Pharlmagen) and/or 2 μg of purified anti-CD3 mAb (145-2C11; BD Pharlmagen) for 5 h. TUNEL staining for DNA fragmentation was applied with a terminal transferase kit (Roche, Indianapolis, IN), according to the manufacturer’s instructions.

Results

High frequency of preapoptotic Ag-specific CD8 T cells in the spleen and lymph nodes during acute LCMV-Armstrong infection

The CD8 T cells from mice inoculated with the lymphotropic LCMV-Armstrong strain were assessed for a preapoptotic phenotype at 9 days postinfection by staining with annexin V. Viral epitope-specific T cells were identified by costaining with MHC-IgG1 Dβ dimers charged with immunodominant peptides NP396 or gp33 or else by tracing Thy1.2+ LCMV-specific transgenic T cells inoculated into Thy1.1+ mice before infection. A large proportion of the LCMV epitope-specific CD8 T cells were preapoptotic in spleen and lymph nodes, as shown previously (21). Fig. 1A shows annexin V reactivity of transgenic T cells in either a wide-open gate (top) or a narrowed lymphocyte gate (bottom). Fig. 1B shows annexin V reactivity of total CD8-, NP396-, and gp33-specific lymphocyte-gated T cells averaged from three experiments. Ag-specific T cells (~40−60%) gated on live lymphocytes reacted with annexin V in the spleen and the three lymph nodes examined. Peripheral tissue CD8 T cells in the peritoneal cavity, visceral fat pad, and lung stained poorly with annexin V. The preapoptotic phenotype in the liver seemed intermediate between the lymphoid and peripheral organs, as noted previously (21). Most annexin V+ transgenic T cells from spleen and lymph nodes were not stained with PI, a nucleic acid dye to stain late apoptotic cells with loss of membrane integrity (Fig. 1A), indicating they were in a preapoptotic state. In contrast, a high proportion of the small number of annexin V+ T cells in peritoneal cavity and lung stained with PI. Thus, the small number of annexin V-reacting cells in the peripheral tissues seems to be further along in the apoptotic pathway.
IFN-γ production by annexin V+ CD8 T cells in lymphoid organs

We questioned the fate of these annexin V-reactive spleen and lymph node T cells that we refer to as being preapoptotic because there was very little staining with markers of more advanced stages of apoptosis, including PI and the TUNEL stain for DNA fragmentation (21). We asked whether preapoptotic CD8 T cells were still functional and able to secrete IFN-γ in response to peptide stimulation. Lymphocytes from acutely LCMV-infected C57BL/6 mice were incubated at 37°C in vitro for 5 h in the presence of brefeldin A. Nearly all of the transgenic spleen and lymph node T cells secreted IFN-γ in response to gp33 peptide, regardless of whether they bound annexin V (Fig. 2A), indicating that those preapoptotic T cells are still functional in cytokine production. In peripheral tissues, peritoneal cavity, and lung, most of the transgenic T cells made IFN-γ, although they reacted less with annexin V. Many host T cells from spleen and lymph node, when stimulated with NP396 peptide, were annexin V− IFN-γ+. Of interest is that in the peritoneal cavity and lung, most of the transgenic T cells made IFN-γ, indicating that those preapoptotic T cells are still functional in the periphery (Fig. 2B). We and others have consistently seen that LCMV epitope-specific spleen T cell frequencies in acute infection are at least as high in intracellular IFN-γ assays as they are with MHC tetramer or MHC dimer assays, consistent with the idea that nearly all of these spleen cells are functional (30, 32).

Delay of DNA fragmentation in annexin V+ CD8 T cells in vitro by IL-2 or anti-CD3 stimulation

