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Gene Expression in Antigen-Specific CD8$^+$ T Cells During Viral Infection[1]

Jason M. Grayson, Kaja Murali-Krishna, John D. Altman, and Rafi Ahmed[2]

Following infection with intracellular pathogens, Ag-specific CD8$^+$ T cells become activated and begin to proliferate. As these cells become activated, they elaborate effector functions including cytokine production and cytolysis. After the infection has been cleared, the immune system returns to homeostasis through apoptosis of the majority of the Ag-specific effector cells. The surviving memory cells can persist for extended periods and provide protection against reinfection. Little is known about the changes in gene expression as Ag-specific cells progress through these stages of development, i.e., naive to effector to memory. Using recombinant MHC class I tetramers, we isolated Ag-specific CD8$^+$ T cells from mice infected with lymphocytic choriomeningitis virus at various time points and performed semiquantitative RT-PCR. We examined expression of: 1) genes involved in cell cycle control, 2) effector and regulatory functions, and 3) susceptibility to apoptosis. We found that Ag-specific CD8$^+$ memory T cells contain high steady-state levels of *Bcl-2*, *Bax*, IFN-γ, and lung Kruppel-like factor (*LKLF*), and decreased levels of p21 and p27 mRNA. Moreover, the pattern of gene expression between naive and memory cells is distinct and suggests that these two cell types control susceptibility to apoptosis through different mechanisms. *The Journal of Immunology,* **2001, 166: 795–799.**

CD8$^+$ T cells play a critical role in controlling viral as well intracellular and parasitic infections. During the acute phase of many infections there is a marked expansion of CD8$^+$ T cells. In humans this is observed during HIV (1), EBV (2), and CMV infections (3). In mice, CD8 expansion occurs during vaccinia virus, vesicular stomatitis virus (4), murine γ-herpesvirus (5), and lymphocytic choriomeningitis virus (LCMV)[3] infection (6). After viral clearance, a death phase ensues where 90–95% of the Ag-specific cells undergo apoptosis. The surviving memory cells persist for the life of the animal and rapidly assume effector functions upon re-encounter with Ag. To understand the mechanisms that control these developmental stages, we examined gene expression as CD8$^+$ T cells progressed through the naive, effector, and memory transitions. Emphasis was placed on genes involved in effector and regulatory functions, cell cycle control, and susceptibility to apoptosis.

Because the predisposition of a cell to apoptosis is a complex, multifactorial process, we examined expression of several members of the *Bcl-2* superfamily. *Bcl-2* and *Bcl-x_L* exert anti-apoptotic effects (7, 8), whereas *Bad* and *Bax* are pro-apoptotic (9, 10). In addition to the *Bcl-2* superfamily, other genes such as lung Kruppel-like factor (*LKLF*) (11) and Fas ligand (FasL; Ref. 12) regulate the magnitude of T cell responses. The size of the memory T cell pool will be influenced by four variables: 1) the number of naive cells recruited into the response, 2) the extent of proliferation following Ag encounter, 3) the amount of death after the response is complete, and 4) the long-term survival and proliferation of the pool of memory cells. Cyclin-dependent kinase inhibitors (CDKi) such as p21$^{Waf1/Cip1}$ (13) and p27^{Kip1} (14) play critical roles in the cell cycle by controlling progression from G_1 to S phase by inhibiting the activities of cyclin-dependent kinases, thus blocking phosphorylation of the retinoblastoma (Rb) protein. It is also critical to understand the regulation of effector molecules such as perforin and IFN-γ during these transitions to understand the mechanisms that allow for a rapid anamnestic response. Using recombinant MHC class I tetramers, we isolated Ag-specific CD8$^+$ T cells from mice infected with LCMV at various time points and performed semiquantitative RT-PCR analysis for the genes discussed above.

Materials and Methods

Virus infection and mice

Six- to 8-wk-old female BALB/c mice purchased from The Jackson Laboratory (Bar Harbor, ME) were infected with 2×10^5 PFU of LCMV-Armstrong i.p. and used at the indicated time points. For secondary challenge experiments, immune mice were injected with 2×10^6 PFU of LCMV-Clone 13 i.v. and used at the indicated time points. Virus stocks were grown and quantitated as described previously (15).

