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Activated and Memory CD8$^+$ T Cells Can Be Distinguished by Their Cytokine Profiles and Phenotypic Markers[1]

Mark K. Slifka[2] and J. Lindsay Whitton

Dissecting the mechanisms of T cell-mediated immunity requires the identification of functional characteristics and surface markers that distinguish between activated and memory T lymphocytes. In this study, we compared the rates of cytokine production by virus-specific primary and memory CD8$^+$ T cells directly ex vivo. Ag-specific IFN-γ and TNF-α production by both primary and long-term memory T cells was observed in ≤60 min after peptide stimulation. Although the on-rate kinetics of cytokine production were nearly identical, activated T cells produced more IFN-γ, but less TNF-α, than memory T cells. Ag-specific cytokine synthesis was not a constitutive process and terminated immediately following disruption of contact with peptide-coated cells, demonstrating that continuous antigenic stimulation was required by both T cell populations to maintain steady-state cytokine production. Upon re-exposure to Ag, activated T cells resumed cytokine production whereas only a subpopulation of memory T cells reinitiated cytokine synthesis. Analysis of cytokine profiles and levels of CD8, LFA-1, and CTLA-4 together revealed a pattern of expression that clearly distinguished in vivo-activated T cells from memory T cells. Surprisingly, CTLA-4 expression was highest at the early stages of the immune response but fell to background levels soon after viral clearance. This study is the first to show that memory T cells have the same Ag-specific on/off regulation of cytokine production as activated T cells and demonstrates that memory T cells can be clearly discriminated from activated T cells directly ex vivo by their cytokine profiles and the differential expression of three well-characterized T cell markers. ***The Journal of Immunology,*** **2000, 164: 208–216.**

CD8$^+$ T cells are critical for controlling many viral infections (1) and are central to vaccine-induced antiviral immunity (2, 3). During the primary response to an acute viral infection, naive CD8$^+$ T cells are driven in a predominantly Ag-specific manner to become effector T cells (4–6). After resolving the infection, the majority of virus-specific T cells die by activation-induced cell death (7, 8), but a population of memory T cells is stably maintained for life; up to 2 years in mice (9) and 35–50 years in humans (10). Several murine surface markers distinguish naive T cells from activated/memory T cells, including CD11a, CD11b, CD44, CD62L, and peanut lectin agglutinin (9, 11–15), but there are currently no surface markers that can be used to distinguish activated T cells from memory T cells in normal mice (16–18).

Infection of adult mice with the natural murine pathogen lymphocytic choriomeningitis virus (LCMV),[3] provides one of the best-characterized model systems for studying antiviral T cell responses and cytokine production (4, 5, 17, 19–26). LCMV infection is cleared within 8–10 days, predominantly by CD8$^+$ T cells (27–30) in a perforin-dependent manner (31, 32). The peak of the effector CD8$^+$ T cell response, as demonstrated by the ability to lyse virus-infected target cells directly ex vivo, occurs at ~8 days postinfection (p.i.). The effector phase of the immune response declines sharply by 20–30 days p.i., but protection against secondary challenge is maintained by a stable population of memory CD8$^+$ T cells (9). In the absence of pre-existing antiviral Abs, these cells can protect against a subsequent viral challenge (21). In BALB/c mice, >95% of the CD8$^+$ T cell response is mounted against a single immunodominant epitope, LCMV NP$_{118-126}$ (33), and studies comparing TCR Vβ usage and CDR3 length in CD8$^+$ T cells indicate that memory T cells are stochastically selected from this primary T cell pool (34). The LCMV model system therefore provides the unique opportunity to compare activated and memory T cell populations that have the same Ag specificity and conserved CDR3 and Vβ usage in a nontransgenic mouse model.

In this study, we compared virus-specific cytokine production by activated T cells at the peak of the effector T cell response to that of long-term memory T cells. After peptide stimulation directly ex vivo, both activated and memory CD8$^+$ T cells initiated cytokine production within 60 min, and the maximum number of responding antiviral T cells was attained within 4–6 h poststimulation. Remarkably, disruption of T cell contact with peptide-coated APCs resulted in the immediate termination of cytokine production in both populations, and re-exposure to specific peptide Ag led to reinitiation of cytokine production by the majority of activated T cells and a subpopulation of memory T cells. Thus, both primary and memory T cells were able to cycle cytokine production on, off, and on again in an exquisitely sensitive response to Ag contact. Virus-specific CD8$^+$ T cells expressed high levels of LFA-1 (CD11a/CD18) at all time points examined, thus confirming its usefulness in distinguishing activated/memory T cells from naive T cells. Direct ex vivo expression of CTLA-4 (CD152) was limited to the first 4–8 days p.i. and declined to baseline levels by 15 days p.i., indicating that it is predominately

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; p.i., postinfection; MFI, mean fluorescence intensity.

