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Cutting Edge: Increased Expression of Bcl-2 in Antigen-Specific Memory CD8$^+$ T Cells[1]

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Bcl-2 plays a critical role in regulating cell survival and apoptosis. We examined Bcl-2 expression in virus-specific CD8 T cells during the expansion, death, and memory phases of the T cell response following infection of mice with lymphocytic choriomeningitis virus (LCMV). Naive CD8 T cells expressed a basal level of Bcl-2 that was down-regulated in effector CD8 T cells just before the death phase. Bcl-2 levels remained low during the death phase but surviving memory CD8 T cells expressed higher levels of Bcl-2 than naive cells. These changes were shown to occur in LCMV TCR transgenic cells as well as virus-specific CD8 T cells in C57BL/6 and BALB/c mice identified by MHC class I tetramers. In all instances, memory CD8 T cells expressed higher levels of Bcl-2, suggesting that increased Bcl-2 expression plays a role in the long-term maintenance of memory CD8 T cells in vivo. ***The Journal of Immunology*****, 2000, 164: 3950–3954.**

It is now well established that memory T cells can persist for extended periods in vivo (1, 2) The pool of memory T cells can remain remarkably stable and this constancy in numbers is maintained by a balance between cell survival, apoptosis, and proliferation (3–5). The mechanisms that regulate the life span of memory T cells are not understood at the molecular level. We have begun to address this question by examining expression of molecules that play a role in cell survival and apoptosis.

One of the most widely studied regulators of cell death is the Bcl-2 superfamily. This family is composed of more than 15 family members in humans, mice, and worms. Some members of the family are proapoptotic (Bad, Bax, Bid) while others (Bcl-2 and Bcl-x_L) exhibit anti-apoptotic functions (6). At any given time, a cell's susceptibility to apoptosis is determined by a ratio of the pro- to anti-apoptotic factors. The prototypic member of this gene family is Bcl-2. Originally identified as a translocation in B cell lymphoma (7), overexpression of Bcl-2 provides protection against cell death by preventing release of cytochrome *c* after mitochondrial damage (8).

Several studies have shown that Bcl-2 plays a role in T cell survival. The most compelling evidence comes from the observation that Bcl-$2^{-/-}$ mice exhibit rapid loss of peripheral T cells (9, 10). Also, it has been shown that activated T cells that exhibit increased susceptibility to apoptosis in vitro express lower levels of Bcl-2 (11). However, very little is known about Bcl-2 expression in Ag-specific T cells in vivo. In this study, we have examined the expression of Bcl-2 in CD8 T cells as they progress through the various stages of the immune response (naive to effector to memory) following infection of mice with lymphocytic choriomeningitis virus (LCMV).[4] Using a transgenic T cell system and MHC class I tetramers to identify virus-specific CD8 T cells in normal mice, we show that memory CD8 T cells express higher levels of Bcl-2 than naive T cells. This finding suggests that Bcl-2 may play a role in the long-term survival of memory CD8 T cells.

Materials and Methods

Virus infection and mice

Six- to 8-wk-old female BALB/c or C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were infected with 2×10^5 PFU of LCMV Armstrong strain i.p. and used at the indicated time points. Virus stocks were grown and quantitated as described previously (12).

Priming of transgenic cells

Priming of P14 transgenic cells has been described before (13). The LCMV TCR P14 transgenic mice (B6 × 129) were obtained from The Jackson Laboratory and then backcrossed to C57BL/6 at Emory University. The transgenic mice used in this study were backcrossed six to eight times. Briefly 2×10^6 naive P14 splenocytes were injected i.v. into naive C57BL/6 hosts. Four hours after transfer, mice were infected with 2×10^5 PFU of LCMV Armstrong i.p. and used at the indicated time points.

Flow cytometry and FACS analysis

Preparation of cells and staining has been described previously (1).

Intracellular staining for Bcl-2

Cells (2×10^6) were surface stained as described above. After washing unbound Ab and MHC class I tetramer, the cells were subjected to intracellular staining for Bcl-2 using the CytoFix/Cytoperm kit according to the

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[4] Abbreviation used in this paper: LCMV, lymphocytic choriomeningitis virus.

