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Molecular and Functional Profiling of Memory CD8 T Cell Differentiation

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Summary

How and when memory T cells form during an immune response are long-standing questions. To better understand memory CD8 T cell development, a time course of gene expression and functional changes in antigen-specific T cells during viral infection was evaluated. The expression of many genes continued to change after viral clearance in accordance with changes in CD8 T cell functional properties. Even though memory cell precursors were present at the peak of the immune response, these cells did not display hallmark functional traits of memory T cells. However, these cells gradually acquired the memory cell qualities of self-renewal and rapid recall to antigen suggesting the model that antigen-specific CD8 T cells progressively differentiate into memory cells following viral infection.

Introduction

Development of long-term CD8 T cell memory is an important goal of vaccination because it can provide protection against reinfection and disease. This protection stems from both the increased number of antigen-specific CD8 T cells present in an immune host as well as from the distinct capability of memory CD8 T cells to proliferate, secrete antiviral cytokines, and kill infected cells more rapidly than naïve CD8 T cells upon exposure to antigen (reviewed in Dutton et al., 1998; Kaech et al., 2002). A typical CD8 T cell response to viral infection or vaccination consists of three characteristic phases—clonal expansion of virus-specific cells and acquisition of effector functions, contraction of the effector cell population through apoptosis, and generation of a long-lived population of memory cells (Dutton et al., 1998; Homann et al., 2001; Kaech et al., 2002; Murali-Krishna et al., 1998). The effector cell population is relatively short-lived as the majority (~90%–95%) of these cells die over the weeks following viral clearance. However, the remaining cells that survive generate a long-lived population of memory CD8 T cells that is stably maintained by steady, yet slow, cell turnover (Jameson, 2002). Although the in vivo dynamics of antigen-specific CD8 T cells have been well characterized in several systems, the mechanisms that determine how and when these memory T cells develop remain largely unknown.

The differentiation of naïve CD8 T cells into effector and memory cells is complex and hence, many fundamental questions remain unanswered. CD8 T cell activation spurs a vast chain of events that include activation of multiple signal transduction pathways, structural reorganization of the membrane and cytoskeleton, chromatin remodeling and expression of new genes, alterations in cell adhesion and migration, and induction of cell division. This transformation is critical for developing functional effector CD8 T cells that can eliminate infectious pathogens and for developing long-lived memory CD8 T cells that can persist in a responsive state; however, it is not known what the mechanisms are that drive memory cell differentiation. In addition, the developmental lineage that is followed during different types of immune responses is also not certain. Several studies suggest that the lineage of memory CD8 T cells development is linear and memory cells directly descend from effector cells (naïve → effector → memory), but recent studies have also suggested that activated CD8 T cells can bypass the effector cell stage and develop into memory cells (Jacob and Baltimore, 1999; Lauvau et al., 2001; Manjunath et al., 2001; Oehen and Brduscha-Riem, 1998; Opferman et al., 1999). Whether short-lived effector cells and long-lived memory cells are generated by different developmental programs or whether the same program is utilized but a fraction of the cells selectively survive and become memory cells is not clear. Lastly, it has not been carefully determined when memory cells arise following antigenic stimulation.

In this study, we have used two distinct but complimentary approaches to better understand memory CD8 T cell differentiation. First, we have tried to uncover molecular mechanisms involved in memory cell development by identifying genes that are differentially expressed in these cells and by analyzing how the pattern of gene expression changes as cells transit from effector and memory cell stages. Second, we have more precisely defined when memory CD8 T cells form during an immune response by examining when antigen-specific CD8 T cells begin to exhibit certain functional properties characteristic of memory T cells. This combination of studies strongly indicated that antigen-specific CD8 T cells acquire memory cell properties several weeks after virus clearance.

Results and Discussion

Characterization and Purification of P14 Transgenic Naïve, Effector, and Memory CD8 T Cells

The P14 transgenic mouse strain was used to analyze the changes in gene expression that occur as naïve antigen-specific CD8 T cells differentiate into effector and into memory CD8 T cells. P14 CD8 T cells express a T cell receptor (TCR) that recognizes the GP33-41 epitope in the LCMV glycoprotein. Naïve P14 CD8 T cells were obtained directly from the spleens of uninfected

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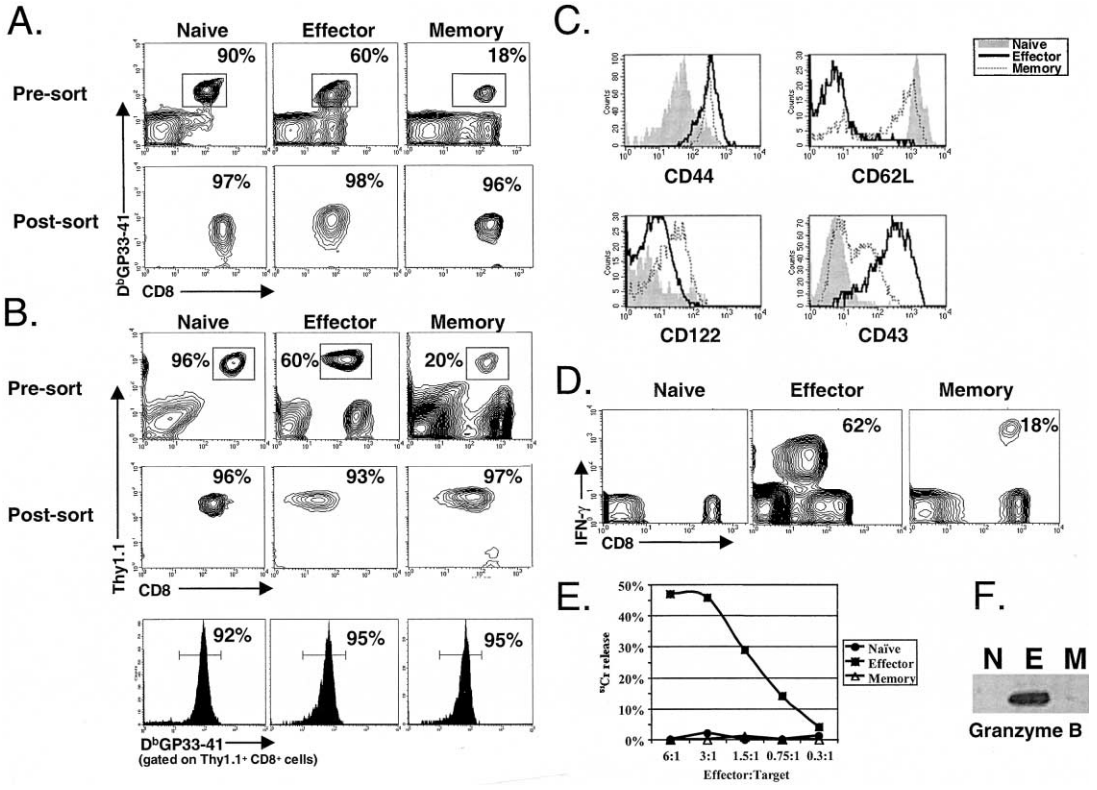

Figure 1. Isolation and Characterization of Naïve, Effector, and Memory P14 CD8 T Cells

(A and B) Naïve P14 splenocytes (Thy1.1$^+$) were adoptively transferred into C57BL/6 mice (Thy1.2$^+$) that were then infected with LCMV. Eight or >40 days later, P14 CD8 T cells were isolated by FACS using CD8α antibodies and D^{b}GP33-41 MHC class I tetramers (A) or CD8α and Thy1.1 antibodies (B). Naïve P14 cells were isolated from uninfected P14 mice. The percent of D^{b}GP33-41$^+$ cells of CD8$^+$ (A) or Thy1.1$^+$ CD8$^+$ (B) T cell populations prior to cell sorting are indicated in the top images and the percent purity post sorting is indicated in the bottom images (data are representative of at least three separate cell isolations). The bottom image in (B) shows that nearly all (~95%) of the sorted Thy1.1$^+$ CD8$^+$ T cells are antigen-specific (D^{b}GP33-41 tetramer^{+ve})

(C) The levels of surface CD44, CD62L (L-selectin), CD122 and CD43 for naïve (filled), d8 effector (thick line), and memory (thin line) D^{b}GP33-41$^+$ P14 CD8 T cells are shown.

(D) Naïve, d8 effector, and memory P14 splenocytes were stimulated for 5 hr with GP33-41 peptide followed by staining for CD8α and IFN-γ. The percent of CD8 T cells producing IFN-γ is indicated. Note that ~100% of the effector and memory P14 CD8 T cells produce IFN-γ.

(E) The direct ex vivo CTL activity of naïve (circle), d8 effector (square), and memory (>day 40 p.i., open triangle) P14 CD8 T cells after 5 hr chromium-release assay. Cultures were normalized to contain equal numbers of P14 CD8 T cells.

(F) Granzyme B levels in purified naïve, d8 effector, or memory P14 CD8 T cells were detected using Western blotting.

P14 mice, whereas effector and memory P14 CD8 T cells were generated by adoptive transfer of either Thy1.2$^+$ or Thy1.1$^+$ P14 splenocytes into C57BL/6 (Thy1.2$^+$) mice that were subsequently infected with LCMV-Armstrong. Pure populations of "resting" naïve, effector, and memory P14 CD8 T cells were isolated using FACS based on either (1) CD8$^+$ and D^{b}GP33-41 MHC class I tetramer or (2) CD8$^+$ and Thy1.1$^+$ staining (Figure 1).