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found in virus-specific $CD8^+$ T cells during the early in vivo stages of T cell activation. CD8 expression on antiviral T cells was high at early time points (4–6 days p.i.) but was substantially down-regulated by 8–15 days p.i. before returning to normal levels on the surviving memory T cells examined between 30 and 300 days p.i. These results demonstrate that although memory T cells are similar to activated T cells in terms of their on/off rates of Ag-specific cytokine production, memory T cells can be clearly distinguished from both activated and naive T cells by the differential expression of CD8, LFA-1, and CTLA-4. Together, these findings document functional and phenotypic changes that occur as the activated Ag-specific $CD8^+$ T cell population matures into the memory T cell pool.

Materials and Methods

Virus, mice, and cell lines

LCMV immune mice were obtained by injecting 5- to 15-wk-old BALB/c mice i.p. with 2×10^5 PFU of LCMV-Armstrong (Arm-53b) and were used at the indicated time points. BALB/cByJ mice were purchased either from The Jackson Laboratory (Bar Harbor, ME) or from The Scripps Research Institute animal facility. The fibroblast cell line, BALB Clone 7, was grown in RPMI 1640 containing 10% FBS, L-glutamine, and antibiotics.

Peptide

HPLC-purified (>95% pure) LCMV $NP_{118–126}$ peptide (PeptidoGenic, Livermore, CA) was dissolved in sterile PBS at 10 mg/ml and stored at −80°C until use.

Intracellular cytokine staining and flow cytometry

Spleen cells from LCMV-infected or naive mice were cultured at 37°C in 6% CO_2 for the indicated amount of time in the presence or absence of 10^{-7} M peptide (LCMV $NP_{118–126}$) in RPMI 1640 containing 10% FBS, 20 mM HEPES, L-glutamine, and antibiotics. Brefeldin A (Sigma, St. Louis, MO) was added at a final concentration of 2 μg/ml. For on-rate experiments, brefeldin A was included during the entire culture period (0.5–6 h). Cells were immediately placed on ice, washed, and stained for CD8 (PharMingen, San Diego, CA) in round-bottom 96-well plates. The cells were washed and permeabilized using the Cytofix/Cytoperm kit (PharMingen) according to the manufacturer's directions. Intracellular Ags were detected using PE-conjugated mAbs specific for TNF-α or CTLA-4 and FITC-conjugated anti-IFN-γ Abs (PharMingen). Samples were resuspended in PBS containing 2% formaldehyde and acquired on either a FACScan or FACSCalibur flow cytometer (80,000–200,000 gated events acquired per sample) and analyzed using Cellquest software (Becton Dickinson, San Jose, CA).

Biotinylation and peptide loading of stimulator cells

BALB clone 7 cells were harvested by trypsinization and counted. After washing once with PBS to remove FBS and other soluble proteins, the cells were resuspended at 10^7/ml and biotinylated for 20 min on ice in PBS supplemented with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and containing 1.0 mg/ml freshly added NHS-biotin (Calbiochem, La Jolla, CA). Biotinylation was quenched by adding RPMI 1640 containing 10% FBS, and the cells were washed twice before being resuspended in RPMI 1640 containing 10% FBS and 10^{-7} M peptide (LCMV $NP_{118–126}$). The cells (2×10^7/ml) were incubated at 37°C for 1 h with occasional mixing and washed extensively to remove unbound peptide. Biotinylation of targets had no effect on peptide presentation over a range of peptide doses (10^{-5}–10^{-8} M).