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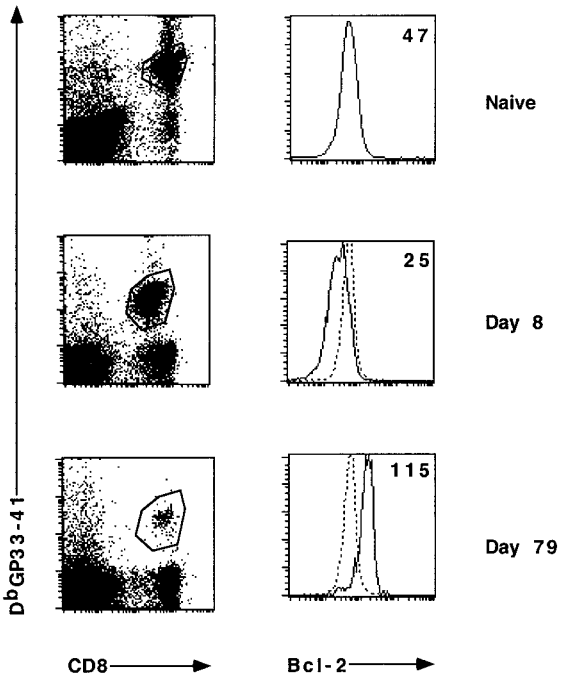

FIGURE 1. Bcl-2 expression in transgenic CD8$^+$ T cells during an immune response. A total of 2×10^6 splenocytes was transferred from naive P14 transgenic mice into nonirradiated naive C57BL/6 hosts. Four hours after transfer, mice were infected with LCMV-Armstrong and used at the indicated time points. Spleen cells were double stained with anti-CD8α and $D^bGP33-41$. The Bcl-2 levels of the gated populations are plotted in histogram format, with the mean fluorescence intensity indicated by the value in the *upper right-hand corner* of the plot. The Bcl-2 levels of naive CD8$^+$ T cells are overlaid on each histogram as the dotted line.

manufacturer's instructions (PharMingen, San Diego, CA). For intracellular Bcl-2 staining, FITC-conjugated hamster anti-mouse Bcl-2 Ab (clone 3F11) or its isotype control Ab (hamster IgG) (PharMingen) was used.

Preparation of H-2D^b and H-2L^d tetramers

The construction and purification of $L^dNP118-126$, $D^bGP33-41$, $D^bNP396-404$, and $D^bGP276-86$ MHC class I tetramers have been described previously (1).

Results

To determine the level of Bcl-2 expression, we performed flow cytometric analysis using P14 transgenic CD8$^+$ T cells. These CD8 T cells express a transgenic TCR specific for the LCMV epitope GP33-41 and allow a comparison of Ag-specific naive, effector, and memory CD8$^+$ T cells. Fig. 1 shows that naive Ag-specific CD8$^+$ T cells had moderate levels of Bcl-2 that decreased in effector cells isolated 8 days postinfection. In addition to decreased levels of Bcl-2, effector cells contained decreased CD8 expression. The decreases in these molecules do not represent a global decrease in staining as effector cells express increased levels of CD44, CD11a, and CD43 (1B11) and intracellular IFN-γ (data not shown). Memory cells (79 days postinfection) contained higher levels of Bcl-2 than those found in naive or effector cells.