It has been questioned whether annexin V+ T cells are in an irreversible apoptotic pathway (22, 33). We showed previously that purified annexin V+ but not annexin V− LCMV-specific transgenic T cells, when transferred into culture, rapidly fragmented their DNA, as monitored by TUNEL assay, if no further stimulation was provided (21). We questioned whether signals through TCR or cytokines would be able to rescue or delay annexin V+ T cells from proceeding into apoptosis. To test for that, annexin V+ and annexin V− splenocytes, which contained activated transgenic T cells 9 days postinfection, were separated by FACS sorting. The purified populations were incubated at 37°C in vitro for 5 h with either IL-2 or anti-CD3 mAb or without any stimulation. TUNEL staining of transgenic Thy1.2+ cells was analyzed on recovered cells with a wide-open gate that would include the very small cells in the final stages of apoptosis (Fig. 3). Culturing of the annexin V+ cells without additives at 4°C for 5 h prevented fragmentation of cell DNA, but when annexin V+ sorted cells were cultured at 37°C in medium but without stimulatory additives, over 50% of the transgenic T cells stained positively in the TUNEL assay. Under the same conditions the sorted annexin V− cells had much less DNA fragmentation. This indicated that reactivity with annexin V
Having shown that the fate of preapoptotic T cells can be variable in vitro, we questioned their fate in vivo. We purified annexin V–/– gp33-specific transgenic CD8 T cells and host NP396-specific T cells from spleen and axillary lymph node (AXL). Splenocytes from Thy1.1 mice reconstituted with Thy1.2 LCMV-specific transgenic CD8 T cells were stimulated with gp33–41 or NP396–404 peptide for 5 h and intracellularly stained with anti-IFN-γ, anti-CD8, anti-Thy1.2, and annexin V. Data were gated on CD8+ Thy1.2+ (A) or host CD8+ cells (B) of total leukocytes (wide-open gate), and the staining of IFN-γ vs annexin V was analyzed. The numbers in the upper left and upper right quadrants represent the percentage of annexin V– and annexin V+ T cells. The numbers below each panel represent the percentage of gp33-specific transgenic T cells and IFN-γ– NP396-specific T cells in each organ. Similar results were seen in nontransgenic experiments with different peptide stimulations in LCMV-infected mice.

Failure of preapoptotic T cells to develop into memory cells in vivo

Having shown that the fate of preapoptotic T cells can be variable in vitro, we questioned their fate in vivo. We purified annexin V–/– CD8+ cells and annexin V– CD8+ cells from day 8 LCMV-infected Ly5.2+ mouse spleens and adoptively transferred them into Ly5.1+ congenic uninfected mice (Fig. 4). At different intervals after transfer, the recipient mice were tail-bleed, and Ly5.2+ donor cells were monitored by FACS staining. As shown in Fig. 4, 9 days after transfer (17 days after the primary LCMV infection), annexin V+ donor cells were present but at 0.05% in PBL, whereas the annexin V– donor cells were at a higher frequency of 0.11%. The percentage of donor cells in PBL is usually between the percentage in spleen and in peritoneal cavity (data not shown), and it is considered be representative of the existence of the donor cells in both lymphoid and nonlymphoid organs. It was noteworthy that some of the annexin V+ donors were found at 9 days posttransfer. A hospitable environment in the naive hosts may have delayed their death. Forty-one days after primary infection, cells from annexin V+ donors could barely be detected, but a small amount of cells from the annexin V– donors (0.04%) could still be observed, indicating enhanced survival capacity in comparison to the annexin V+ cells. To examine whether the remaining donor cells were part of a functional memory pool, we challenged the recipient mice with LCMV. Six days later, spleen cells and PEC were analyzed with Ly5.2 and CD8 staining (Fig. 4). CD8 T cells derived from the annexin V+ donor cells were quickly expanded in percentage during the recall response. Only a small response from T cells derived from annexin V+ donor cell populations (0.01%) was observed under those conditions, and it is not clear whether those surviving were truly derived from annexin V+ cells or from a low level annexin V– contaminant of that population. We did not

FIGURE 2. Production of IFN-γ by annexin V+ gp33-specific transgenic donor T cells and host NP396-specific T cells from spleen and axillary lymph node (AXL). Splenocytes from Thy1.1 mice reconstituted with Thy1.2 LCMV-specific transgenic CD8 T cells were stimulated with gp33–41 or NP396–404 peptide for 5 h and intracellularly stained with anti-IFN-γ, anti-CD8, anti-Thy1.2, and annexin V. Data were gated on CD8+ Thy1.2+ (A) or host CD8+ cells (B) of total leukocytes (wide-open gate), and the staining of IFN-γ vs annexin V was analyzed. The numbers in the upper left and upper right quadrants represent the percentage of annexin V– and annexin V+ T cells. The numbers below each panel represent the percentage of gp33-specific transgenic T cells and IFN-γ– NP396-specific T cells in each organ. Similar results were seen in nontransgenic experiments with different peptide stimulations in LCMV-infected mice.

is an indicator that CD8 T cells will undergo DNA fragmentation and apoptosis.