Effector CD8 T cells were isolated eight days postinfection (d8 p.i.), at the peak of the effector CD8 T cell response, and memory CD8 T cells were isolated at least 40 days p.i. Naïve P14 CD8 T cells were CD44^{lo}, CD62L^{hi}, CD122^{lo}, and CD43^{lo} and did not immediately produce IFN-γ or cytotoxic molecules, whereas d8 effector cells were CD44^{hi}, CD62L^{lo}, CD122^{hi}, and CD43^{hi}, rapidly secreted IFN-γ, were highly cytotoxic in direct ex vivo assays, and contained high levels of Granzyme B (Figures 1C–1F). Memory P14 CD8 T cells were CD44^{hi}, mostly CD62L^{hi} (60%–95% are CD62L^{hi}), CD122^{hi}, CD43^{int}, and rapidly produced IFN-γ upon restimulation, but were not immediately cytotoxic ex vivo and did not contain high levels of Granzyme B (Figures 1C–1F).

Gene Expression Profile of Effector CD8 T Cells

To analyze the first phase of CD8 T cell differentiation (naïve → effector CD8 T cell), gene expression patterns were compared between naïve and d8 effector P14 CD8 T cells on DNA microarrays from Incyte Genomics and Affymetrix that contain ~8,700 and ~12,400 murine genes, respectively. The relative abundance of specific transcripts from each cell type was quantitated and a differential expression ratio was calculated. Genes that had been previously identified or were highly similar to known genes were putatively assigned to functional categories (Table 1). An expanded version of Table 1 and the remaining ESTs and unassigned genes can be found in the Supplemental Data, Tables S1–S3, available at http://www.cell.com/cgi/content/full/111/6/837/DC1. The genes identified in these experiments revealed several traits of effector CD8 T cells; some are novel and

some are similar to those previously described by separate gene expression studies on polyclonal populations of activated T cells (Liu et al., 2001; Teague et al., 1999).

As naïve CD8 T cells differentiate into effector cells, they acquire the ability to migrate from lymphoid to nonlymphoid tissues and this is largely attributed to the increased cell surface levels of chemokine receptors and cell adhesion molecules (for review, see Moser and Loetscher, 2001). We observed that genes encoding chemotactic proteins CCR2, CCR5, neuropilin, and semaphorin 4D, and cell adhesion proteins MAC-2, CD11c, CD18, CD44, and P-selectin ligand were increased in effector CD8 T cells (Table 1, Cell adhesion and migration). The concerted action of these molecules can permit effector cells to migrate toward sites of inflammation, adhere to the endothelial cell linings, and extravasate into tissues. A corollary increase in surface protein levels was observed in effector CD8 T cells for several of these genes (see Supplemental Data, Figure S2 available at http://www.cell.com/cgi/content/full/111/6/837/DC1).

The expression of lymph node homing proteins, such as L-selectin (CD62L) and CCR7 also regulate effector cell migration because their expression is reduced on effector CD8 T cells and thus, their ability to migrate to the lymph nodes is compromised. L-selectin expression can be regulated by both transcriptional and posttranscriptional mechanisms such as proteolytic cleavage after TCR activation (Chao et al., 1997). L-selectin mRNA levels were depressed $\sim$10-fold in d8 effector CD8 T cells compared to naïve cells, indicating that transcriptional repression of L-selectin occurs in vivo. Consequently, nearly all (95%–99%) of the d8 effector cells display low surface levels of L-selectin as observed by flow cytometry (Figure 1C). It remains to be determined when this locus becomes silenced following infection and why two mechanisms exist to decrease L-selectin levels, but together these data highlight the importance of maintaining low L-selectin levels on effector cells.

The mRNAs of several other membrane-spanning or GPI-linked proteins, whose functions have not been fully defined, were increased in effector cells; these included CD97, Ly116 (chandra), Glvr-1 and Ly-6A/E, Ly-6C, and Thy-1 (Table 1, Membrane proteins). The role of Ly-6C and Ly-6A/E in T cell function is not clear and interestingly, the responses of T cells deficient of GPI-linked proteins including Thy-1, Ly-6A, and Ly-6C did not appear to be affected in vitro or in vivo (Takahama et al., 1998).

Killing of infected cells is a critical effector CD8 T cell function that is mediated by the release of perforin and granzymes. Expression of perforin and granzymes A, B, D, and K mRNA was highly elevated in effector CD8 T cells compared to naïve cells (Table 1, T cell effector functions). For granzyme B, this correlated with increased protein levels as observed by Western blotting (Figure 1F). The expression levels of other CD8 T cell effector molecules, such as IFN-γ, RANTES, and Fas ligand were also increased (Table 1).

We also observed that the mRNA levels of many genes encoding signaling molecules were elevated in effector CD8 T cells. Most of these genes could be placed into several well-characterized signal transduction pathways—(1) TCR signaling, such as CD45 phosphatase, grb-2, lck, fyn, lck-interacting adaptor protein (LIME), and PEP phosphatase; (2) intracellular Ca^{2+} signaling, such as calcyclin, calcineurin catalytic subunit, annexin A2 and A6, and Ca^{2+} transporting ATPases; and (3) cytokine signaling, such as JAK1, STAT4, and SOCS-5. A corollary increase in lck and fyn protein in effector cells was observed by intracellular staining (see Supplemental Data, Figure S1 available at above website). Increased expression of the genes that act positively in these signaling pathways may enhance signal transduction, alleviate the dependence on costimulation, or lead to faster responses to antigen than that observed in naïve cells (Dutton et al., 1998; Iezzi et al., 1998). However, negative regulation of TCR and cytokine signaling was also evident because expression of PEP phosphatase and SOCS-5 was increased and expression of the IL-4, IL-7, and IFN-γ receptor α chains was decreased.

Several genes that regulate actin polymerization were increased in effector CD8 T cells, including members of the ARP2/3 complex, talin, filamin, and cdc42 rho GTPase (see Table 1, Cytoskeleton regulation). These gene products may be important for the increased formation of actin-based structures found in effector CD8 T cells, such as filopodia and lipid raft microdomains that could lead to increased cell motility and signaling, respectively. This is similar to previous reports showing that activated CD8 T cells have higher actin and lipid raft content than naïve cells (Liu et al., 2001; Tuosto et al., 2001).

We observed that several genes involved in protein translation, such as ribosomal proteins S5, P2, L23, S15, and L35 were reduced in effector CD8 T cells (Table 1, Protein degradation, modification and translation). Perhaps, this represents a general reduction in translation that may contribute to the effector cell apoptosis that will occur over the proceeding days. Additionally, a decrease in cytokines coincides with viral clearance and this deprivation may induce an energetic crisis that leads to decreased protein synthesis (Rathmell et al., 2001).

Finally, a few genes predicted to impact mitochondrial function were differentially expressed between effector and naïve CD8 T cells, possibly illuminating differences in their respiratory capacity. Genes encoding several glycolytic enzymes, the mitochondrial uncoupling protein 2, and glutathione reductase were upregulated in effector cells as well as genes encoding three subunits of mitochondrial complex I NADH dehydrogenase (Table 1, Energy metabolism).

In summary, the above data confirm that multiple cellular processes are involved in the differentiation of naïve CD8 T cells into effector cells. Most notably, genes involved in signal transduction, actin regulation, cell adhesion/migration, and translation were altered during effector cell development. Finally, given the recent attention of L-selectin expression on subsets of antigen-specific CD8 T cells, this study found that reduced surface expression of L-selectin correlated with decreased mRNA levels in effector CD8 T cells.

Memory CD8 T Cell Gene Expression Profile

During the second phase of a CD8 T cell immune response, the majority of the effector cells die, but those that survive constitute the pool of memory cells. Memory

Table 1. Differential Gene Expression in Effector and Memory CD8 T Cells as Compared to Naïve Cells

Gene Name[c]	Accession Number	fold change[b] E/N	M/N	Description
T cell signal transduction				
JAK1	W29699.1	2.5		binds cytokine receptors
CD45	AA201097.1	2.4		phosphatase
cdc42	AA266975.1	2.1		rho GTPase
FAN	AA013700.1	2.0		TNFR1 adapotor protein
Dok2	AA008417.1	1.9		downstream of tyrosine kinase 2
Calcineurin catalytic chain 3	AA178283.1	1.8		ser/thr phosphatase
IL-4 receptor α chain	AA286506.1	−1.7		cytokine signaling
IFN-γ receptor α chain	AA541842.1	−1.7		binds IFN-γ
Smoothened homolog	AA008222.1	−1.7		hedgehog signaling
SOCS-5	AA270263.1	−1.8		cytokine signaling inhibitor
Toll-like receptor 1	AA177549.1	−2.0		innate immunity
IL-7 Rα[a]	M29697	−2.4		cytokine signaling
similar to AHNAK	AA518354.1		2.4	activates PLC-γ
MKK4/SEK1	AA413508.1		2.0	JNK signaling
Calmodulin binding protein SHA1	AA416426.1		2.0	Ca^{++} signaling
MEL91	W18484.1		1.8	binds PKC
Ier-2/pip92	AA038052.1		1.9	JNK/p38 signaling
Eph receptor A2	AI325344.1		1.8	Receptor tyrosine kinase
CIS1/SOCs	AI893893.1		1.7	inhibits cytokine signaling
TOPK	AA415579.1		1.7	ser/thr kinase
Calcyclin	AA267952.1	7.7	6.1	binds annexin II
Annexin A2	W89518.1	4.8	1.9	Ca^{++} signaling
Annexin VI (A6)	AA499296.1	3.0	1.7	Ca^{++} signaling
Fyn	W62969.1	2.8	2.1	TCR signaling
racGAP1	AA140523.1	2.5	1.7	ras signaling
STAT4	AA175477.1	2.0	1.7	cytokine signaling
Lck	AA008016.1	1.9	1.7	TCR signaling
T cell specific GTPase	AA509565.1	−2.2	−1.7	induced by interferons
Gene expression and chromatin regulation				
Tbx-2	AA087036.1	2.0		transcription factor
HMG-2	AA275111.1	1.7		transcription factor
c-Jun[a]	X12761	−2		transcription factor
fos[a]	X14897		2.6	transcription factor
ATF-2	AA414544.1		1.9	transcription factor
B-ATF[a]	AF017021	4.2	2.4	transcriptional repressor
ROR-α	W34685.1	3.1	2.4	nuclear hormone receptor
Jun B[a]	U20735	−1.9	1.9	transcription factor
LEF-1	AA119479.1	−2.3	−1.9	binds b-catenin