In addition to examining Bcl-2 expression in a monoclonal response, we examined it during the CD8 response in BALB/c mice at various times postinfection. Fig. 2*A* shows that naive CD8$^+$, LFA-1^{low} T cells expressed a basal level of Bcl-2. Following infection with LCMV, Bcl-2 expression was essentially unchanged in Ag-specific cells at 5 days postinfection. However, at the peak of the response on day 8 postinfection (i.e., peak before the death phase), Bcl-2 levels were clearly lower in the Ag-specific population. These changes were observed in four independent experiments. Bcl-2 levels remained lower in virus-specific CD8 T cells during the death phase (days 8–20). The day 13 time point is shown in Fig. 2*A*. Memory cells that survived this death phase expressed higher levels of Bcl-2. Similar increased levels of Bcl-2

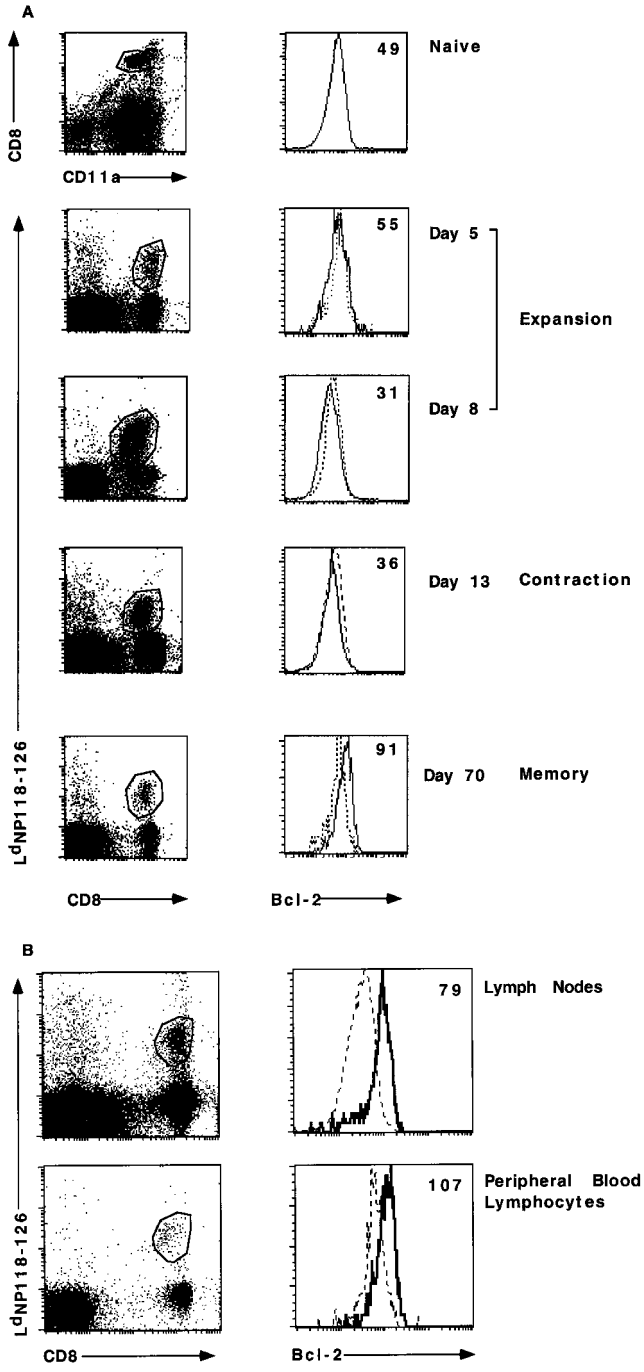

FIGURE 2. Bcl-2 levels in Ag-specific CD8 T cells identified by MHC class I tetramers. *A*, Spleen cells from naive and LCMV-infected (days 5, 8, 13, and 70 post infection) mice were double stained with anti-CD8α and either anti-CD11a/LFA-1 (naive) or MHC class I tetramer $L^dNP118-126$ (infected). *B*, PBL and lymph node cells from immune mice (120 days postinfection) were incubated with anti-CD8α and $L^dNP118-126$. The Bcl-2 levels of the gated populations are plotted in histogram format, with the mean fluorescence intensity indicated by the value in the *upper right-hand corner* of the plot. The Bcl-2 levels of naive CD8$^+$ T cells are overlaid on each histogram as the dotted line.