The dynamics of this process were changed, however, if the annexin V+ cells were incubated with either IL-2 or mAb to CD3. In either case, there was a substantial reduction or delay in the DNA fragmentation, indicating a postponement of the apoptotic process. These experiments indicate that the fates of “preapoptotic” T cells expressing reactivity with annexin V can, at least in vitro, dramatically differ.

FIGURE 3. TUNEL staining of sorted annexin V– and annexin V+ T cells after in vitro incubation. Splenocytes from Thy1.1 mice reconstituted with Thy1.2 LCMV-specific transgenic CD8 T cells were stained with annexin V 9 days after infection. Annexin V– and annexin V+ cells were sorted and cultured at 4°C or 37°C with IL-2 or anti-CD3 for 5 h. The TUNEL assay was applied to cells after each incubation, and data were gated on CD8+ cells of the total recovered cells with a wide-open gate that would include small lymphocytes in late stages of apoptosis. The numbers represent the percentage of TUNEL+ cells within the Thy1.2+ transgenic CD8 T cells. Similar results were seen in two additional experiments.
detect memory cells in the peritoneal cavity of recipients reconstituted with annexin V+ cells, indicating that the preapoptotic T cells could not be rescued in a peripheral environment, which normally houses T cells undergoing only low levels of apoptosis. Hence, preapoptotic cells do not necessarily rapidly die in vivo, but they were excluded from generating memory.

More preapoptotic NP396-specific than gp33-specific CD8 T cells during acute LCMV infection

Data in our previous study suggested that CD8 T cells with different specificities reacted with annexin V differently, although this phenomenon was not investigated (21). We compared the preapoptotic phenotype of NP396-specific with gp33-specific T cells in each organ of day 9 LCMV Armstrong-infected mice (Fig. 1B). In every lymphoid organ we examined, NP396 dimer+ cells exhibited 6–27% more annexin V+ cells than gp33 dimer+ cells. Even in nonlymphoid tissues, in which there were lower levels of preapoptotic cells, this trend was usually observed. Data in Fig. 1B were the average of six mice from three experiments, and each mouse had higher annexin V staining on NP396-specific than gp33-specific T cells in every spleen and lymph node, but the percentage of annexin V+ cells varied between mice. Considering that the staining was done under the same conditions, and at the same time, these differences of annexin V binding on different epitope-specific T cells were not likely to be an artifact from the staining. This observation was seen in each of more than 10 individual experiments and suggested that T cells specific to different epitopes had different sensitivities to apoptosis during the infections.

More preapoptotic NP396-specific than gp33-specific CD8 T cells in clone 13-infected or VV-NP- and VV-gp-infected mice

Having demonstrated that the preapoptotic phenotype is higher in lymphoid organs than in peripheral organs and that the magnitude of the preapoptotic phenotype varies with the epitope, we questioned whether these phenomena were peculiar to the Ag presentation associated with the Armstrong strain of LCMV. This strain of LCMV replicates mostly in lymphoid organs, in which Ag display may render the T cells more susceptible to apoptosis. In addition, the nucleoprotein, from which the NP396 epitope is derived, is the most abundant protein expressed in LCMV-infected cells (35), and this high level of protein expression may stimulate the T cells to a high level of apoptosis.