Gene Name	Accession Number	fold change E/N	M/N	Description
T cell effector functions				
Granzyme A[a]	M13226	5.1		cytotoxic killing
Uteroglobin	AA432689.1		2.1	cytokine
Granzyme B	AA183327.1	15.4	4.9	cytotoxic killing
Lipocortin 1[a]	M69260	6.3	4.2	secreted molecule
Granzyme K[a]	AF011446	6	4.4	cytotoxic killing
IFN-γ[a]	K00083	5.7	7.5	cytokine
Fas ligand[a]	U06948	5.4	4.7	cytotoxic killing
Rantes[a]	AF065947	5.2	5.2	cytokine
MIP-1β	AA178155.1	3.5	6.3	chemotaxis
perforin[a]	X12760	2.3	2.1	cytotoxic killing
Cell cycle				
MCM6	AA016759.1	2.1		DNA synthesis licensing factor
Telomerase binding prot. p23	AA174980.1		1.9	telomerase binding protein p23
Btg-2	AA154848.1		1.9	anti-proliferative
Cullin 3	AA162800.1		1.9	regulates cyclin E levels
Cyclin B1	AA426917.1		1.9	G2/M
Cyclin E1	AA465987.1		1.7	G1/S
Cyclin E2	AA414293.1		1.7	G1/S
Membrane proteins				
CD94	AA161895.1	2.1		NK inhibitory receptor
Ly6C	AA000712.1	15.8	12.4	GPI-linked protein
Ly6A/E	AA145865.1	8.5	7.2	GPI-linked protein
KLRG1[a]	AF097357	6.5	4.2	NK inhibitory receptor
NKG2-D[a]	AF030313	4.9	5.4	NK activating receptor
Ly116	AA161823.1	2.0	3.1	CHANDRA, Th1 cell-specific
Cytoskeleton regulation				
Filamin	AA466198.1	2.1		crosslinks actin filaments
Talin	AA208883.1	2.0		links integrins to cytoskeleton
ARP2/3 complex subunit 4	AA518369.1	1.9		regulates actin polymerization
ARP2/3 complex subunit 5	W47753.1	1.7		regulates actin polymerization
Apoptosis				
Akt2	W82557.1	1.9		anti-apoptotic
Akt	AA237366.1	1.8		anti-apoptotic
Bcl2-interacting killer-like	AA064058.1	1.7		pro-apoptotic-binds Bcl-2 and Bcl-xL
Nur77 (aka N10)	AA209882.1		3.9	pro-apoptotic
Caspase-11	AA175651.1		1.7	pro-apoptotic/ IL-1 and IL-18 synthesis

(*continued*)

Table 1. Continued

Gene Name[c]	Accession Number	fold change[b] E N	M N	Description
Cell adhesion and migration				
Galectin-3/ MAC-2	AA403841.1	2.6		cell adhesion
CD62P ligand, P-selectin ligand	AA475311.1	2.4		cell adhesion
Semaphorin 4D	AA278090.1	1.8		binds CD72
CXCR4	W88094.1	1.7		cell migration
CCR2	AA289476.1	11.0	6.5	cell migration
CD11c	AA178276.1	8.7	3.0	cell adhesion
CCR5	AA144482.1	3.2	2.5	cell migration
CXCR3[a]	AF045146	3.1	4	cell migration
CD18	AA467489.1	2.8	1.8	cell adhesion
CD44	AA396152.1	2.4	3.3	cell adhesion
Glycam-1	AA467267.1	2.3	1.7	cell adhesion
Neuropilin	AA432934.1	1.9	2.0	cell migration
CD62L, L-selectin	AA183698.1	−9.6	−2	cell adhesion
T cell recognition				
MHC Class I H2-Kb	W14540.1	2.4	1.7	MHC class I (H-2Kb)
β-2 microglobulin	AA109951.1	1.7	1.7	MHC class I heavy chain
MHC Class II I-A alpha chain	AA272807.1	−1.9	−2.6	MHC class II (I-Aa)

Gene Name	Accession Number	fold change E N	M N	Description
Energy metabolism				
GAPDH	AA122891.1	2.1		glycolysis
NADH-ubiquinone B22 Subunit	AA415725.1	1.9		e-transport
α-enolase	AA204262.1	1.9	1.7	glycolysis
γ-glutamyl hydorlase	AA259661.1	1.9	1.9	glycolysis
NADH-ubiquinone ASHI subunit	W83085.1	1.9	1.6	e-transport
Glutathione reductase	AA177872.1	1.9	1.6	maintains glutathione levels
Protein degradation, modification, and translation				
spi12	AA173013.1	3.4		serine proteinase inhibitor
Ribosomal protein L23	AA220584.1	−1.7		translation
60S ribosomal protein L39	AI604200.1	−1.8		translation
40S ribosomal protein S15	AA033398.1	−1.9		translation
60S ribosomal protein L35	AA221110.1	−2.0		translation
eEF1-beta chain	AA268148.1	−2.2		translation
Sialyltransferase 1	AI323095.1	−2	−1.7	protein glycosylation

[a] Data from Affymetrix array U74A (values in $\log_2$).

[b] Gene expression ratios represent the average value of three independent hybridizations to Incyte microarrays (mouse GEM 1) or a single hybridization to Affymetrix array U74A. For Incyte arrays, 240 and 187 genes were found differentially expressed at least 1.7-fold in effector and memory CD8 T cells, respectively, as compared to naïve cells. This selection criterion was based on the report that a 1.7-fold difference can be detected with 95% confidence on these cDNA microarrays (Yue et al., 2001). The majority of these genes were upregulated in effector (191/240) and memory (174/187) CD8 T cells as compared to naïve cells.

[c] Genes in bold indicate that they are preferentially expressed in memory cells as compared to effector cells.

An expanded Table can be found in Supplemental Data, Supplemental Table S1 available at http://www.cell.com/cgi/content/full/111/6/837/DC1.

cells are qualitatively distinct from naïve cells and can proliferate and acquire effector functions much more rapidly upon exposure to antigen. Also, memory cells, in contrast to naïve cells, undergo a slow antigen-independent homeostatic proliferation to maintain their numbers in the periphery. To better understand these functional differences, the gene expression profile of memory CD8 T cells was compared to naïve cells and the results of this comparison are shown in Table 1 and Supplemental Data (available at http://www.cell.com/cgi/content/full/111/6/837/DC1). This study revealed sets of genes and potential pathways that may be important for generating memory cell phenotypes as well as confirmed features of memory CD8 T cells that have been previously recognized.

Recently, it has been observed that memory CD8 T cells reside in both lymphoid and non-lymphoid tissues, and hence, several of the genes involved in cell migration and adhesion that were upregulated in effector cells were also increased in memory cells (Table 1); this included CCR2, CCR5, the semaphorin ligand neuropilin, CD44, CD18, CD11c, and glycam-1. However, CXCR4 was selectively upregulated in memory CD8 T cells as compared to effector cells and may indicate that memory CD8 T cells are inclined to migrate toward stromal-cell derived factor-1 (SDF-1) expressing cells. In addition, L-selectin (CD62L) mRNA was reduced ~2-fold in memory CD8 T cells compared to naïve cells, but this was significantly less than the ~10-fold decrease observed in effector cells. This observation suggested that transcription was reinitiated at the L-selectin locus as effector cells differentiated into memory CD8 T cells. Recently, functional differences between the $CD62L^{lo}$ and $CD62L^{hi}$ subsets of memory CD8 T cells have received much attention, but it is not clear how these different subsets are generated (Masopust et al., 2001; Reinhardt et al., 2001; Sallusto et al., 1999). This study suggests, however, that during LCMV infection the precursors of the $CD62L^{hi}$ subset of memory CD8 T cells transit through a $CD62L^{lo}$ state because nearly all the LCMV-specific CD8 T cells exhibit low levels of both L-selectin mRNA and protein d8 p.i. (Figure 1 and Table 1).

The two most highly differentially expressed genes in memory CD8 T cells compared to naïve cells encode family members of the GPI-linked proteins Ly-6C and Ly-6A/E. Likewise, these proteins are expressed at very high levels on memory CD8 T cells (see Supplemental Data, Figure S1 available at http://www.cell.com/cgi/content/full/111/6/837/DC1). As previously mentioned, the role of these proteins in memory T cell function is unclear and requires further study.

Several genes involved in signal transduction were elevated in memory CD8 T cells. Indeed, the expression of some of these genes were similarly increased in effector cells, such as calcyclin, fyn, lck, racGAP1, LIME, PEP phosphatase, annexin A2 and A6, and STAT4 (see Table 1); however, other genes were preferentially found in memory CD8 T cells, such as members of the p38 and jun kinase (JNK) signaling pathways MKK4, Ier-2, junB, fos, and ATF-2. Recent antigen contact is an unlikely cause of the increased expression of these genes, but growth factors, cytokines, or other stimuli may play a role. Elevation of signal transduction proteins may increase sensitivity to TCR signals and expedite memory cell recall responses. This may also contribute to why memory CD8 T cells can persist in the absence of MHC class I interactions and rely less on costimulation for activation than naïve cells (Dutton et al., 1998; Jameson, 2002).