Off-rate kinetics of cytokine production

Spleen cells (10^6/well) from LCMV-infected mice were stimulated with 2×10^5 biotinylated fibroblasts coated with 10^{-7} M peptide (LCMV $NP_{118–126}$) for 5 h at 37°C in 6% CO_2. Streptavidin-coated magnetic beads (Perceptive Biosystems, Framingham, MA) were added at a concentration of 40 beads per biotinylated stimulator cell and mixed by pipetting. After 20 min at 37°C, the cultures were resuspended by pipetting, and biotinylated cells were removed by placing the 96-well plate over a magnet (Perceptive Biosystems) for 2 min. This resulted in >95% removal of biotinylated stimulator cells. (To ensure that a stimulatory amount of peptide was not released from these cells, we cultured peptide-coated cells at 37°C for up to 5 h, collected the supernatant, and mixed this 1:1 with fresh spleen cells from LCMV-infected mice (at day 8 p.i.). No IFN-γ production by virus-specific $CD8^+$ T cells was observed.) The nonbiotinylated splenocytes were recovered by collecting 100 μl of supernatant which was immediately added to wells containing 4×10^5 peptide-coated or uncoated fibroblast APCs (prewarmed to 37°C) to observe the off rate of cytokine production. Brefeldin A (Sigma) was added to a final concentration of 2 μg/ml for the final 60 min of culture to facilitate intracellular cytokine accumulation. Cycloheximide (Sigma) was added to a group of peptide-stimulated cultures at a final concentration of 100 μg/ml. One group of cultures was stimulated with soluble anti-CD3 (10 μg/ml) and anti-CD28 (2.5 μg/ml; PharMingen) and assayed under the same conditions as the peptide-stimulated cultures described above. At the indicated time points, cells were removed from the culture plates, washed, and placed at 4°C overnight in PBS containing 10% FBS and anti-CD8. The next day intracellular cytokine staining was performed according to the manufacturer's directions.

Statistics

Statistical differences between cytokine levels of activated (day 8) and memory T cells were determined by the Student *t* test using SigmaStat (SPSS, Chicago, IL) software.

Results

Kinetics of virus-specific cytokine production

Adult BALB/c mice were infected with LCMV and were used either at 8 days postinfection to study activated T cell responses at the peak of the effector T cell response, or at 280–300 days p.i. to study memory T cell responses. Fig. 1 shows FACS analysis of IFN-γ production by these two T cell populations following direct ex vivo stimulation with the immunodominant peptide LCMV $NP_{118–126}$. IFN-γ was undetectable in both day 8 and memory $CD8^+$ T cells immediately ex vivo, suggesting that cytokines are not constitutively expressed by either cell population. In contrast, IFN-γ production was readily detectable in both T cell populations within 60 min of peptide stimulation. The maximum number of responding T cells detected by intracellular IFN-γ staining was attained within 6 h and was similar to that previously identified using peptide-tetramer staining (4). Cytokine production was not observed after peptide stimulation of naive T cells, indicating that only primed virus-specific T cells were activated to produce cytokines under these in vitro conditions (data not shown and see Ref. 4). More detailed analyses of cytokine production by day 8 and memory T cells are shown in Fig. 2. As shown previously (26), IFN-γ production by day 8 T cells began within 30 min poststimulation, and the maximum number of IFN-γ^+ $CD8^+$ T cells was observed by 6 h poststimulation (Fig. 2*A*). Likewise, TNF-α production was detectable within 30 min poststimulation and peak numbers were attained within 6 h (Fig. 2*B*). Memory T cells responded with nearly identical kinetics, and maximal production of both cytokines was observed by 6 h poststimulation (Fig. 2, *A* and *B*). To compare the on-rate kinetics of day 8 and memory T cell cytokine production on the same relative scale, the number of peptide-specific $CD8^+$ T cells producing IFN-γ or TNF-α at each time point was plotted as a percentage of the maximum response. As shown in Fig. 2*C*, the kinetics of IFN-γ production was strikingly similar in day 8 and memory T cell populations. In each case, the time required to reach 50% of the maximal number of cytokine-producing cells was between 90 and 120 min poststimulation. Similar analysis of TNF-α production (Fig. 2*D*) showed again that both T cell populations reached 50% maximum cytokine production within 2 h of stimulation directly ex vivo. These results demonstrate that, upon encountering the appropriate peptide/MHC complex, memory T cells express antiviral cytokines with kinetics that are nearly superimposable with those of activated T cells analyzed during the peak of the effector T cell response.

To further characterize the cytokine responses of activated and memory T cells, we compared the amounts of IFN-γ and TNF-α