Preparation of MHC class I tetramers

The construction of L^{d}NP118-126 MHC class I tetramers has been described previously (6).

Sorting of naive and Ag-specific CD8$^+$ T cells

Freshly explanted splenocytes from naive or LCMV-infected mice (days 5, 8, and 105 post primary infection and 4 days post secondary infection) were surface stained with PE-conjugated anti-CD8α Ab (clone 53-6.7; PharMingen, La Jolla, CA) and either FITC-conjugated rat anti-mouse monoclonal CD11a (clone 2D7; PharMingen) or APC-conjugated L^{d}NP118-126 tetramer in PBS containing 1% BSA (FACS buffer) for 30 min at 4°C. After unbound Ab and tetramer were removed by washing, cells were sorted into either CD11a high or low, and CD8-positive populations or CD8-positive and tetramer-positive populations. Samples were acquired on a FACSVantage instrument (Becton Dickinson, San Jose, CA), and data were analyzed using CellQuest software (Becton Dickinson).

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus, CDKi, cyclin-dependent kinase inhibitor; *LKLF*, lung Kruppel-like factor; FasL, Fas ligand; HPRT, hypoxanthine phosphoribosyltransferase.

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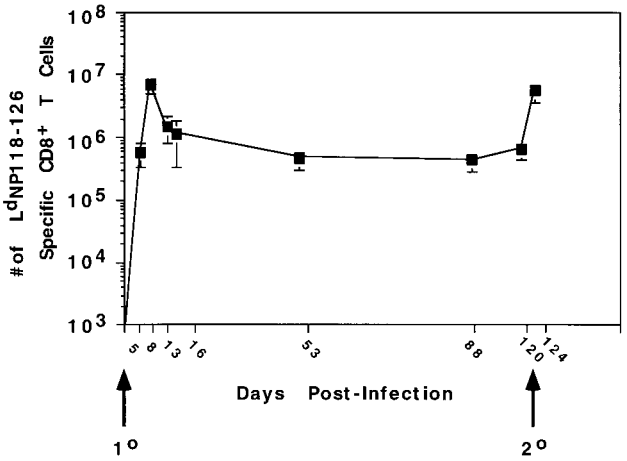

FIGURE 1. Quantitation of Ag-specific CD8$^+$ T cells during LCMV infection. BALB/c mice were examined at the indicated days postinfection for the number of virus-specific CD8$^+$ T cells in the spleen by MHC class I tetramer staining. Data represent the average values obtained from three to six mice. Arrow 2° indicates challenge of immune mice with LCMV clone 13. The bars represent the SD of each time point.

RT-PCR analysis

Sorted cells were isolated and total RNA was extracted. cDNA synthesis was primed with a $(dT)_{15}$ oligonucleotide. The resulting cDNA was amplified using the following primers: p21, 5′-CCGTGGACAGTGAG CAGTTG-3′ and 5′-TGGGCACTTCAGGGTTTTCT-3′; FasL, 5′-CGT-GAGTTCACCAACCAAAGC-3′ and 5′-CCCAGTTTCGTTGATCACA AG-3′; *Bax*, 5′-ACAGATCATGAAGACAGGGG-3′ and 5′-CAAAGTAGAA GAGGGCAACC-3′; hypoxanthine phosphoribosyltransferase (HPRT), 5′-GTAATGATCAGTCAACGGGGGAC-3′ and 5′-CCAGCAAGCTTGCA ACCTTAACCA-3′; *Bcl-2*, 5′-TTCTCCTTCCAGCCTGAGAGCAA-3′ and 5′-ATGACCCCACCGAACTCAAAG-3′; *Bcl-x_L*, 5′-CATCAATGG CAACCCATCCTG-3′ and 5′-TGTTCCCGTAGAGATCCACAAAAG-3′; *Bad*, 5′-TCCGGAGCCTGGGGAGCGACGCGGG-3′ and 5′-CTCATCGC TCATCCTTCGGAGCTC-3′; IFN-γ, 5′-AGCGGCTGACTGAACTCAG ATTGTAG-3′ and 5′-CGTCTAGAGTCACAGTTTTCAGCTGTATAG G-3′; PERF, 5′-TCTCGCATGTACAGTTTTCGCCTGGTA-3′ and 5′-TGTGAGCCCATTCAGGGTCAGCTG-3′; LKLF, 5′-CTGGAGGCCAA GCCCAAACGCGGC-3′ and 5′-CGTTGGGGACAGTAAACTCAAAG GCA-3′; p27, 5′-CCCGCCCGAGGAGGAAGATGTCAAAC-3′ and 5′-CCC TTTTGTTTTGCGAAGAAGAATCT-3′.