A unique property of memory CD8 T cells is homeostatic proliferation that can replenish the pool of memory cells. We found several cell cycle genes upregulated in memory CD8 T cells such as cyclin E1, E2, and B1 (Table 1, Cell cycle). The gene encoding telomerase binding protein p23 was also upregulated which supports our findings that resting LCMV-specific memory CD8 T cells contain telomerase activity in vivo (Hathcock et al., 2003). Lastly, IL-15 is required for memory cell homeostatic proliferation, and in accordance, memory cells expressed the IL-15Rα chain ~1.5-fold higher than naïve cells. This is similar to that reported by Goldrath et al. (2002) and correlates with increased protein levels on memory cells (Schluns et al., 2002).

The expression of several genes encoding effector molecules such as IFN-γ, fas ligand, RANTES, MIP-1β, perforin, and granzymes B, K, and M were substantially higher in memory cells than in naïve cells (Table 1). Interestingly, for granzyme B, this expression profile did not correlate with significant increases in protein as compared to d8 effector cells (Figure 1F). The uncoupling of transcription from translation has also been observed for IFN-γ and IL-2 in antigen-specific CD8 T cells and may signify a general mechanism to simultaneously maintain memory CD8 T cell preparedness while preventing the improper release of cytotoxic and inflammatory molecules (Bachmann et al., 1999; Grayson et al., 2001; Slifka et al., 1999; Veiga-Fernandes et al., 2000).

In summary, several genes involved in signal transduction, cell migration, and cell division are differentially regulated in memory CD8 T cells as compared to naïve CD8 T cells. Furthermore, in combination with recent studies, our results confirm that the mRNA expression of key effector molecules (i.e., granzyme B and IFN-γ) is constitutive in memory CD8 T cells but that production of the protein is regulated by antigen contact and this may facilitate rapid memory cell recall responses. Finally, these studies show that several genes continue to be differentially expressed in memory CD8 T cells, as compared to naïve cells, and illuminate that following the effector cell stage, gene expression patterns are permanently altered in antigen-specific memory CD8 T cells.

Comparison of Effector and Memory CD8 T Cell Gene Expression Profiles

Since effector and memory P14 CD8 T cells were compared to the same reference naïve P14 CD8 T cell population, the similarities and differences between effector and memory cell gene profiles could be assessed by aligning the differentially expressed genes. This created a set of ~350 genes that were differentially expressed at least 1.7-fold in either effector or memory CD8 T cells as compared to naïve cells. Of this set of genes, ~30% were commonly upregulated in both effector and memory CD8 T cells (see Table 1 and Supplemental Data, Figure S2 and Table S4, available at http://www.cell.com/cgi/content/full/111/6/837/DC1). This implied that

many of the gene regulatory changes that occurred during effector cell development were maintained in memory cells and supported the model that a direct lineage exists between effector and memory CD8 T cells. Second, closer examination of the differential expression ratios revealed that the magnitude of expression was commonly greater in effector cells than in memory cells which implied that effector cells may be more transcriptionally active (compare in Table 1). Third, and perhaps most important, subsets of genes were found to be preferentially expressed in either effector or memory cells (e.g., actin regulators and cell cycle genes, respectively). The appearance of effector or memory-specific genes suggests that memory CD8 T cells are not simply "resting effectors" but are a distinct cell population. This interpretation invoked the model that memory cell development occurs in two stages; a first phase where naïve cells differentiate into effector cells, followed by a second phase where effector cells differentiate into memory CD8 T cells.

Changes in Gene Expression between Effector and Memory Cell Stages

The above proposal led us to examine how gene expression patterns change over the weeks following infection when memory cells form. Thus, P14 CD8 T cells were isolated at the peak of the immune response (d8 p.i.), during the death phase (d15 and d22 p.i.), and after formation of a memory CD8 T cell population (d40+ p.i.). mRNA from these populations was compared to that of naïve P14 cells to identify genes that were differentially expressed during this time period. A K-means clustering algorithm was used to identify coordinately regulated genes and six major patterns were observed (Figure 2). Several of the genes placed into each group are listed in Table 2, but a complete list can be found in the Supplemental Data (Table S6 available at http://www.cell.com/cgi/content/full/111/6/837/DC1). In the first group, the expression levels of many genes (~60%) were set at d8 p.i. and did not significantly change over the following weeks; this suggested that most gene regulatory changes imprinted during effector cell differentiation are maintained in memory cells. The second group (Groups 2a and 2b, Table 2) contained genes that tended to be most highly expressed at d8 p.i., but their expression gradually decreased over the next several weeks to the level in memory cells. Perhaps, this reflects a global reduction in transcriptional activity associated with memory CD8 T cell development. This group included granzyme B, CCR2, CD11c, Ly-6C, and Ly-6A/E. Group 3 displayed genes that remained downregulated in antigen-specific CD8 T cells following infection and included the transcription factor LEF-1, T cell-specific GTPase, and sialytransferase-1. Groups 4, 5, and 6 included genes that displayed more dynamic patterns during the effector to memory transition period. In group 4, the gene expression levels showed minimal change at d8 but then increased at days 15–22 p.i., whereas in groups 5 and 6 the expression levels initially decreased during this interval and then increased as the memory CD8 T cell population was stabilized. A key example was the change in L-selectin (CD62L) expression that steadily increased from d8 to day 40 p.i. (see Group 6b, Figure 2). Other examples include CXCR4, several ribosomal protein genes (P2, S5, S15, and L39), and the forkhead transcription factor FKHR.

Progressive Development of Memory CD8 T Cell Properties

Identification of Memory Cell Precursors at d8 Postinfection

Because the expression pattern of many genes continued to change following the peak of the CD8 T cell response (d8 p.i.), this led us to consider when are memory CD8 T cells being generated? This gene expression data may suggest that the virus-specific CD8 T cells continue to differentiate and coordinately acquire memory cell attributes over the weeks following viral clearance and proposes the model that functional, long-lived memory cells develop several weeks postinfection. This is in contrast with the model that memory CD8 T cells are generated simultaneously with effector cells and exist by the peak of the CD8 T cell response. To examine these models more closely, we first inspected whether virus-specific CD8 T cells that can give rise to memory CD8 T cells exist by d8 p.i. As shown in Figure 3, LCMV-specific CD8 T cells were purified at d8 p.i., labeled with CFSE, and transferred into naïve mice. The kinetics of effector cell contraction in the recipients mirrored that seen normally; that is, ~50%–70% of the donor cells died within the first week and ~10%–20% became long-lived memory cells by one month posttransfer. The memory cells that arose from the transferred d8 effector CD8 T cells were indistinguishable from those generated normally based on expression of surface markers and cytokines and could protect against viral rechallenge with the virulent strain of LCMV-clone 13 (Figure 3B and data not shown; Kaech and Ahmed, 2001). In combination with similar results from other antigenic systems, these data strongly indicate that precursors to memory CD8 T cells exist early during an infection (Hu et al., 2001; Jacob and Baltimore, 1999; Opferman et al., 1999; Voehringer et al., 2001).

It is possible that the memory CD8 T cell population is generated by a few precursor cells that expand while the effector cells disappear following viral clearance. This idea was investigated by examining the level of CFSE fluorescence in the transferred d8 effector population 1, 11, and 30 days after transfer (Figure 3C). Interestingly, we observed minimal to no division of this cell population within one months time; more than 90% of the memory cells generated from the transferred d8 population had not divided (Figure 3C). This showed that the memory CD8 T cell population does not arise from a small subset of effector cells that expand during the death phase. Rather, the memory CD8 T cells descend directly from effector cells. This is similar to that recently reported for development of the memory CD4 T cells (Hu et al., 2001).

It has not been carefully determined when fully functional memory CD8 T cells develop during an immune response. The above results indicated that cells present 8 days p.i. have the potential to generate a stable and protective memory cell population, but do these cells display functional properties ascribed to memory CD8 T cells? To better delineate when memory cells are