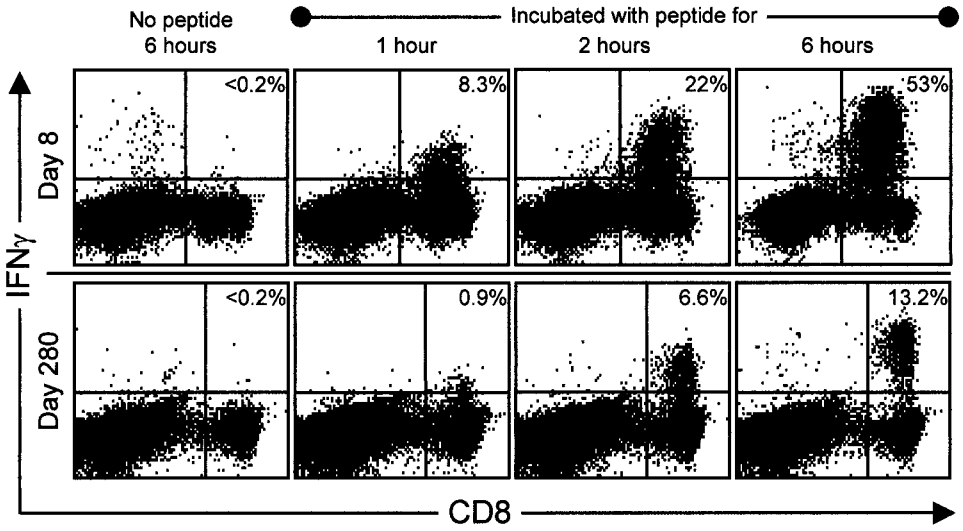

FIGURE 1. Detection of peptide-specific cytokine production by intracellular cytokine staining. Total spleen cells from acutely infected mice (day 8) or LCMV-immune mice (day 280) were cultured directly ex vivo in the presence or absence of the immunodominant peptide LCMV $NP_{118-126}$. At the indicated time points, the cultures were harvested and cells were stained with anti-CD8 (*x*-axis) and anti-IFN-γ (*y*-axis; both axes are $\log_{10}$ scales). The number shown in the *upper right quadrant* of each FACS profile indicates the percentage of $CD8^+$ T cells that scored positive for IFN-γ production after subtracting the background number of events from identical cultures that were not stimulated with peptide. At least 80,000 events were acquired for each sample and the results are representative of eight independent experiments.

that were produced after peptide stimulation directly ex vivo (Fig. 3). $CD8^+$ T cells from mice at 8 days p.i. and memory T cells from mice at 295 days postinfection were stimulated in parallel for 6 h with peptide, and the levels of IFN-γ and TNF-α were compared by quantitating the mean fluorescence intensity (MFI) of cytokine staining. As shown in Fig. 3, day 8 T cells produced more IFN-γ than did memory T cells ($p = 0.02$). In contrast, TNF-α production was much higher in memory T cells than in day 8 T cells ($p < 0.001$). It is striking that, although activated and memory $CD8^+$ T cells have similar on rates of cytokine synthesis (Fig. 2), they plateau at significantly different levels.

Rapid termination of cytokine production upon disruption of Ag contact

The regulatory requirements that determine when cytokine production is turned off is a largely unappreciated and unexplored aspect of T cell-mediated immunity. Without proper control mechanisms in place, unrestrained cytokine production might be harmful or even lethal to the host. Our study (Figs. 1 and 2), as well as others (4, 35, 36) have found that without peptide stimulation, the vast majority of virus-specific $CD8^+$ T cells are not expressing cytokines when analyzed directly ex vivo, even at the peak of the