The cDNA from each sample was subjected to one round of PCR amplification consisting of an initial melting step (94°C for 8 min) and 30 repetitive cycles (94°C for 1 min); 52°C (p21, FasL, and *Bax*), 55°C (HPRT, *Bcl-2*, *Bcl-x_L*, and *Bad*), or 59°C (IFN-γ, LKLF, and p27, and Perf) for 1 min; and 72°C for 1 min. The PCR was performed using Ampli-*Taq* Gold (Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions.

Results

Infection of BALB/c mice by LCMV causes a massive expansion (>10,000 fold) of Ag-specific CD8$^+$ T cells. The vast majority of this response is directed against the L^d-restricted NP118-126 epitope (6). On days 5, 8, and 105 postinfection there were 5.8×10^5, 9×10^6, and 4.3×10^5 Ag-specific CD8$^+$ T cells in the spleen. Upon secondary challenge there was a re-expansion of Ag-specific cells to 6×10^6 (see Fig. 1). We used this to our advantage to isolate Ag-specific effector and memory cells. Naive cells were isolated on the basis of staining CD8$^+$ and CD11a^{low} (see Fig. 2*A*). We chose this marker instead of CD44, which is expressed in a constitutive fashion in BALB/c mice (16). Ag-specific cells were isolated by staining with both CD8α Ab and L^{d}NP118-126 tetramer. In all cases, the purity of the post-sort population was greater than 95%. The fluorescence decrease in the day 8 postsort sample is most likely due to quenching as the cells pass through the cytometer a second time. The specificity of postsort populations was confirmed by restimulation with NP118-126 peptide in IFN-γ ELISPOT assays where a 1:1 correlation between tetramer and IFN-γ-producing cells was observed.

After isolation of pure populations (>95%) of Ag-specific cells, total RNA was extracted and first-strand synthesis was performed using an oligo$(dT)_{15}$ primer. We examined the expression of four types of genes, those involved in T cell effector (perforin and IFN-γ) and regulatory functions (LKLF and FasL), and apoptosis (*Bcl-2*, *Bad*, *Bax*, *Bcl-x_L*), and cell cycle control (p21 and p27).