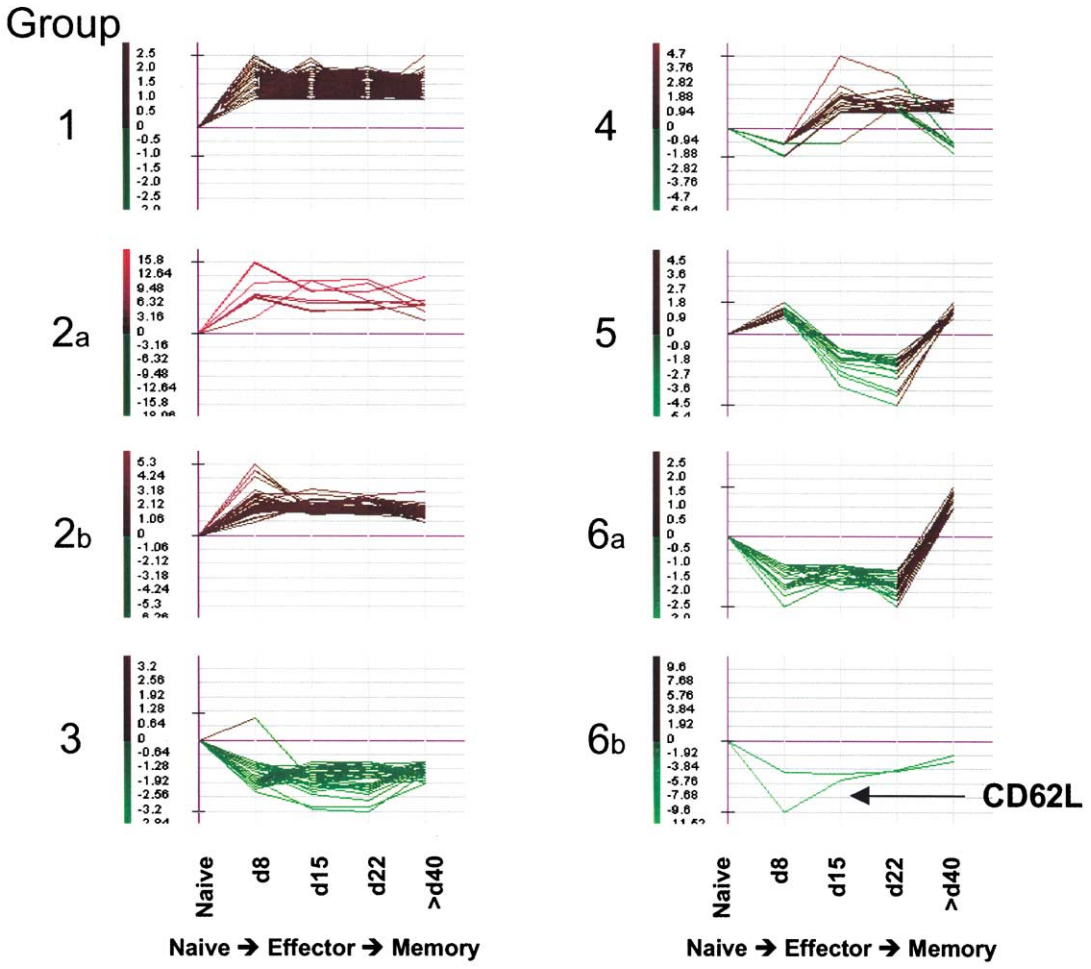

Figure 2. Repatterning Gene Expression between Effector and Memory Cell Stages

The gene expression profiles of P14 CD8 T cells isolated 8, 15, 22, and >40 days p.i. were compared to naïve cells. Genes with coordinated expression patterns were grouped as determined by K-means algorithm using 10 estimated sets and six representative patterns are presented. Scales to left of graphs indicate expression levels. Genes were selected for analysis if their average expression level deviated from that of naïve cells by at least a factor of 1.7 in at least one of the time points and gave reproducible measurements across multiple hybridizations (431 genes met this criteria).

formed, we examined when virus-specific CD8 T cells began to exhibit two quintessential memory cell qualities—the ability to proliferate in response to homeostatic signals and the ability to rapidly proliferate and survive in response to antigenic signals.

Impaired Homeostatic Proliferation in Effector CD8 T Cells

Memory CD8 T cells can undergo homeostatic proliferation that is regulated by cytokines such as IL-15 and IL-7 (Jameson, 2002). As shown in Figure 3C, the d8 effector cell population showed minimal to no proliferation after transfer into naïve mice suggesting that memory cell precursors present at d8 p.i. cannot respond to homeostatic proliferative signals as effectively as memory CD8 T cells present 40 days p.i. To examine when following infection the antigen-specific CD8 T cells begin to proliferate in response to homeostatic signals, P14 CD8 T cells from d8, d15, d22, and >d40 p.i. were CFSE-labeled and transferred into non-irradiated naïve mice and the extent of cell division was examined ~30 days later (Figure 3D). As expected, memory CD8 T cells from d40 p.i. divided substantially within the month whereas cells from d8 p.i. divided minimally. Interestingly, cell division was seen in the population of cells transferred from d15 and d22 p.i., demonstrating that between days 15–22 p.i. antigen-specific CD8 T cells acquire the property to undergo homeostatic proliferation. This indicated that at d8 p.i., the memory cell precursors are initially unresponsive to homeostatic proliferative signals, but over the next 2–3 weeks they become responsive. Effector and memory cells display similar levels of IL-15Rα chain; therefore, this may not account for their proliferative differences (Schluns et al., 2002). Thus, in accordance with changes in gene expression, the ability to proliferate to homeostatic signals is gradually acquired over the weeks following viral clearance.

Impaired Effector CD8 T Cell Proliferation and Survival in Response to Antigen

Another salient memory T cell trait is their high proliferative potential in response to antigen. Upon secondary

Table 2. Coordinate Regulation of Gene Expression during Memory CD8 T Cell Development

Group[a]	Accession Number	Gene Name	Description	d8 p.i.	d15 p.i.	d22 p.i.	>d40 p.i.
2a	AA289476.1	CCR2	cell migration	11	11.8	12	6.5
	AA000712.1	Ly-6C	membrane protein	15.8	9.4	9.2	12.4
	AA183327.1	Granzyme B	cytolysis	15.4	9.3	11	4.9
	AA178276.1	CD11c	cell adhesion	8.7	7.2	6.9	3
	AA145865.1	Ly-6A/E	membrane protein	8.5	6.6	6.6	7.2
	AA472994.1	sim to Ly6A/E	membrane protein	8.2	5.1	5.1	6.4
	AA267952.1	Calcyclin	Ca^{++} signaling	7.7	4.9	5.3	6.1
2b	AA265396.1	Stathmin	cytoskeleton regulation	4.8	1.9	2.6	2.1
	W62969.1	Fyn	signal transduction	2.8	1.8	1.9	2.1
	AA161823.1	Ly116/CHANDRA	membrane protein	3.1	1.9	1.9	2
	W89518.1	Annexin A2	Ca^{++} signaling	4.8	2.3	3	1.9
	AA467489.1	CD18 (beta-integrin)	cell adhesion	2.8	2.1	2.3	1.8
	AA146265.1	EST		2.9	1.9	1.9	1.8
	W82294.1	EST		2.9	1.6	1.7	1.8
	AA140523.1	rac-GAP	signal transduction	2.5	1.9	2.2	1.7
	AA245492.1	EST		5.3	1.7	2.2	1.7
	AA173013.1	Spi12 proteinase inhibitor	ser. protease inhibitor	3.4	2.1	1.9	1.6
	AA475311.1	P-selectin ligand	cell migration	2.4	1.7	1.6	1.6
	AA008417.1	Dok2	signal transduction	1.9	1.8	2	1.5
	AA276837.1	EST		2.8	2.2	2.1	1.4
	AA403841.1	Galectin-3	cell adhesion	2.6	1.9	2.2	1
3	W87149.1	p53		−1.2	−1.6	−1.7	−1
	AA268148.1	eEF-1b2	translation	−2.2	−1.4	−1.5	−1
	AA444490.1	TIMP2	metalloproteinase inhibitor	−1.4	−1.9	−2.1	−1.2
	AA221110.1	60S ribosomal protein L35A	translation	−2	−1.4	−1.6	−1.3
	AA276764.1	Integrin alpha E	cell adhesion	−1.2	−2.1	−2.4	−1.3
	AA261454.1	EST		−1.4	−2.4	−2.7	−1.3
	AA118626.1	EST		−1.8	−3.1	−3.2	−1.5
	AA008222.1	Smoothened homolog	signal transduction	−1.7	−1.3	−1.1	−1.6
	AA509565.1	T cell specific GTPase	signal transduction	−2.2	−1.6	−1.2	−1.7
	AI323095.1	sialytransferase 1	glycosylation	−2	−1.9	−1.7	−1.7
	AA098196.1	IgA heavy chain C region		−1.4	−2.2	−1.9	−1.8
	AA521593.1	EST		−1.8	−1.2	−1.2	−1.9
	AA119479.1	LEF-1	gene expression	−2.3	−3	−3	−1.9
	AA272807.1	MHC class II I-A α chain		−1.9	−1	1.2	−2.6
4	AI893893.1	CIS1/SOCS	cytokine signaling	−1	1.5	2.2	1.7
	AA437891.1	EST		−1.1	2.2	1	1.6
	AA108880.1	Ca^{++} channel γ-subunit	Ca^{++} signaling	−1	2	2.6	1.4
	AA212893.1	EST		−1	2.7	1	1.4
	AA521764.1	RAMP-2	regulates calcitonin receptor	−1	2	1.5	−1
	AA060880.1	EST		−1	4.7	3.4	−1
	AA177218.1	EST		−1	2.3	1.5	−1
5	W18484.1	MEL91	signal transduction	1.5	−3.3	−4.5	1.8
	AA007970.1	stannin	detoxification	1.3	−1.8	−2.3	1.5
	AA014889.1	sim to alpha 1,2-mannosidase		1.4	−2.1	−2.8	1.4
	AA178361.1	Lysosomal acid lipase 1		1	−2.6	−3.9	1.4
	W99918.1	EST		1.3	−2.4	−3.6	1.3
6a	W88094.1	CXCR4	cell migration	−1	−1.7	−1.3	1.7
	AA080443.1	EST		−1	−1	−2.1	1.6
	AA097980.1	Cytochrome P450	detoxification	−1	−1	−2.3	1.6
	AA068750.1	SDF-1	cell migration	−1.2	−1	−1.9	1.4
	AA047991.1	60S ribosomal protein P2	translation	−1.7	−1.1	−1.2	1.3
	AA073904.1	Dickkopf-3	signal transduction	−1	−1	−2.5	1
	AA240279.1	40S ribosomal protein S5	translation	−1.7	−1	−1.3	1
	W36356.1	Forkhead (FKHR)	gene expression	−1	−1.7	−1.7	1
	W41682.1	Glucosamine fruct.-6-PO_4 transaminase	hexosamine pathway	−2.1	−1.5	−1.7	1
	W65070.1	EST		−2.5	−1.5	−1.7	1
	AA033398.1	40S ribosomal protein S15	translation	−1.9	−1.3	−1.4	1
	AI604200.1	60S ribosomal protein L39	translation	−1.8	−1.6	−1.6	1
6b	AA183698.1	L-selectin (CD62L)	cell migration	−9.6	−5.3	−3.9	−2
	W82894.1	EST		−4.3	−4.5	−4.1	−2.7

[a] Groups according to those outlined in Figure 2.

Group 1 list of genes and an expanded table can be found in Supplemental Data, Supplemental Table S6 available at http://www.cell.com/cgi/content/full/111/6/837/DC1.