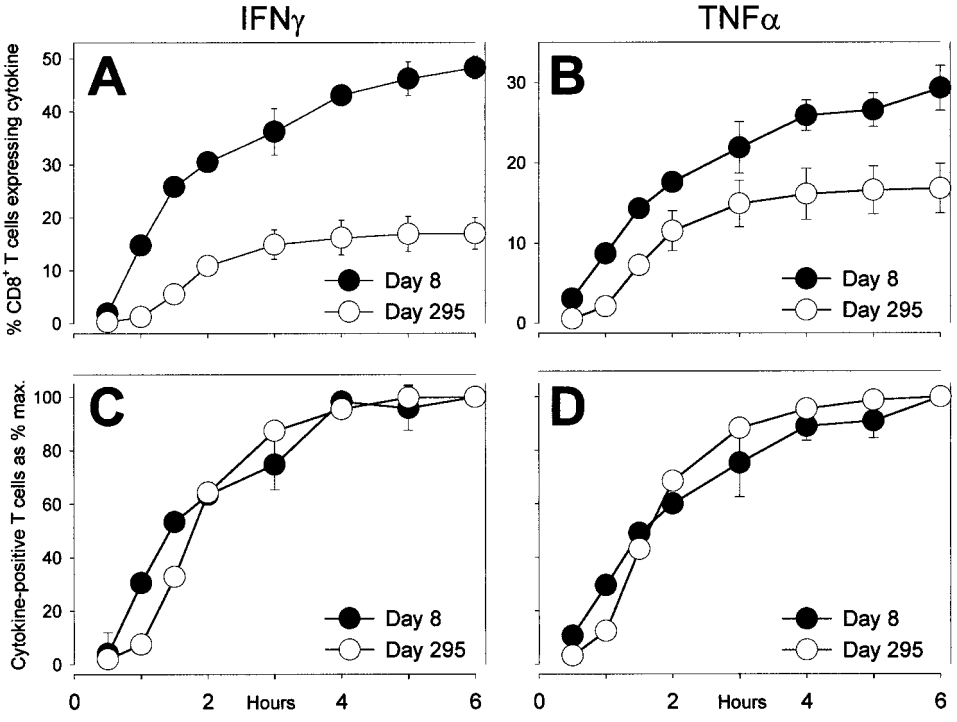

FIGURE 2. Kinetics of antiviral cytokine production by activated and memory T cells. Spleen cells from mice at 8 days (●) or 295 days (○) after LCMV infection were stimulated with peptide for the indicated periods of time and analyzed by flow cytometry. The percentage of IFN-γ^+ (*A*) and TNF-α^+ (*B*) $CD8^+$ T cells were calculated after subtracting the small number of background events from unstimulated cultures assayed in parallel. The percent maximum response for IFN-γ (*C*) or TNF-α (*D*) was calculated by dividing the number of cytokine-positive T cells at each time point by the number of cytokine-positive T cells observed at the 6-h time point multiplied by 100. Each time point shows the average of four mice ± SD.

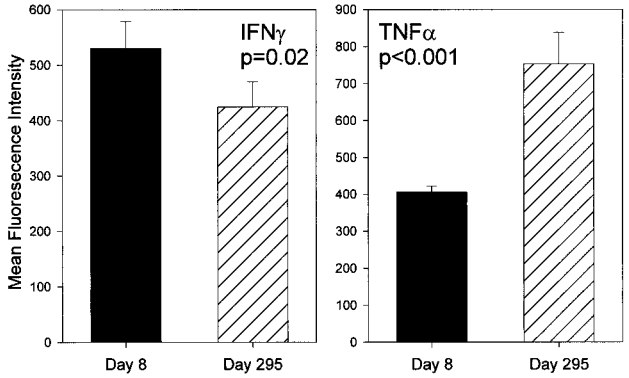

FIGURE 3. Levels of cytokine produced by activated T cells and memory T cells. Spleen cells from mice at either 8 days or 295 days p.i. were stimulated with peptide for 6 h directly ex vivo, and the levels of intracellular IFN-γ and TNF-α were quantitated by measuring the MFI using flow cytometry. The samples were stimulated, stained for the respective cytokines, and assayed in parallel. The data represent the average of four mice per group $\pm$ SD, and statistical significance was determined using the Student *t* test.

antiviral immune response. One explanation of these findings is that T cells may rapidly terminate cytokine production in the absence of continuous Ag contact. To ascertain the role of peptide/MHC contact in the maintenance of sustained cytokine production, we developed a novel technique to quickly and synchronously separate responding T cells from peptide-coated APCs using magnetic bead depletion. This allowed us to determine how rapidly both day 8 and memory T cells were able to down-regulate cytokine synthesis upon disruption of Ag contact. LCMV-specific T cells were stimulated for 5 h with biotinylated, peptide-coated fibroblasts as APCs to generate a high level IFN-γ production (Fig. 4, $t = 5$). One group was left unmanipulated and samples were harvested at the indicated time points. The cognate interaction between the T cells and the APCs in two other groups was disrupted by pipetting and the APCs were removed from the cultures using streptavidin-magnetic beads. The recovered spleen cells were then plated either into wells containing uncoated APCs, to monitor the down-regulation of IFN-γ production, or into wells containing peptide-coated APCs, to determine whether cytokine production could be restored if the $CD8^+$ T cells again came into contact with their cognate Ag. As shown for day 8 cells in Fig. 4*A*, if stimulated cultures were left unmanipulated, then cytokine production by virus-specific T cells was maintained at a steady-state level for the duration of the experiment. Addition of cycloheximide, a protein synthesis inhibitor, resulted in a rapid decline in the number of IFN-γ-producing T cells. Disruption of T cell contact with peptide-coated APCs resulted in an equally rapid reduction (Fig. 4*A*, on/off), indicating that cytokine production ceased immediately upon Ag disengagement. Down-regulation of IFN-γ production was not a procedural artifact, since T cells stimulated with soluble anti-CD3/anti-CD28 showed no alterations in cytokine production after an identical regimen of in vitro manipulations. In addition, if T cells were restimulated on peptide-coated APCs (Fig. 4*A*, on/on), then the percentage of IFN-γ^+CD8^+ T cells rebounded to the initial numbers observed before APC removal. In parallel experiments, memory T cells from mice at 280 days p.i. were stimulated with peptide-coated APCs and then treated as described above (Fig. 4*B*). Similar to day 8 T cells, memory T cell cultures maintained nearly steady-state IFN-γ production while in contact with peptide-coated APCs. After removal of the stimulatory cells, the number of cells producing IFN-γ dropped by >90%, a decline almost identical to