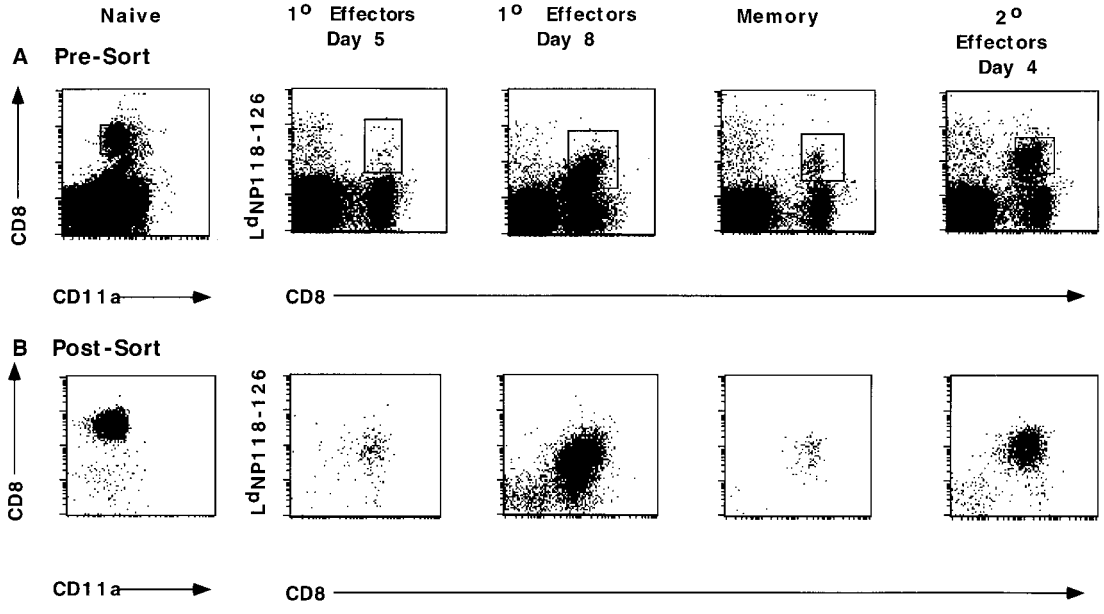

FIGURE 2. Isolation of pure populations of Ag-specific CD8 T cells during viral infection. Spleen cells from naive and LCMV-infected BALB/c mice days 5, 8, and 105 primary infection, and day 4 secondary infection were double-stained with anti-CD8α and either anti-CD11a or L^{d}NP118-126. CD11a^{low} and CD8-positive and CD8 and tetramer double-positive cells were sorted based on the gates indicated by the rectangles. The presort analysis of each population is presented in (*A*), whereas the postsort analysis is presented in (*B*).

Analysis of HPRT, a housekeeping gene for nucleotide biosynthesis, was included as a normalization control.

Naive CD8$^+$ T cells do not produce cytokines or lyse cells until they encounter their cognate Ag and become activated. This is reflected in Fig. 3*A*, which shows this population of cells contained very little perforin or IFN-γ mRNA. After infection with LCMV, these cells became activated and elaborated their effector functions. These effector CD8$^+$ T cells expressed IFN-γ mRNA on both days 5 and 8 during the primary response, whereas perforin mRNA is highly expressed on day 8 at the peak of the effector response. After viral clearance and a return to homeostasis, Ag-specific CD8$^+$ T cells enter the memory phase. These cells do not spontaneously produce cytokines nor do they lyse cells unless they re-encounter Ag. Surprisingly, memory cells contained high levels of IFN-γ, but no detectable perforin mRNA. After reinfection with virus, memory cells redifferentiated into secondary effector cells. These cells share similar abilities as primary effectors in that they produce cytokines and lyse infected cells. These cells are also similar to primary effector cells on a molecular level; they expressed comparable levels of IFN-γ and perforin mRNA. Negative controls for all reactions failed to produce amplification products.