We first considered whether the lymphotropic nature of the Armstrong strain of LCMV predisposed to higher levels of apoptosis in lymphoid organs by examining preapoptotic CD8 cells in mice infected with the highly disseminating clone 13 variant of LCMV. In contrast to the Armstrong strain, this virus grows to high titers throughout the body in lymphoid and nonlymphoid organs (36). Infection with $5 \times 10^2$ PFU of clone 13 induces similar kinetics of the CD8 T cell response as Armstrong in C57BL/6 mice. At day 9 of the response, the annexin V reactivities of NP396-dimer+ and gp33-dimer+ CD8 T cells were analyzed (Fig. 5). Virus-specific T cells in the spleens and lymph nodes expressed much higher reactivity with annexin V than did those in peripheral tissue, indicating that T cells in the periphery resisted apoptosis even when virus replicated to high levels in the periphery. The differential apoptosis between epitope-specific T cells also remained, as T cells from spleen and lymph nodes had many more NP396-specific cells than gp33-specific T cells bound with annexin V. Thus, the tissue-dependent and epitope-dependent variations in LCMV-specific preapoptotic cells were similar under highly varied conditions of infection.

We questioned whether these epitope-specific and tissue site-dependent differences in annexin V binding would be seen under conditions of Ag presentation where similar amounts of nucleoprotein and glycoprotein were made and presented in different cells. We coinfect mice with equal amounts of VV-recombinants

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**Figure 4.** Failure of annexin V+ CD8 cells to generate memory. Annexin V+ and annexin V+ CD8 splenic cells were sorted from day 8 LCMV-infected Ly5.2 mice and adoptively transferred into Ly5.1 hosts. Donor cells were traced at different days after infection by Ly5.2 staining from blood. After secondary LCMV challenge, donor cells from spleen and peritoneal cavity (PEC) were analyzed by FACS. The number in the upper right quadrant represents the percentage of donor cells. Data are representative of 10 mice for each group in separate experiments.
expressing either LCMV-NP or LCMV-gp, such that there would be more comparable expression of the proteins, which were under control of the same promoter. Studies have shown that only a small percentage of APCs get infected with similar doses of VV, so these viruses should at least initially infect and express their Ags in different APCs (37). Lymphocytes from various organs were stained with NP396- or gp33-charged dimers and annexin V. The staining and frequencies of annexin V+ epitope-specific CD8 T cells are shown in Fig. 6. The LCMV epitope-specific T cell frequencies, as expected, were much lower than in the acute LCMV infection, but again, substantially higher frequencies of preapoptotic cells were detected in the spleen than in the peritoneal cavity, and the differences between the epitopes remained. There were 61% of T cells scoring as annexin V+ in the spleen NP396-dimer+ population, but only 38% in gp33-dimer+ cells. Thus, the tissue-dependent and epitope-dependent preapoptotic properties remained the same when the two proteins were produced comparably and when they were presented presumably by separate APCs.

### Duration of epitope-dependent annexin V reactivity

To further investigate whether the epitope-dependent preapoptotic phenotype is dependent on exposure of Ag, we examined the annexin V reactivity of NP396- and gp33-specific T cells at different time points during primary infection, the resting memory state, and secondary infection. As shown in Fig. 7, this differential preapoptotic phenotype was detected at the day 8–9 peak of the primary T cell response, but also at day 7 when some Ag is present (Fig. 7A) and at day 12 when Ag is mostly cleared and the T cells have greatly contracted (Fig. 7B). These epitope-dependent differences remained at 2 (Fig. 7C) or 4 mo, and even 14 mo (data not shown) after infection, when the host was in a resting memory state, with little if any Ag present to stimulate the T cells. If immune mice were challenged with LCMV and examined 6 days later (Fig. 7D), similar differences in the preapoptotic state could be seen in both spleen and lymph nodes. The data in Fig. 7 suggest that epitope-dependent differences in apoptosis are determined early in the T cell response and that the differential level of the preapoptotic phenotype remains imprinted on these T cells as Ag is cleared and as the host goes into the long-term memory state.

We wondered whether the differential apoptosis induced more profound contraction of NP396-specific T cells than gp33-specific T cells, perhaps changing the hierarchy of these two epitope-specific responses. The percentages of NP396- and gp33-specific CD8 T cells identified with MHC dimer staining at different days postinfection were plotted in Fig. 7E. Greater expansion of NP396-specific T cells was often seen at peak response, but more gp33-specific T cells were observed in the memory state. This exchange of immunodominance between NP396 and gp33 is associated with a more moderate attrition of gp33-specific T cells than NP396-specific T cells. This is consistent with less annexin V staining of gp33-specific T cells (Fig. 7, A–C).