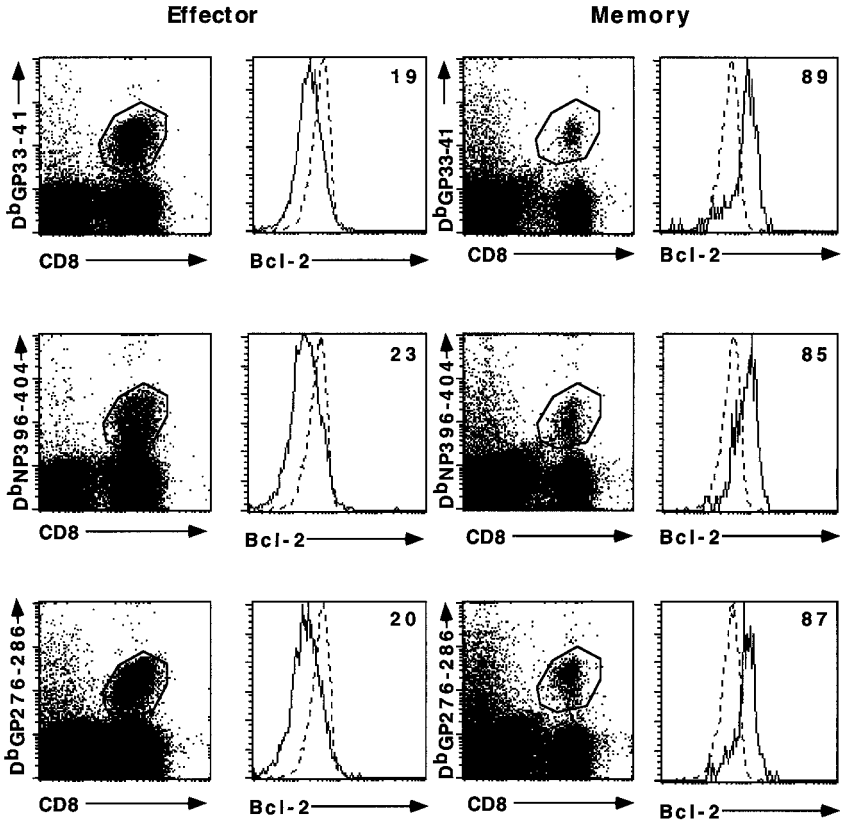

FIGURE 3. Analysis of Bcl-2 expression among CD8 T cells of different Ag specificities identified by MHC class I tetramers. Spleen cells were isolated from C57BL/6 mice at days 8 and 186 postinfection with LCMV. Cells were stained with anti-CD8α and either D^bGP33-41, D^bNP396-404, or D^bGP276-86 tetramer. The Bcl-2 levels of the gated populations are plotted in histogram format, with the mean fluorescence intensity indicated by the value in the *upper right-hand corner* of the plot. Additionally, the Bcl-2 levels of naive ($CD8^+CD44^{low}$) T cells are overlaid on each histogram as the dotted line.

protein were observed in Ag-specific memory CD8 T cells isolated from pooled lymph nodes and PBLs (Fig. 2*B*).

In BALB/c mice, the majority of the antiviral CD8 T cell response is specific for one immunodominant epitope (NP118-126). We also tracked how Bcl-2 expression changes in C57BL/6 mice that make sizable CD8 T cell responses to three different LCMV epitopes. Ag-specific CD8 T cells were examined (Fig. 3) on day 8 (effector) and day 186 (memory) postinfection with LCMV. Using MHC class I tetramers, we examined Bcl-2 levels in cells specific for the D^b-restricted epitopes GP33-41, NP396-404, and GP276-86. Ag-specific effector cells for all three epitopes showed remarkably similar decreases in Bcl-2 expression compared with naive $CD8^+CD44^{low}$ cells. More important, memory cells for all three epitopes contained higher levels of Bcl-2 compared with naive CD8 T cells. Our observations of cells with different epitope specificities demonstrate that high Bcl-2 levels are a generalized feature of CD8 memory cells.