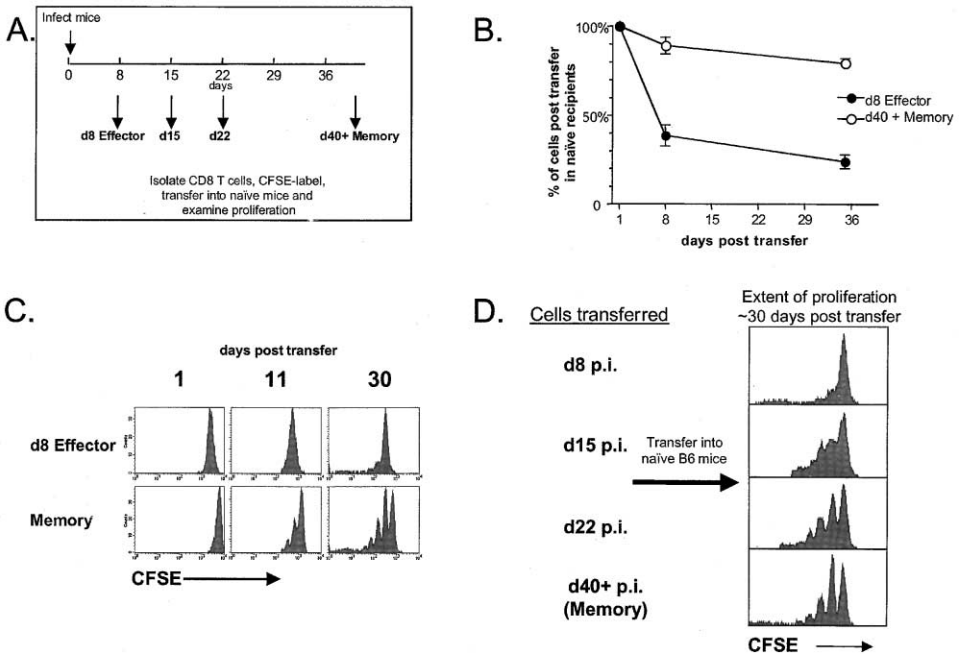

Figure 3. Memory Cell Precursors Exist by d8 p.i. but Have Not Acquired the Ability to Proliferate to Homeostatic Signals

(A) P14 CD8 T cells were purified at d8, 15, 22, and >d40 p.i., labeled with CFSE, and transferred into non-irradiated naïve B6 mice and the extent of cell division was examined at later time points.

(B) The number of d8 effector or d40+ memory cells present 7 and 35 days posttransfer are shown as a percentage of donor cells present one day after transfer. Note that the number of transferred memory CD8 T cells remains relatively constant, but the number of d8 effector cells contracts similar to that observed normally between days 8–30 p.i.

(C) The extent of cell division, based on CFSE fluorescence, in the d8 effector and d40+ memory cells was examined 1, 11, and 30 days posttransfer. Histograms displaying CFSE fluorescence are gated on $D^bGP33\text{-}41^+$ $CD8^+$ P14 T cells.

(D) P14 CD8 T cells from d8, d15, d22, and >d40 p.i. were CFSE-labeled and transferred into naïve mice and cell division was examined 30 days later as described in (C).

contact with antigen, memory CD8 T cells divide rapidly and extensively to generate a second burst of effector cells. Therefore, we examined when following an acute viral infection antigen-specific CD8 T cells display a memory cell-like proliferative capacity in response to antigen.

First, we compared the capacity of P14 CD8 T cells from mice infected 8, 15, 22, or >40 days previously to proliferate to antigen in vitro. P14 CD8 T cells were labeled with CFSE and stimulated with antigen-presenting cells (APCs) pulsed with GP33-41 peptide for 72 hr. This showed that memory P14 CD8 T cells (d40 + p.i.) divided the most extensively, whereas d8 and d15 cells divided the least and d22 cells showed an intermediate pattern (Figure 4A). The progeny of stimulated memory cells (d40 + p.i.) also survived better in culture than did those of d8 effector cells. Typically, cultures containing memory P14 CD8 T cells expanded ~6-fold, whereas those containing d8 cells dropped ~3-fold despite the observed proliferation (data not shown). These results indicated by d40 p.i., and to an extent by d22 p.i., the capacity of LCMV-specific CD8 T cells to extensively proliferate and survive when stimulated in vitro has increased, but these qualities had not developed by d15 p.i.

To determine if a similar phenomena occurred in vivo, CD8 T cells from mice infected 8, 14, 22, and >40 days previously were transferred into LCMV carrier mice that have been persistently infected with LCMV since birth. Every week after adoptive transfer serum viral titers were quantitated. In carrier mice that received adoptive immune therapy using memory CD8 T cells, viral titers plummeted within two weeks and were maintained at undetectable levels (Figure 4B). In contrast, carrier mice that received adoptive immune therapy using cells from d8 p.i., viral titers initially decreased, indicating effector functions were intact, but virus was not controlled and levels eventually returned to that seen prior to transfer (Figure 4B). A similar outcome was observed when d14 cells were transferred (Figure 4D). d22 cells, on the other hand, could control the virus in most carrier mice, but not all (data not shown). If d8 and memory cells were cotransferred into carrier mice, the virus was cleared rapidly, thus, eliminating the possibility that a second population of "regulatory" cells existed at d8 p.i. that inhibited effector CD8 T cell function (Figure 4B). In summary, these experiments demonstrate that d8 and d15 effector CD8 T cells do not sustain effector functions as well as memory CD8 T cells (d40 + p.i.) in response to antigen in vivo.

The inability of d8 effector cells to control viremia may be largely attributed to decreased proliferation in carrier mice. Equal numbers of d8 effector and memory $Thy1.1^+$ LCMV-specific CD8 T cells were labeled with CFSE, independently transferred into $Thy1.2^+$ LCMV carrier mice, and examined 4 and 7 days later. Within this time, the memory CD8 T cells expanded significantly and were

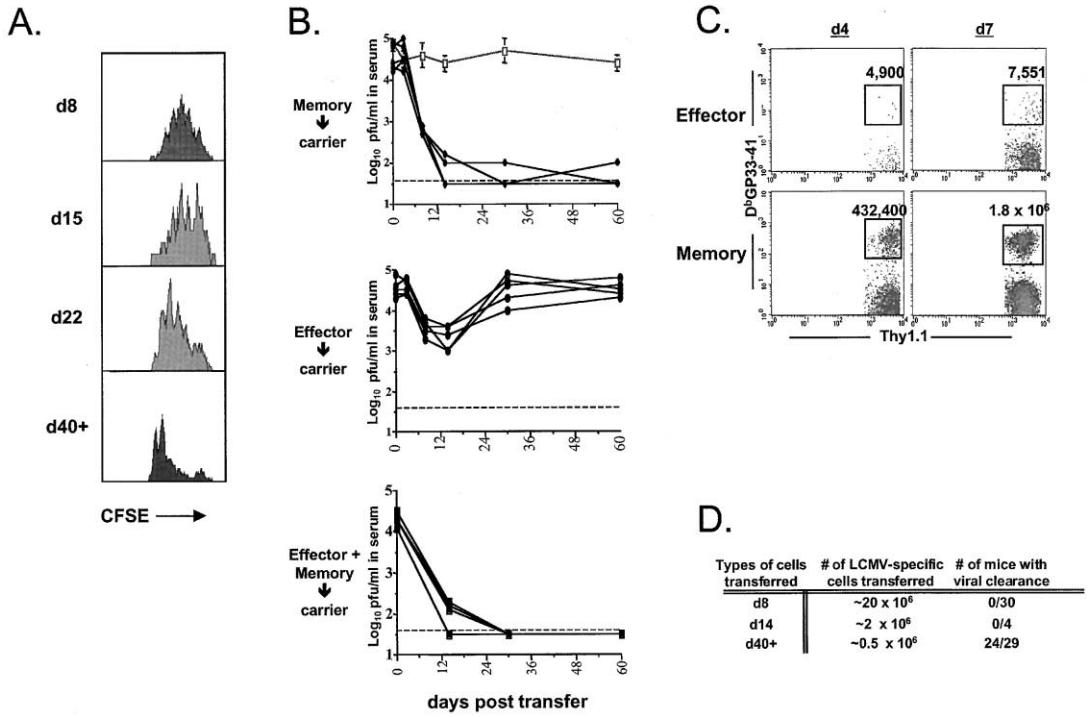

Figure 4. Responses of LCMV-Specific CD8 T Cells to Antigen during Memory CD8 T Cell Development

(A) P14 CD8 T cells were isolated at the indicated times after infection, labeled with CFSE, and stimulated with GP33-41 peptide for three days in vitro. Cells were stained with CD8α antibodies and $D^bGP33\text{-}41$ tetramers and CFSE fluorescence was analyzed by flow cytometry. Histograms displaying CFSE fluorescence are gated on $D^bGP33\text{-}41^+$ $CD8^+$ T cells.

(B) The kinetics of viral clearance in LCMV carrier mice that received adoptive immune therapy. Splenocytes containing memory CD8 T cells (d40+ p.i., top) or effector CD8 T cells (d8 p.i., middle) or both (bottom) were transferred into carrier mice and serum viral titers were determined by plaque assay at the indicated times posttransfer. Viral titers in untreated LCMV carrier mice are shown (open squares). Dashed line indicates threshold of detection.

(C) LCMV-specific CD8 T cell proliferation in carrier mice. $\sim 1 \times 10^6$ $Thy1.1^+$ LCMV-specific CD8 T cells from d8 (top) or d40+ infected mice (bottom) were CFSE-labeled and transferred into $Thy1.2^+$ carrier mice and four and seven days later their expansion was examined. Dot plots are gated on $Thy1.1^+$ cells. Numbers of $D^bGP33\text{-}41$-specific CD8 T cells per spleen are indicated.