#### Reduced IL-7Rα expression in preapoptotic cells

We sought to determine whether the preapoptotic phenotype correlated with the expression of other surface Ags on the CD8 T cells. Our initial studies with virus-specific spleen CD8 cells indicated that annexin V+ cells expressed higher levels of activation markers such as CD44, Ly6C, CD11a, CD11b, and CD122 than annexin V− cells (data not shown). Given our observation that memory cells in vivo are derived from annexin V+ cells (Fig. 4) and the observation by others that cells expressing high levels of IL-7Rα preferentially enter the memory state (19), we examined
FIGURE 7. Higher annexin V-binding of NP396-specific cells from different times after LCMV infection and less attrition of gp33-specific T cells. Lymphocytes from spleen and axillary lymph node (AXL) of mice infected with LCMV for 7 days (A), 12 days (B), 2 mo (C), or 6 days (D) after secondary LCMV infection were stained with anti-CD8, MHC dimer, and annexin V. FACS plots were gated on CD8+ cells. The numbers in the upper right quadrant represent the percentage of annexin V+ cells of Ag-specific T cells. E, Percentage of dimer+ cells at different days after infection. Data were average of six mice at day 9 postinfection and of two mice at rest of time points, and analysis has been repeated three times with three mice in each experiment.

the expression of IL-7Rα in the systems being studied. Lymphocytes from spleen and peritoneal cavity were stained with anti-CD127 (IL-7Rα) mAb combined with NP396 or gp33 tetramer and annexin V. Higher levels of IL-7Rα were expressed on CD8 T cells from the peritoneal cavity (32%) than from the spleen (20%) 8 days after infection with LCMV-Armstrong (Fig. 8A). Within the spleen, annexin V− cells had more cells (28%) that were IL-7Rα− than did preapoptotic annexin V+ cells (19%) (Fig. 8B). Some of those annexin V− cells had high expression of CD8, indicating they were not highly activated. Of note is that gp33-specific

FIGURE 8. Greater expression of CD127 (IL-7Rα) on peritoneal cavity, annexin V−, and gp33-specific CD8 T cells. Lymphocytes from spleen and peritoneal cavity (PEC) of day 8 LCMV-infected mice were stained with anti-CD8, anti-CD127, and MHC tetramer or annexin V. A, More CD8 cells from peritoneal cavity express IL-7Rα than cells from spleen. The numbers in the upper right quadrant represent the percentage of IL-7Rα+ in CD8+ cells. B, More annexin V− CD8 cells express IL-7Rα than annexin V+ cells. CD8− annexin V+ (12%) and CD8+ annexin V− (26%) splenocytes were gated, and the staining with IL-7Rα for one of each group was shown. The numbers in the upper right quadrant represent the percentage of IL-7Rα− cells. C, More gp33-specific cells express IL-7Rα than NP396-specific cells. Data were gated on CD8+ tetramer− cells (among CD8+ cells, 16% of NP396+ and 9.3% of gp33+). The numbers in the upper right quadrant represent the percentage of IL-7Rα+ in the Ag-specific T cells. Similar results were seen in MHC dimer-stained cells and have been repeated in four separated experiments.
T cells had 2-fold more IL-7Rα+ cells from both spleen and peritoneal cavity than NP396-specific cells (Fig. 8C). Thus, in both tissue-dependent and epitope-dependent patterns of apoptosis, high IL-7Rα expression correlated with resistance to apoptosis.

Discussion

A surprisingly high number of Ag-specific CD8 T cells are pre-apoptotic during the acute and memory response against LCMV. In this report we show that although preapoptotic T cells could be rescued at least temporarily from rapid death in vitro, the preapoptotic phenotype prevented memory development in vivo. The preapoptotic phenotype differed, as shown before, in tissue sites, and as shown here, between T cells specific for different epitopes. This seemed to be an intrinsic property of the epitope that was independent of where and how the protein encoding the epitope was expressed. There was greater attrition over time of epitope-specific T cells that had higher annexin V reactivity (Fig. 7E). In all cases the preapoptotic phenotype inversely correlated with expression of IL-7Rα, consistent with the idea that IL-7-dependent signals may inhibit apoptosis.