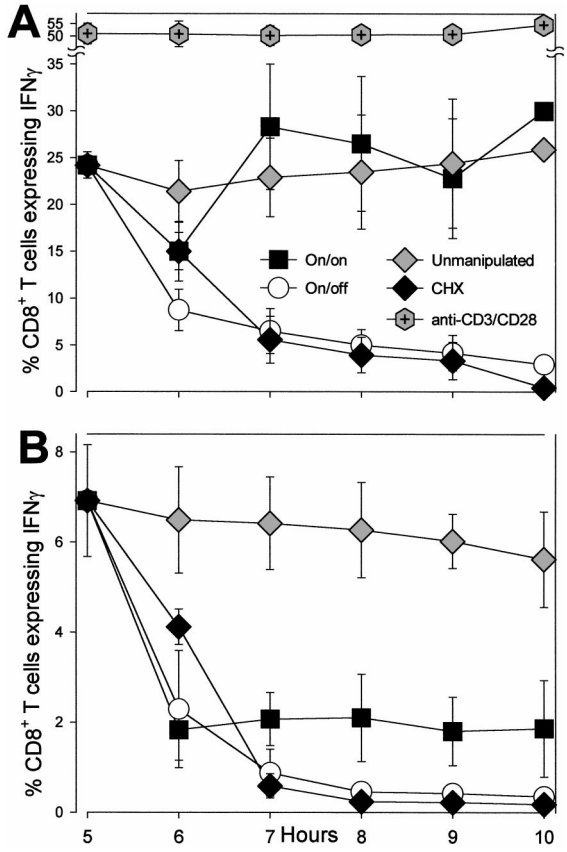

FIGURE 4. Down-regulation of cytokine production after the removal of Ag. Spleen cells (10^6/well) from mice at 8 days p.i. (*A*) or 280 days p.i. (*B*) were stimulated for 5 h with peptide-coated, biotinylated BALB clone 7 cells (2×10^5/well), at which time (designated $t = 5$), $CD8^+$ T cells produced high levels of IFN-γ. Thereafter, the cells were either left unmanipulated, treated with cycloheximide (CHX; 100 μg/ml), or were separated from antigenic contact by removal of the biotinylated stimulator cells using streptavidin-coated magnetic beads. After APC depletion, $\sim 4 \times 10^5$ of the recovered T cells were immediately transferred to wells containing 4×10^5 APCs that were either coated with peptide (on/on) or were untreated (on/off). More than 95% of the biotinylated target cells were removed by the magnetic bead depletion (data not shown). The mechanical disruption and removal of APCs did not have an effect on cytokine production by day 8 spleen cells stimulated with soluble anti-CD3 and anti-CD28. The data show the average of three to four mice $\pm$ the SD except for the 10-h time point in *A* which represents the cytokine response of an individual mouse. The data are representative of four experiments.

that observed following the administration of cycloheximide. In contrast to day 8 T cells, only about 30% of the original IFN-γ^+ memory T cells could be recovered if they were restimulated on fresh peptide-coated targets. The results of these experiments indicate that if Ag-specific T cells are not in direct contact with a target cell presenting a stimulatory level of peptide, then cytokine production is rapidly turned off until the appropriate peptide/MHC complex is encountered.

Patterns of cytokine production and CD8 expression

There are several activation/memory markers that distinguish between naive and Ag-experienced T cells (9, 11–15). However, functional and phenotypic markers that specifically distinguish between activated T cells and memory T cells in normal nontransgenic mice have not been described previously. In this study, we evaluated patterns of cytokine production and CD8 expression levels over the course of both primary and secondary LCMV infections to determine whether either of these criteria corresponded