(D) The number of LCMV-specific CD8 T cells transferred in the experiments shown in (B) and the number of carrier mice where viremia was absent following immune therapy are shown.

CFSE-negative indicating they had divided at least 7–10 times (data not shown); whereas, the d8 effector cells expanded minimally or not at all (Figure 4C). Thus, by day 40 p.i., the LCMV-specific CD8 T cells could proliferate and survive in response to antigen in vivo, and consequently, control viral loads, whereas these properties were not exhibited by d8 p.i.

Quantitative Perspective on Adoptive Immune Therapy of Carrier Mice

The qualitative differences observed between d8 effector and d40+ memory cells in the LCMV carrier mice experiments suggest that memory cell precursors that exist by d8 p.i. have not fully developed memory cell properties. This point is better illustrated when these experiments are analyzed quantitatively. Typically, the number of LCMV-specific memory CD8 T cells formed equals ~5%–10% the number of effector cells at the peak of the CD8 T cell response (d8 p.i.) (Murali-Krishna et al., 1998). As shown in Figure 4D, ~40-fold more LCMV-specific effector CD8 T cells were transferred than memory cells into carrier mice (20×10^6 versus 0.5×10^6) and still the effector cell population was strikingly ineffective at reducing viral levels in vivo. Therefore, if a functional memory CD8 T cell population had existed by d8 p.i., then $\sim 1\text{–}2 \times 10^6$ memory cells (5%–10% of 20×10^6) would have been transferred, and this should have been sufficient to control viremia since as few as 0.5×10^6 LCMV-specific memory CD8 T cells from d40+ p.i. were required. This strongly suggests that although the precursors to memory CD8 T cells exist in the d8 effector population they have not fully acquired the protective qualities of memory cells.

Differential TCR Signaling Capabilities in Effector and Memory CD8 T Cells

To better understand why d8 effector CD8 T cells can not proliferate and survive as well as memory CD8 T cells when stimulated with antigen, we examined the ability to transduce TCR signals and to activate the downstream mitogen-activated protein kinases ERK1 and 2 (Extracellular signal-Regulated Kinase). ERK1/2 are ser/thr kinases that when phosphorylated translocate to the nucleus where they activate transcription factors involved in cell cycle regulation and effector cell differentiation. The ability of P14 CD8 T cells to activate ERK1/2 at different times postinfection was assessed by flow cytometry using antibodies that specifically rec-

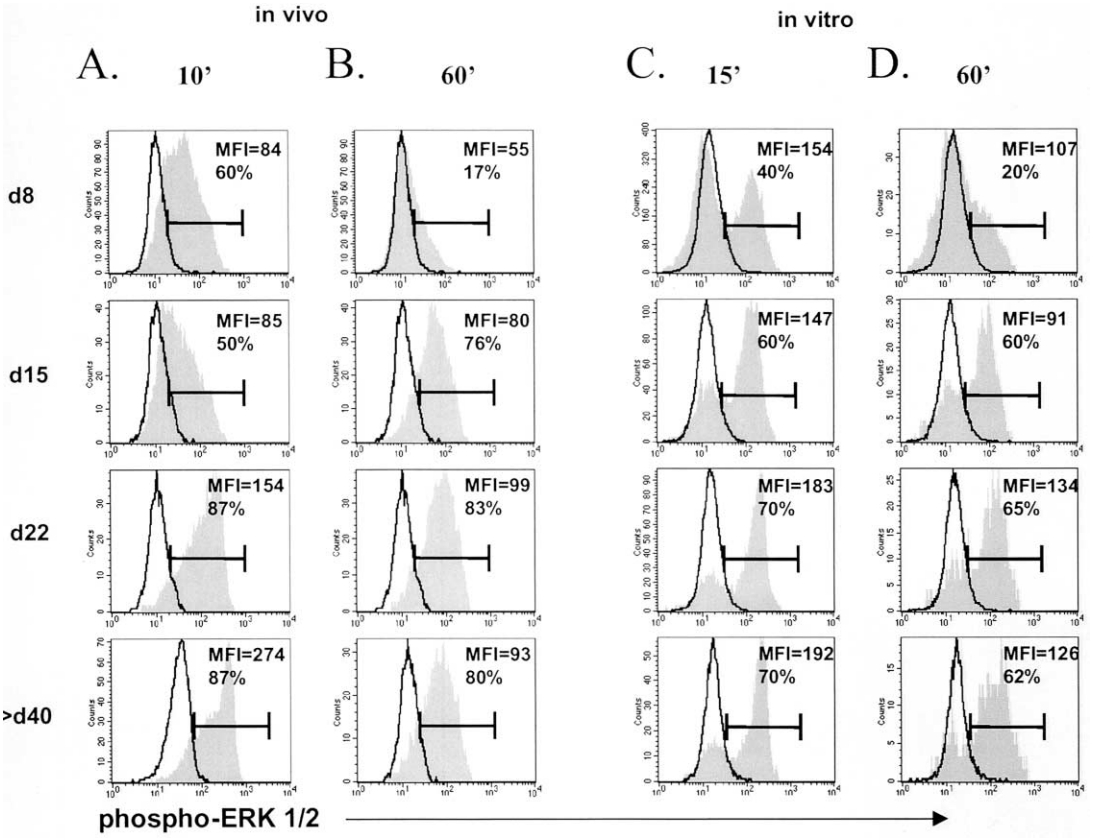

Figure 5. Decreased Ability to Phosphorylate ERK1/2 in Activated Effector CD8 T Cells

(A and B) Phosphorylation of ERK1/2 in vivo. Thy1.1+ P14 chimeric mice were infected 8, 15, 22, or >40 days previously with LCMV, were injected with 100 μg GP33-41 peptide, and the spleens were isolated 10 and 60 min later. Splenocytes were stained with Thy1.1 and phospho-ERK1/2 (pERK) antibodies and analyzed by flow cytometry. Histograms indicate pERK1/2 fluorescence in peptide-stimulated (shaded) and unstimulated (bold line) Thy1.1$^+$ cells and represent one of five experiments. The percent of cells containing high levels of pERK1/2 and the mean fluorescent intensity (MFI) is indicated.

(C and D) Splenocytes were isolated from mice at the same days p.i. as in (A), but cells were stimulated with peptide for the indicated lengths of time in vitro.

ognize phosphorylated ERK1/2 (pERK 1/2) (Chow et al., 2001). Mice containing Thy-1.1$^+$ P14 CD8 T cells were infected with LCMV and 8, 15, 22, or >40 days postinfection; the cells were restimulated in vivo by injection of GP33-41 peptide. After 10, 30, and 60 min, the splenocytes were fixed and stained with antibodies to Thy-1.1 and pERK1/2. After 10 min of stimulation nearly all the memory cells had high levels of pERK1/2 staining that was sustained over the next hour, although the level of pERK1/2 fluorescence had decreased (Figures 5A and 5B). In contrast, a smaller percentage of d8 effector cells contained high levels of pERK1/2 and the mean fluorescent intensity (MFI) was substantially lower, indicating that on a per cell basis d8 cells contained fewer pERK1/2 molecules than memory cells. In addition, d8 cells did not sustain pERK1/2 levels as well as memory cells. The signaling capacity of d15 cells more closely resembled that of d8 cells, whereas d22 cells behaved more like memory cells. A very similar trend was observed when P14 CD8 T cells from days 8, 15, 22, and >40 p.i. were stimulated with GP33-41 peptide in vitro (Figures 5C and 5D). Combined, these data suggest that d8 effector CD8 T cells can not phosphorylate ERK1/2 as efficiently as memory CD8 T cells, but that this property is gradually acquired over the next several weeks. This may account for the reduced proliferative potential and survival observed in effector CD8 T cells in response to antigenic stimulation.

Models of Memory CD8 T Cell Differentiation

Elucidating the mechanisms that drive development of memory CD8 T cells and delineating when these cells form during an immune response are answers that have long been sought after. Two models of memory CD8 T cell development are described in Figure 6B. In the first model, functional memory CD8 T cells are generated in the presence of antigen and exist by the peak of the CD8 T cell response (e.g., d8 p.i. with LCMV). These fully formed memory cells then selectively survive the death phase and are maintained. The second model proposes memory cell precursors are generated during the expansion phase but initially do not display functional memory cell traits. Several weeks following antigen clearance, however, the cells gradually acquire memory cell properties.