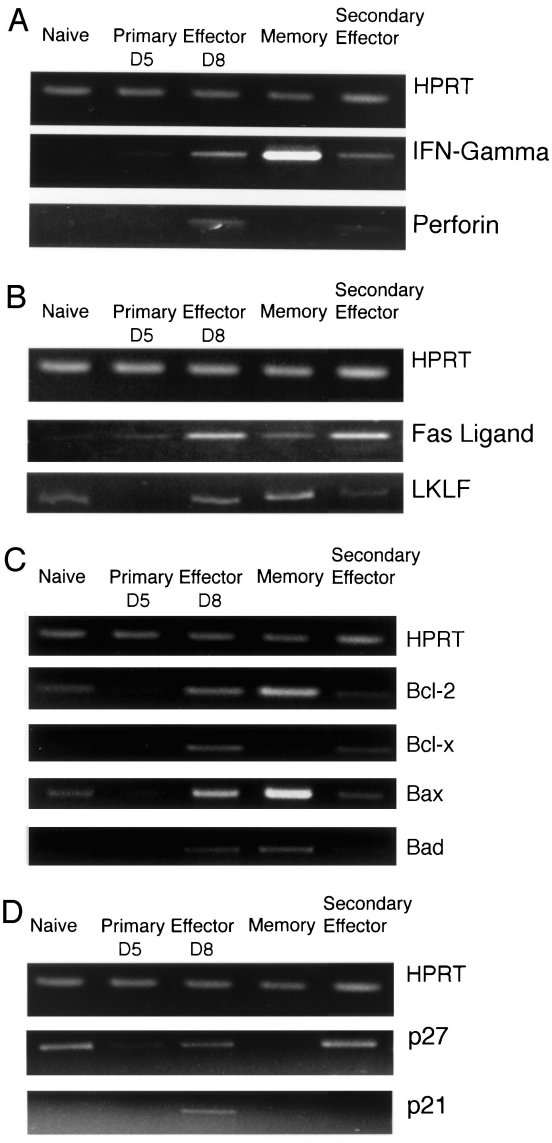

FIGURE 3. Analysis of gene expression in Ag-specific CD8$^+$ T cells during viral infection. Total RNA was isolated from naive and Ag-specific CD8$^+$ T cells 5, 8, and 105 days post primary infection and 4 days post secondary infection. First-strand cDNA synthesis was performed using a $(dT)_{15}$ primer, and products were amplified by 30 rounds of amplification using primers specific for murine IFN-γ and perforin (*A*), FasL and LKLF (*B*), *Bcl-2*, *Bcl-x$_L$*, *Bad*, and *Bax* (*C*), p21$^{Waf1/Cip1}$, p27^{Kip1} (*D*), and HPRT was included as a normalization control. Products were resolved on a 2% agarose gel, stained with ethidium bromide, and photographed. Data are representative of two experiments performed with pools of three mice in each group.

In addition to expressing effector molecules during an antiviral response, CD8$^+$ T cells express regulatory molecules that help to control the size of the response. We first addressed expression of indirect regulators in naive cells (Fig. 3*B*). As shown in Fig. 3*B*, these cells expressed little FasL mRNA but high levels of LKLF. After infection and differentiation of naive cells into effectors, an increase in FasL mRNA was observed in effectors on both days 5 and 8. LKLF levels appear to have decreased in the early effectors isolated on day 5, but have returned to normal levels in day 8 effector cells. After viral clearance and deletion of the effector cells, Ag-specific cells enter the memory phase. Memory cells are maintained at relatively constant numbers and undergo a slow proliferative renewal. Memory, unlike naive, cells do not require MHC class I TCR interactions for their division (17). These cells expressed little FasL but increased levels of LKLF. After reinfection, secondary effectors express similar levels of LKLF and FasL as primary effectors.

Because apoptosis plays a key role in downsizing the T cell response we also examined the expression of several members of the *Bcl-2* superfamily (see Fig. 3*C*). Naive cells, which are relatively resistant to apoptosis, contained moderate amounts of *Bcl-2* and *Bax* mRNA, whereas *Bad* and *Bcl-x$_L$* were expressed at extremely low levels. After activation, T cells become more susceptible to apoptosis. In effector cells isolated early in the response, *Bcl-2* mRNA decreased slightly, whereas *Bad*, *Bax*, and *Bcl-x$_L$* remained below the limit of detection. By day 8, at the peak of the response, before the onset of the death phase, expression of all four members of the *Bcl-2* superfamily increased. After completion of the death phase, Ag-specific cells enter the memory phase and reacquire resistance to apoptosis. This resistance to apoptosis is reflected by increased levels of *Bcl-2* mRNA, whereas *Bcl-x$_L$* remains below the limit of detection. Expression of *Bad* and *Bax*, two proapoptotic genes, was also increased in these cells. Upon rechallenge and generation of secondary effectors, these cells again become sensitive to apoptosis. This sensitivity is accompanied by decreased levels of *Bcl-2*, *Bad*, and *Bax* mRNAs, and increased levels of *Bcl-x$_L$*.