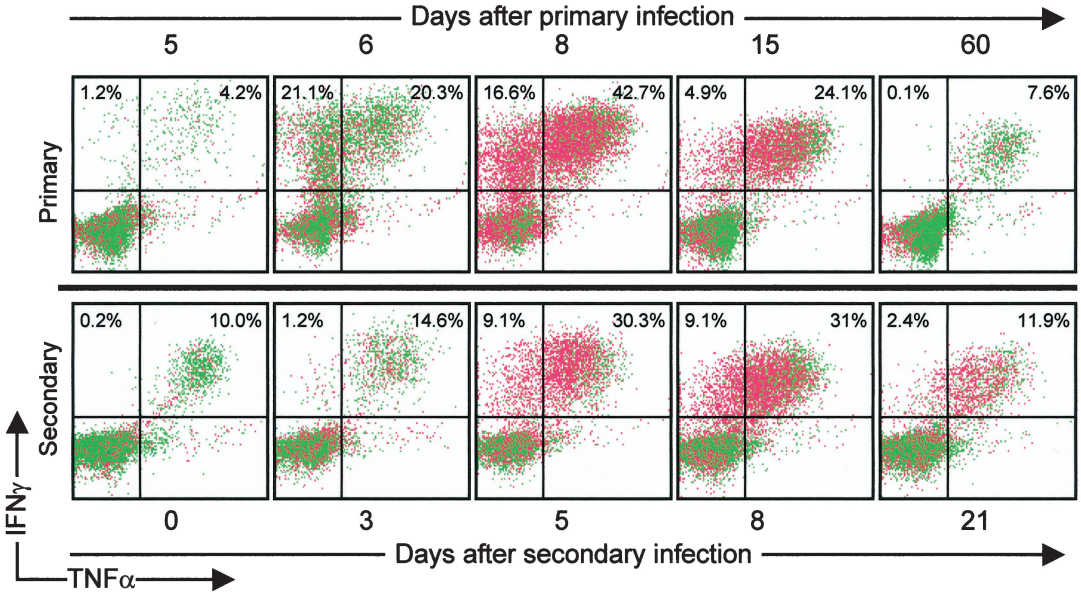

FIGURE 5. Cytokine profiles and CD8 levels during primary and secondary responses to viral infection. Naive BALB/c mice were infected with 2×10^5 PFU LCMV at staggered time points to allow $CD8^+$ T cell-mediated cytokine responses to be assayed on the same day. LCMV-immune mice were infected with 2×10^6 PFU LCMV-Armstrong at staggered time points from 114 to 132 days after primary infection to assay them together at 135 days postprimary infection. Total spleen cells were stimulated directly ex vivo with peptide for 6 h and analyzed by intracellular cytokine staining for IFN-γ and TNF-α. The flow cytometry plots were gated on $CD8^+$ T cells using a large gate (fluorescence range, 120-3400). The number in the *upper left quadrant* of each dot plot represents the percentage of $CD8^+$ T cells that are IFN-γ^+TNF-α^-, and the number in the *upper right quadrant* shows the percentage that are IFN-γ^+TNF-α^+. The events in the *lower right quadrants* were present also in unstimulated cultures and have not been subjected to further analysis. To distinguish $CD8^{high}$ cells from $CD8^{low}$ cells, the events are colored either green (representing the high CD8 levels (fluorescence range, 930-3400) observed in naive T cell controls) or red (representing T cells with down-regulated levels of CD8 (fluorescence range, 120–930)). The MFI of CD8-negative spleen cells was <50. Each plot shown is representative of two to four individual samples.

with an activated phenotype or a memory phenotype. At the indicated days p.i., IFN-γ and TNF-α production by virus-specific $CD8^+$ T cells were assayed after 6 h of peptide stimulation (Fig. 5). At 5–8 days p.i., the virus-specific T cell population was a mixture of both IFN-γ^+TNF-α^- cells and IFN-γ^+TNF-α^+ cells at a ratio of ~1:1–1:3. By 15 days p.i., the ratio of IFN-γ^+TNF-α^- to IFN-γ^+TNF-α^+ cells was about 1:5. This trend continued until day 60, at which point the ratio had shifted to at least 1:50, indicating that 98% of the responding memory T cells produced both IFN-γ and TNF-α simultaneously. This maturational shift in cytokine profiles is not unique to BALB/c mice; an identical shift in cytokine production was observed following acute LCMV infection of C57BL/6 mice (data not shown). Likewise, recombinant vaccinia virus infection resulted in a similar maturational shift in cytokine profiles during the virus-specific $CD8^+$ T cell response (data not shown). Therefore, with the aid of intracellular cytokine staining directly ex vivo, we have identified a previously unrecognized shift in cytokine profiles which appears to be common to several virus infections and is not influenced by the strain or MHC haplotype of the mice. To determine whether the IFN-γ^+TNF-α^+ memory phenotype would persist if the T cells were re-exposed to Ag in vivo, LCMV-immune mice were rechallenged with virus and assayed directly ex vivo for cytokine production (Fig. 5). Before secondary challenge, ~10% of $CD8^+$ T cells were virus specific. By 3 days p.i., this number had increased to ~15%, and by 5 days p.i., ~40% of $CD8^+$ T cells were epitope specific (Fig. 5). Moreover, the increase in total virus-specific $CD8^+$ T cells was even greater than indicated by the above percentages due to cell proliferation; the total number of virus-specific T cells in the spleen had doubled by 3 days p.i. and increased by >10-fold by 5 days p.i. The ratio of IFN-γ^+TNF-α^- to IFN-γ^+TNF-α^+ T cells at day 135 was 1:50 (Fig. 5, secondary, day 0) but, by 3 days postsecondary infection, IFN-γ^+TNF-α^- cells were more numerous, and the ratio had shifted to 1:10. By day 5 postsecondary infection, the ratio had shifted further to 1:3, but by 21 days the ratio began to revert to a memory phenotype and >80% of virus-specific T cells produced both IFN-γ and TNF-α. Therefore, at the peak of the antiviral response (during primary or secondary infection), there are two populations of virus-specific T cells, IFN-γ^+TNF-α^- and IFN-γ^+TNF-α^+, but after resolving the infection, the memory T cell pool becomes double positive.