Since Ag-specific memory $CD8^+$ T cells contained higher levels of Bcl-2 than naive cells, this prompted us to examine Bcl-2 levels in total $CD44^{high}$ memory phenotype cells. Fig. 4 confirms that memory phenotype ($CD8^+CD44^{high}$) CD8 cells of undefined specificity contain higher levels of Bcl-2 than naive phenotype cells ($CD8^+CD44^{low}$). Interestingly, this was not a

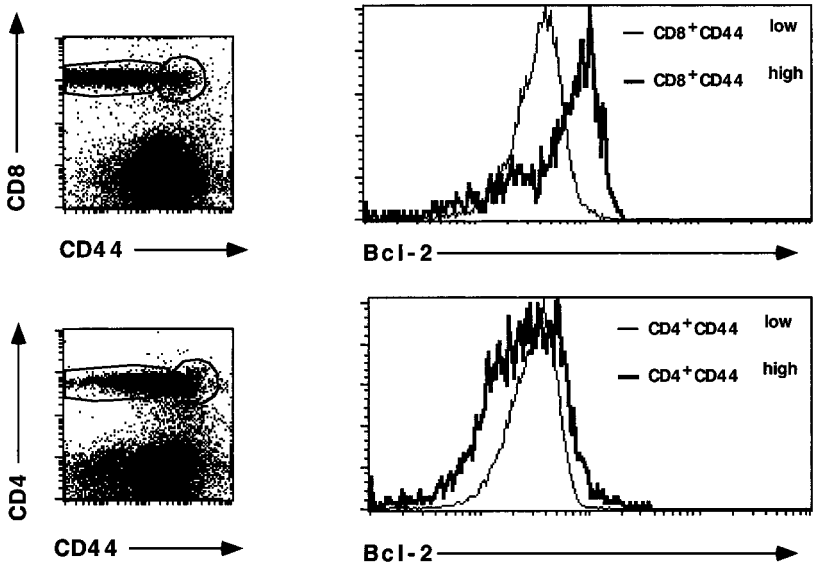

FIGURE 4. Bcl-2 is expressed at high levels in memory phenotype $CD8^+$, but not $CD4^+$ T cells. Spleen cells from naive C57BL/6 stained with anti-CD44 and anti-CD8α or anti-CD4. The Bcl-2 levels of the gated populations are plotted in histogram format.

characteristic of CD4 T cells and $CD4^+CD44^{high}$ cells exhibited no difference in their Bcl-2 levels when compared with $CD4^+CD44^{low}$ cells.

Discussion

In this study, we tracked expression of Bcl-2 through all phases of a CD8 T cell response: activation/expansion, contraction, and memory phases. We found that naive CD8 T cells express a basal level of Bcl-2 that does not decrease in early effector cells (5 days postinfection) during the expansion phase. However, Ag-specific cells at 8 days postinfection (at the peak of the expansion phase and at the beginning of the death phase) express lower levels of Bcl-2. Throughout the death phase Bcl-2 remains low in Ag-specific CD8 T cells. Surviving memory cells contain higher levels of Bcl-2 than naive cells. These findings were observed in both monoclonal and polyclonal CD8 responses. Additionally, they were observed in multiple LCMV epitopes in both BALB/c and C57BL/6 mice.

What are the implications of higher Bcl-2 expression in CD8 memory T cells? To understand what Bcl-2 may be doing, it is critical to note some differences between memory and naive cells. Memory cells survive for extended periods in vivo and exhibit a higher rate of homeostatic proliferation than naive cells (3). Little is known about the rate of progression through the phases of the cell cycle in these cells. Previous studies of Bcl-2 overexpression have shown that higher levels of Bcl-2 correlate with an extended G_1 phase (14). Additionally, Ag-induced cell death in vitro has been demonstrated to occur from a late G_1 phase cell cycle checkpoint that can be blocked by expression of $p16^{INK4a}$ or by inactivation of the retinoblastoma tumor suppressor protein (pRb) (15). The Bcl-2 levels in memory cells could increase G_1 phase, allowing the cell to repair damage accrued over its long life span and then to rapidly progress through the rest of the cycle. The higher rate of homeostatic proliferation observed in memory cells may simply be a consequence of increased survival of the G_1 phase transition. The increased levels of Bcl-2 found in memory cells may also influence survival of these cells after they encounter Ag upon reinfection. The higher levels of Bcl-2 may lead to a diminished death phase after secondary infection, resulting in a net increase of memory cells.