Cell surface reactivity with annexin V has long been recognized as a reliable predictor of apoptosis, but some reports have suggested that annexin V may stain cells that are not committed to the death pathway (22, 33). We previously demonstrated that preapoptotic annexin V+ LCMV-specific CD8 T cells fragment their DNA after a short incubation in vitro. Whether such completion of the apoptotic process would occur so quickly in vivo was uncertain, as our study shows that the DNA fragmentation of preapoptotic cells could be postponed under certain conditions of culture. Signaling through CD3 or IL-2R pathways delayed their death (Fig. 3). Such signaling in vivo may help the annexin V+ effectors to stay alive and functional long enough to defend against infections. These annexin V+ cells were shown in Fig. 2 to be perfectly capable of producing IFN-γ on recognition of their ligand. More than half of the Ag-specific spleen cells have a preapoptotic phenotype before virus is cleared and when cytokines such as IL-2 are produced in vivo, so the death of T cells might be delayed under those conditions. When the Ags are gone and the IL-2 is no longer present, these preapoptotic effectors may fragment their DNA and die, and the total CD8 T cell number would decrease dramatically. The death of T cells and silencing of the immune response at the end of infection correlates with the shut down of IL-2 synthesis (38).

This preapoptotic phenotype of annexin V+ PI− TUNEL− seems to preclude the development of memory cells in vivo. Adoptive transfer experiments showed that only the annexin V− effectors gave rise to long-lived memory and a recall response (Fig. 4). These activated annexin V+ T cells tend to be eventually discarded, but their rates of disappearance may vary. Some annexin V+ CD8 cells did not die immediately after transfer into naive hosts and could be detected over a week later (Fig. 4). We have shown that the leukocyte environment in the periphery can inhibit apoptosis (21), but did not clarify whether it could rescue annexin V+ cells or only prevent annexin V− cells from dying. In our study, although some transferred annexin V+ T cells survived for a few days, their numbers ultimately decreased substantially, and virtually no recall response was detected on secondary challenge. No survival or recall of these cells could be observed in the peritoneal cavity either, suggesting that annexin V+ cells cannot survive over time in the apoptosis-resistant periphery. All these indicate that once T cells advance into the preapoptotic annexin V-reactive stage, their death can be postponed, but they die eventually and cannot provide immunological memory.

A striking observation was that T cells with different specificities have different apoptotic phenotypes and that these phenotypes appear to be imprinted early in infection and last long after infection into the memory state, in which very little if any Ag is present. This preapoptotic phenotype seems to be an intrinsic property of each epitope, as similar results were seen whether the Ag was presented by different LCMV strains with different tropisms or by VV recombinants, which most likely were presenting the two studied epitopes in different APCs. The titers of VV are low in these infected animals, reducing the opportunity of coinfection of the same APC with the two different recombinants. The epitope-based differences in apoptosis appear to be imprinted on the T cells when Ag is present, but they remain during the silencing phase when Ag is cleared, and they remain in the resting memory response. Re-exposure to Ag in a recall response simply increases the number of epitope-specific cells, but their differences in preapoptotic phenotype remain. Long-term imprinting on CD8 T cells has been shown in studies documenting how help from CD4 T cells during the early part of infection will influence how well CD8 T cells will respond to Ag in a recall response (39–41), and some comparable factors may be occurring here.

Conversely, this may not be due to imprinting at all but may instead be a function of self-Ags that cross-react with T cells specific to different epitopes, perhaps sending different levels of apoptotic signals to T cell populations specific to different Ags. It has been speculated that lipid raft formation may be different in influenza-specific T cells reactive with a distinct epitope that is similar to a self-Ag (42, 43). Our recent analyses have shown that NP396- and gp33-specific T cells in 129×1 (129sJ) mice, which have H2b MHC alleles on a different genetic background, did not display the preapoptotic differences seen in C57BL/6 mice. The percentage of annexin V− of NP396+ vs gp33+ CD8 T cell populations of five mice tested in three separate experiment were: mouse 1, 41 vs 43%; mouse 2, 46 vs 41%; mouse 3, 40 vs 46%; mouse 4, 50 vs 53%; mouse 5, 49 vs 46%. The average value of NP396+ annexin V− cells was 45.2% ± 2.0%, and the average value of gp33− annexin V− cells was 45.8% ± 2.0%. This suggests that the genetic background of mice can influence the preapoptotic phenotype.