Our study heavily supports the latter model because several of the salient memory cell properties examined

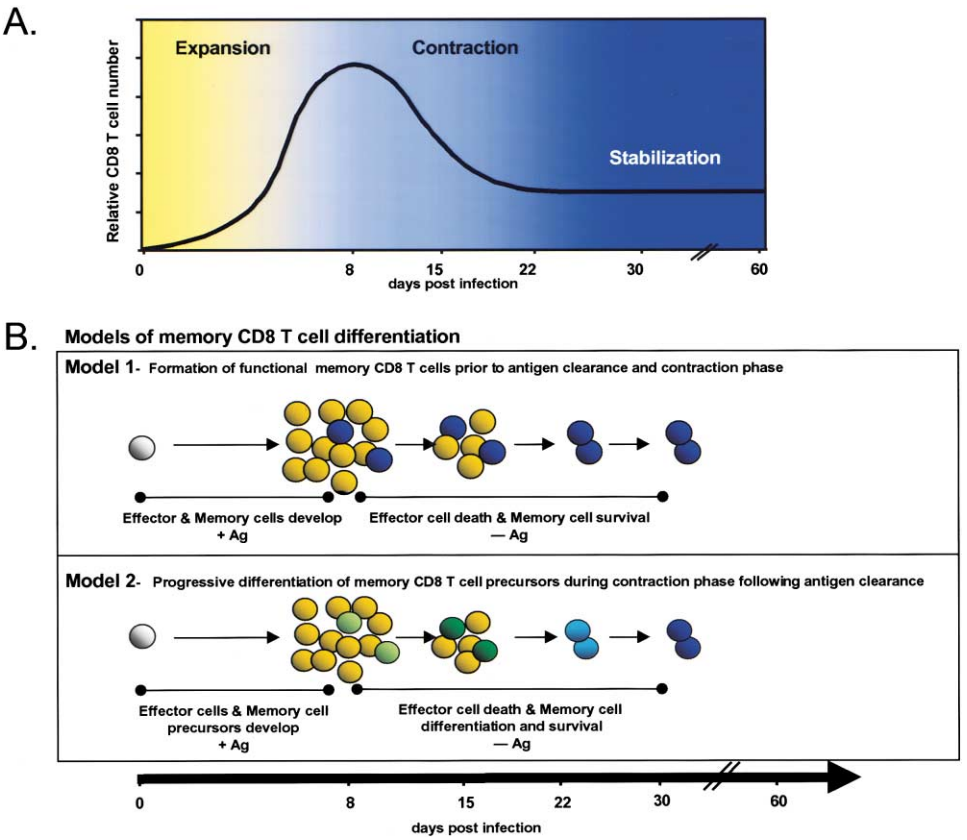

Figure 6. Models of Memory CD8 T Cell Development during an Immune Response

(A) Schematic illustration of the three phases of an antigen-specific CD8 T cell response following immunization.

(B) Model 1 proposes that long-lived memory CD8 T cells develop simultaneously with short-lived effector CD8 T cells during the primary immune response in the presence of antigenic stimulation. Following antigen clearance, the short-lived effector cells die and the memory CD8 T cells survive and are maintained. Model 2 suggests that memory CD8 T cell development occurs in two stages. First, antigenic stimulation induces the development of effector CD8 T cells that function to eliminate the infectious pathogen. Initially following elimination of antigen, the majority of the antigen-specific effector CD8 T cells die but the surviving memory cell precursors (in bold lines) have not yet acquired a complete set of memory CD8 T cell phenotypes and gene expression pattern. Second, successive changes in gene expression and other cellular processes occur over the next several weeks that gradually transform these cells into functional memory CD8 T cells that become long-lived and can effectively respond to antigenic and homeostatic signals. Model 2 is strongly supported by the data described herein.

here were not exhibited by the virus-specific CD8 T cells until several weeks following infection. Although d8 effector cells were cytolytic and could secrete IFN-γ, their ability to survive, to proliferate in response to antigenic and homeostatic signals, and to activate ERK1/2 was impaired compared to memory CD8 T cells (d40+ p.i.). By d22 p.i., the cells behaved more like memory cells indicating that memory cell qualities were being acquired between days 8–22 p.i. Another memory CD8 T cell phenotype that is gradually acquired is the heightened expression of the anti-apoptotic molecule Bcl-2. Bcl-2 levels are low in LCMV-specific effector CD8 T cells at d8 p.i., but increase by day 40 p.i. (Grayson et al., 2000). The gene expression analysis done in this study also revealed that the profile of gene expression of memory CD8 T cells differs from that of d8 effector cells and demonstrated that the expression of many genes changes between d8-40 p.i. Further examination of these genes may identify key signals involved in memory CD8 T cell differentiation. Nearly 30 years ago, classic studies in the LCMV model had noted functional differences between "early" (d9) and "late" (d30) LCMV-specific CD8 T cells (Johnson and Cole, 1975; Volkert et al., 1974). Our present study now provides a cellular and molecular basis for the differential behavior of antigen-specific CD8 T cells at these two time points. A detailed explanation for the behavioral differences between these two cell populations is provided by our results that indicated that at d8 p.i. memory cell precursors exist but they do not display all memory cell attributes.

Similar to memory CD8 T cells, naïve cells display a high capacity to proliferate and to activate ERK1/2 (data not shown). Thus, initially these properties are intrinsic to CD8 T cells but after prolonged antigenic stimulation these functions become impaired. This phenomenon, previously termed antigen-induced non responsiveness (AINR), has been observed in other experimental systems (Bikah et al., 2000; Deeths et al., 1999). Our results demonstrate that in vivo AINR is transient and that memory cell precursors regain the ability to respond to antigen. How the T cells responses are "reset" is not clear,

but may be linked to the ability to transduce and sustain TCR signals. Also, regaining responsiveness may require a period of rest from antigen because under continuous antigenic stimulation, such as during chronic viral infections and malignant melanomas, virus-specific T cells often are deleted or become dysfunctional and lose effector functions (Lee et al., 1999; Zajac et al., 1998). It is not clear if long-lived, functional memory CD8 T cells can be generated under these conditions.

Characterization of a gene expression profile of functional memory CD8 T cells will hopefully aid discovery of the mechanisms that regulate development and maintenance of these cells, which would prove invaluable for optimizing vaccination. Moreover, comparing gene expression profiles of functional and dysfunctional CD8 T cells may uncover the source of effector cell dysfunction that, in turn, could lead to novel immunotherapeutic approaches to fight chronic infections and tumors. Also, delineating when memory CD8 T cells form in vivo and acquire a high proliferative capacity has considerable implications for vaccine regimens that involve boosting for efficacy, because our results suggest that vaccine boosters should be separated by a significant length of time to allow the effector cells generated to differentiate into memory cells and reset their responsiveness to antigen. Finally, it is tempting to speculate about similarities between immunological and neuronal memory and whether common molecular mechanisms may be involved in regulating these two types of "memory".

Experimental Procedures

Viral Infection and Mice

C57BL/6 and B6.PL-Thy1^a/Cy mice were purchased from Jackson Laboratory (Bar Harbor, ME). The P14 transgenic mice (Thy1.2$^+$ or Thy1.1$^+$) were previously described (Kaech and Ahmed, 2001). Effector (d8 p.i.) and memory (>d40 p.i.) P14 CD8 T cells were generated by adoptive transfer of $\sim 2 \times 10^5$ naïve P14 CD8 T cells into non-irradiated C57BL/6 mice followed by intraperitoneal infection with 2×10^5 pfu of LCMV-Armstrong. LCMV carrier mice were derived as described (Ahmed et al., 1987) and were bled from the periorbital venous plexus and serum viral titers were quantitated as described (Ahmed et al., 1987).

Cell Surface and Intracellular Staining and CFSE-Labeling

All antibodies were purchased from Pharmingen (San Diego, CA), except fyn and lck antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and phospho-ERK1/2 antibodies from Cell Signaling Technology (Beverly, MA). Cells were stained for surface proteins or intracellular cytokines as described in Murali-Krishna et al. (1998) and for phospho-ERK1/2 as described in Chow et al. (2001). Cells were labeled with CFSE (Molecular Probes, Eugene, OR) as described in (Kaech and Ahmed, 2001).

FACS Cell Sorting

Cells were isolated by FACS cell sorting as described (Grayson et al., 2001). Cells were stained with CD8α and Thy1.1 antibodies or CD8α antibodies and D^{b}GP33-41 MHC class I tetramers on ice in PBS containing 1% BSA and sorted using a FACS Vantage (Beckton Dickinson, San Diego, CA).

Direct Ex Vivo CTL Assays

GP33-41-specific CTL activity was determined by a 5 hr ^{51}Cr-release assay as previously described (Murali-Krishna et al., 1998).

DNA Microarray Hybridization and Analysis

Total RNA was isolated from cells in Trizol (GIBCO/BRL Life Technologies, Rockville, MD) according to manufacture's protocol. cDNA was synthesized using SuperScript Choice cDNA synthesis kit (GIBCO/BRL) and an oligo(dT) primer containing a T7 promoter. The MEGAscript T7 kit (Ambion, Austin, TX) was used to amplify cRNA from the cDNA. The cRNA was reverse transcribed with either Cy3 or Cy5 fluorescently labeled nucleotides and hybridized on mouse GEM 1 microarrays at Incyte Genomics (St. Louis, MO) as described in Yue et al., (2001). Samples compared: naïve versus d8 effector (n = 3) or memory cells (n = 3) or d15 (n = 2) or d22 cells (n = 2). Expression pattern clusters were defined using hierarchical tree and K-means clustering algorithms in J-Express v. 1.1 (Dysvik and Jonassen, 2001). Hybridization of biotin-labeled cRNA to Affymetrix U74A chips occurred according to manufacturer's protocols and similar to that described (Teague et al., 1999).

Western Blotting

Protein lysates from 1×10^6 FACS sorted naïve, d8 effector, and memory P14 Thy1.1$^+$ CD8 T cells were resolved by SDS-PAGE. Granzyme B was detected by Western blotting using rabbit anti-granzyme B antibodies at 1:1000 (a gift of Dr. Tim Ley, Washington University, St. Louis, MO).

Antigen-Driven and Homeostatic Proliferation Assays

For in vitro proliferation assays P14 CD8$^+$ T cells from infected mice were isolated at 8, 15, 22, or greater than 40 days p.i. and cultured with GP33-41 peptide as previously described (Kaech and Ahmed, 2001). In LCMV carrier mice, splenocytes from Thy1.1$^+$ B6 mice infected 8 and >40 days previously were CFSE-labeled and adoptively transferred separately into LCMV carrier mice and analyzed 4 and 7 days later. A total of 1×10^6 D^{b}GP33-41 and D^{b}NP396-404-specific CD8 T cells from each population were transferred. For homeostatic proliferation assays, splenocytes from P14 chimeric mice infected 8, 15, 22, or >40 days previously with LCMV were CFSE-labeled and transferred into naïve, non-irradiated C57BL/6 hosts. The donor cells were examined $\sim$1, 11, and 30 days post-transfer.

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