In addition to apoptosis, the magnitude of a CD8 response will also be controlled by proliferation. Naive cells, which are mostly resting, expressed high levels of the inhibitor CDKi p27. The expression of another CDKi, p21 was below the limit of detection. After infection and activation, Ag-specific CD8$^+$ T cells begin a period of rapid Ag-driven proliferation. When effector cells were examined at day 5 they expressed decreased levels of p27 and no detectable p21. By day 8, at the peak of the response and the end of the Ag-driven proliferation, these cells expressed high levels of both p27 and p21. Memory cells also undergo homeostatic proliferation that is far slower than the Ag-driven proliferation. Surprisingly, these cells did not express detectable levels of either molecule. Upon reinfection, another period of Ag-driven proliferation is induced that reached a maximum at 4 days postinfection. Cells isolated at this point expressed high levels of p27 but not p21 mRNA.

Table I. *Summary of gene expression in Ag-specific naive, effector, and memory CD8$^+$ T cells*[a]

	Naive	Effectors		Memory	Comments
		D5	D8		
Effector molecules					Increased expression of IFN-γ mRNA may account for the more rapid cytokine response of memory cells.
IFN-γ	−	+	++	+++	
Perforin	−	+/−	+	−	
Regulatory molecules					LKLF expression decreases upon TCR stimulation (D5 early effectors) but returns to normal levels by D8 (late effectors). This underscores the fact that LKLF expression correlates with recent TCR stimulation. LKLF expression remains high in memory cells.
LKLF	+	−	+	++	
Fas L	+/−	+	++	+/−	
Apoptosis control					Increased expression of *Bcl-2*, *Bad*, and *Bax* in memory cells suggests these cells control sensitivity to apoptosis differently than naive cells.
Bcl-2	+	−	+	++	
Bad	+/−	−	+	++	
Bax	+	+/−	++	+++	
Bcl-x$_L$	+	−	++	+/−	
Cell cycle control					Differential expression of p27 between naive and memory cells suggests that these cells control proliferation through different mechanisms.
p21	−	−	+	−	
p27	+	+/−	+	−	

[a] A minus (−) symbol indicates expression below the limit of detection. The ± symbol indicates expression at the limit of detection. Increases in expression are indicated by + symbols.

Discussion

In this paper we have analyzed gene expression in Ag-specific effector and memory CD8$^+$ T cells isolated during a viral infection. A summary of our observations is presented in Table I. On a molecular level naive, effector, and memory cells can be distinguished by their genetic programs. Of particular interest are the differences observed between naive and memory cells; memory cells contained increased *Bcl-2*, *Bad*, *Bax*, LKLF, IFN-γ, and decreased p27 mRNA relative to naive cells. What are the consequences of these differences, and how do they affect the ability of memory cells to mount a rapid secondary response?

Our first major observation is that memory cells contain increased levels of *Bcl-2*, *Bad*, and *Bax* mRNAs when compared with naive cells. In addition to quantitative changes in gene expression, memory cells express all three genes, whereas naive cells primarily express *Bcl-2* and *Bax*. Using an adoptive transfer system of transgenic CD4$^+$ T cells, Garcia and colleagues (18) observed similar differences between naive and memory CD4$^+$ T cells. They observed that memory CD4$^+$ T cells contained increased *Bcl-x$_L$* and *Bax* mRNAs. Differential expression of *Bcl-2* protein in memory CD8$^+$ and CD4$^+$ cells has been documented before (19). The function of the increased *Bcl-2* found in CD8$^+$ memory T cells is unclear; does it render these cells more resistant to apoptosis or serve as a counter to the increased levels of *Bad* and *Bax*? Although the *Bcl-2* superfamily is important in controlling susceptibility to apoptosis, other factors, such as loss of mitochondrial potential and production of reactive oxygen species, will influence cell survival. The influence of these factors on CD8$^+$ T cell survival is currently being addressed in our laboratory.