Our studies confirm and extend previous findings of Bcl-2 expression in activated T cells in vitro and in vivo. Activation of T cells in vitro with strong mitogenic stimuli and IL-2 results in slightly increased levels of Bcl-2 (11, 16, 17). In LCMV infection, we only observe decreases in Bcl-2 after prolonged expansion and proliferation. Ag-specific cells 5 days postinfection contain basal levels of Bcl-2 but with further proliferation contain low levels by 8 days postinfection. This maintenance of basal levels of Bcl-2 after early TCR activation makes teleological sense. If Bcl-2 decreased immediately after TCR activation, then cells would be deleted and a chronic infection would ensue. Additionally, our findings extend observations made in CD8 T cells from EBV and HIV patients (18, 19). CD8 T cells from patients generally had Bcl-2 levels lower than those found in uninfected controls. In these studies, the cells are of undefined specificity but they exhibit lower levels of Bcl-2 similar to Ag-specific cells at the peak of LCMV infection.

We also addressed differences between naive and memory phenotype cells of undefined specificity for both CD8 and CD4 cells. CD8 cells of a memory phenotype ($CD8^+CD44^{high}$) contained higher levels of Bcl-2 than the naive phenotype ($CD8^+CD44^{low}$), mirroring our observations with Ag-specific memory cells. Examination of CD4 naive and memory phenotype ($CD4^+CD44^{low}$ vs $CD4^+CD44^{high}$) revealed no major differences between these two populations in Bcl-2 expression. This is consistent with the findings of Garcia et al. (20). Using transgenic CD4 T cells (specific to C5 complement protein), they monitored a CD4 response in vivo and found that Bcl-2 levels decrease in effector T cells and then return to normal levels in memory CD4 T cells (similar to those seen in naive cells). The difference in Bcl-2 expression between memory CD4 and CD8 T cells can be potentially explained through two mechanisms. Bcl-2 may be more of a critical survival factor for CD8 than CD4 T cells. Null mutants of the Bcl-2 gene lose most of their lymphocytes by the fourth week of life. Additionally, lymphocytes from these animals show impaired survival in vitro. Both of these defects are manifested more prominently in CD8 than in CD4 T cells (10). Another possible reason for the discrepancy in Bcl-2 levels is differences in cycling rate. As mentioned earlier, Bcl-2 is expressed more highly in Ag-specific memory than naive CD8 T cells. Memory CD8 T cells also have a higher rate of homeostatic proliferation than memory CD4 cells (K. Murali-Krishna and R. Ahmed, unpublished observations), suggesting a potential link between division rate in vivo and Bcl-2 expression. However, it should be noted that definitive analysis of Bcl-2 levels in Ag-specific memory $CD4^+$ T cells will require the use of MHC class II tetramers to directly identify the Ag-specific cells.

Apoptosis is a complex multifactorial process controlled by interactions between pro- and anti-apoptotic genes. In this study, we have focused on the expression of one of the most important genes controlling apoptosis but future studies are needed to understand the roles of other genes such as *Bcl-x*, *Bad*, and *Bax* in controlling CD8 responses. We observed that Ag-specific memory cells contain increased Bcl-2, although effector cells are $Bcl\text{-}2^{low}$. Understanding the mechanism by which a population of cells expressing high levels of Bcl-2 (memory) is generated from cells with low levels (effector) is a current endeavor in our laboratory. In addition, determining whether Bcl-2 is necessary for the generation and maintenance of CD8 memory T cells will require the generation of chimeras with CD8 T cells that lack Bcl-2. Finally, further studies are needed to determine whether the higher levels of Bcl-2 found in memory cells renders them more resistant than naive cells to apoptotic stimuli.

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