It could, however, be expected that T cells specific to the NP396 epitope may be more apoptotic in the presence of Ag. NP396 binds to the MHC with much higher avidity than does gp33, and the T cell population binding to NP396-expressing APC is a high affinity population (28, 44). In the very early stage of infection, NP396-specific T cells proliferate slightly better than gp33-specific T cells (45), and as early as 5 days postinfection show differences in mitochondrial ion potential, which is a harbinger of apoptosis (46). The higher rate of apoptosis is associated with a greater attrition of NP396-specific T cells during the silencing phase (Fig. 7E). This is associated with a change of relative immunodominance between NP396 and gp33 in acute response and memory state. Under conditions of high dose clone 13 infection, which results in a persistent infection and clonal exhaustion of T cells, the NP396-specific T cells are completely eliminated from the system, whereas the gp33-specific T cells remain present, but in a dysfunctional energized state (5, 47–50). It has been thought that the high levels of nucleoprotein Ag and the high avidity of APC-NP396-T cell interactions act to send strong AICD signals to the NP396+ cells. This much is understandable, but our results in this study indicate differences between these epitope-specific T cell populations even when Ag is no longer around. We conclude that populations of T cells specific to different epitopes can have different personalities that are linked to an intrinsic property of the epitope. This may affect their long-term stability in vivo, as shown in Fig. 7E.
reactive T cells are IL-7R for memory CD8 T cell precursors, but not all the annexin V non-reactive between annexin V – cells (55). What causes some cells to express IL-7R and annexin V non-reactivity are markers for memory CD8 T cell precursors, but not all the annexin V non-reactive t cells are IL-7Rα+.

IL-7 is a critical factor for cell survival and can prevent apoptosis of T cells (15). Although it is not clear whether signaling through IL-7R is directly involved in prevention of apoptosis of the virus-specific CD8 T cells during LCMV infection (19), the enhanced expression of IL-7Rα in peritoneal cells over spleen cells, in spleen annexin V– cells over annexin V+ cells, and in gp33-specific over NP396-specific cells correlates IL-7Rα expression with cell survival (Fig. 8). This correlation is not exact, as the difference in annexin V staining between spleen and peritoneal CD8 T cells is very large, but PEC only have ~10% more IL-7Rα cells than do splenocytes. The difference of IL-7Rα staining between annexin V+ and annexin V– splenocytes is convincing but not dramatic. gp33-specific T cells have about twice as many IL-7Rα cells as do NP396-specific cells in both the spleen and peritoneal cavity, and this correlates with the difference in annexin V staining of these two epitope-specific T cells. Our preliminary microarray genechip analyses have revealed a lower amount of IL-7Rα mRNA between spleen and peritoneal transgenic cells and between annexin V+ and annexin V– cells (data not shown). Activated T cells may selectively down-regulate their surface expression of IL-7Rα (15), and this down-regulation has been suggested to be an indicator that the TCR has recently been triggered (19).

One, therefore, might expect that, in the presence of Ag, there would be more triggering of the NP396-specific T cells and subsequent IL-7Rα receptor down-modulation.

Both IL-7Rα positivity and annexin V nonreactivity are markers for memory CD8 T cell precursors, but not all the annexin V non-reactive T cells are IL-7Rα+. The apoptosis is a dynamic process and many of the annexin V– cells might ultimately become annexin V+ cells that cannot become memory cells. In fact, only 10–20% of the activated CD8 T cells are IL-7Rα+ (Fig. 8A) (19). However, at the least partial correlation between IL-7Rα expression and annexin V nonreactivity is consistent with the concept that both tissue-dependent and epitope-dependent differences in apoptosis may be partially due to their sensitivity to cytokines such as IL-7. Other factors may contribute to the regulation of apoptosis during virus infection, as well. One study suggests that Bim, a proapoptotic Bcl-2 family member, may be important for shutting down the immune response. Wild-type CD8 T cells died rapidly, but Bim−/− cells were resistant to apoptosis after withdrawal of IL-7 in culture (55). What causes some cells to express IL-7Rα and others not to remains unclear.

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References

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