In addition to the *Bcl-2* superfamily, other regulatory molecules such as LKLF and FasL help to control the CD8 response. Originally identified as a transcription factor, null mutants of LKLF rapidly lose all peripheral CD8$^+$ T cells. The few remaining T cells express an activated phenotype (11). Additional evidence from in vitro and in vivo stimulation demonstrates that LKLF levels decrease upon T cell activation (11, 20). Our study and others by Schober and colleagues demonstrate that memory cells contain LKLF levels equivalent to or greater than those found in naive cells (20). It is important to note that during the effector response LKLF levels are below the limit of detection on day 5, but have returned to levels comparable to those found in naive cells by day 8 of infection. A critical difference between these two time points is that on day 5 Ag-specific cells may have recently encountered Ag, whereas at day 8 very few cells will have done so. This observation underscores the fact that LKLF expression correlates more with recent TCR stimulation than with effector status. In recent years, a large amount of data has accrued about the role of FasL in controlling T cell death (12). The role of this molecule in controlling Ag-specific responses remains less clear. Lpr mice, which contain a mutation in the *Fas* gene, contain more Ag-specific CD8$^+$ T cells than wild-type mice 15 days postinfection (M. Galvan, J. Whitmire, and J. G., unpublished observations). This defect in deletion is not permanent, as by the time memory is established lpr and wild-type mice contain similar numbers of memory cells (21). In our study we observed increased expression of FasL in effector cells that decreases in memory cells to a level slightly above that of naive. Whether this increased expression in memory cells has any functional consequences is under investigation.

CD8$^+$ T cells undergo two different types of proliferation, homeostatic and Ag driven. Homeostatic proliferation is a slow process that is MHC class I dependent for naive cells and independent for memory cells (17). In conditions of severe lymphopenia induced by irradiation, homeostatic proliferation will continue until the lymphoid compartment is full (22, 28). Ag-driven proliferation is extremely rapid as cells can complete one full cycle in 4–6 h. Our observation that memory cells do not express p27 or p21 mRNA, whereas naive cells express high levels of p27, suggests the mechanisms that control homeostatic proliferation in these two populations are different. Animals that contain a targeted mutation in the p27 gene display a gross hypertrophy with increased numbers of naive lymphocytes underscoring the role of this gene in maintaining naive T cell homeostasis (23). The decreased levels of p27 in memory cells suggest that different CDKi may affect homeostasis of different populations. Considering that there are multiple members of the CDKi family, other molecules such as p15, p16, and p57 could be controlling cell cycle decisions in these cells. In this study we confirm and extend previous observations

that p27 levels decrease in cells activated by TCR stimulation (14). It is important to note that when the Ag-driven proliferation slows down on day 8, both p21 and p27 are highly expressed consistent with a role for these molecules in blocking T cell cycle progression.

In addition to differences in the expression of molecules related to cell cycle and apoptosis control we observed differences between naive and memory cells in the expression of molecules related to effector function. Memory cells, unlike naive cells, will rapidly produce IFN-γ after Ag encounter. The IFN-γ mRNA levels in memory cells were higher than those in naive cells and comparable to those found in effector cells. Our results confirm one aspect of other studies of effector and memory cells (24, 25). In these studies the authors observed roughly equivalent levels of IFN-γ and perforin mRNA between effector and memory cells. One potential caveat to our study is that gene expression may be altered by incubation with MHC class I tetramers. Although studies have shown that prolonged incubation with tetramers can lead to activation of T cells (26), the conditions used in this study to isolate cells have been shown to be insufficient to induce cytolytic activity (27) or IFN-γ production in ELISPOT assays in Ag-specific memory cells isolated directly ex vivo. Our results also differ from previous studies of naive and memory cell gene expression in that we cannot detect perforin mRNA in memory cells. Although memory cells can produce cytokines within 3 h of stimulation these same cells require 12 h or more to lyse cells, suggesting differential gene expression may underlie the functional properties of these cells.

Using MHC class I tetramer-sorted cells we have documented key differences in gene expression between naive and Ag-specific effector and memory $CD8^+$ T cells. Our study shows that memory cells express a distinct profile of apoptosis-related genes and differing levels of effector and regulatory molecules than naive cells that may provide a molecular basis for the functional differences between these cells.

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