In analyzing the cell populations described in Figs. 1 and 2, we noted that CD8 expression by virus-specific day 8 T cells was significantly lower than that seen in responding memory T cells analyzed at 295 days p.i. (66% reduction, $p < 0.001$). We therefore conducted a more extensive analysis of CD8 expression over the course of both primary and secondary virus infections (Fig. 5). CD8 expression on virus-specific T cells was visualized using two colors to depict different levels of CD8; green events represent high CD8 expression (within the range of CD8 levels on naive T cells assayed in parallel) and red events represent down-regulated levels of CD8 expression. At 5–6 days p.i., CD8 levels were high (MFI, 1610 and 1160, respectively) but by the peak of the primary T cell response at 8 days p.i., CD8 levels were sharply down-regulated on almost 90% of the virus-specific T cells (MFI, 695). By 60 days p.i., cytokine production had matured to an IFN-γ^+TNF-α^+ memory phenotype and the level of CD8 had returned to high levels (MFI, 1230). Following reinfection with LCMV, CD8 levels on virus-specific T cells were down-regulated by ~50% at day 5 postsecondary infection. By 21 days postsecondary infection, CD8 levels had started to increase as the T cell population reverted to the IFN-γ^+TNF-α^+ memory phenotype. The low levels of CD8 observed on activated T cells at day 8 p.i. was not due to the in vitro stimulation/staining procedure since CD8 levels

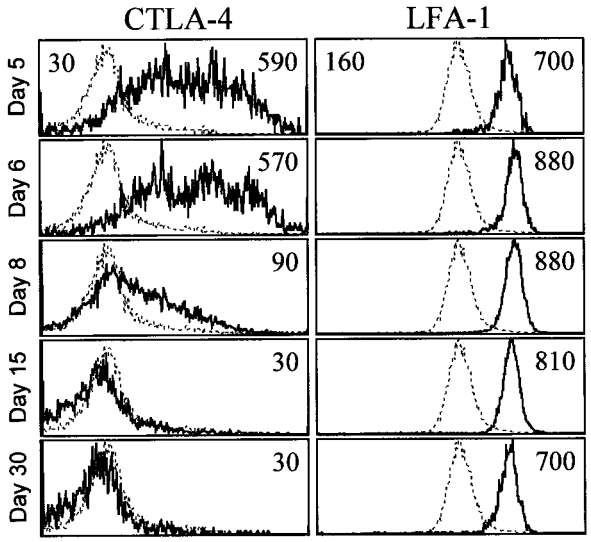

FIGURE 6. CTLA-4 and LFA-1 expression on virus-specific $CD8^+$ T cells. Total spleen cells from naive or LCMV-infected mice were cultured directly ex vivo for 6 h with LCMV $NP_{118–126}$ peptide. The cells were then surface stained for CD8 and LFA-1 and assayed for intracellular IFN-γ and CTLA-4 expression. Naive T cells (dotted line) were gated on $CD8^+$ cells, and virus-specific T cells (solid lines) were gated on $CD8^+$IFN-γ^+ cells. The number in the *upper left corner* of each *top histogram* is the MFI of naive $CD8^+$ T cells, and the number in the *upper right corner* of each histogram shows the MFI of virus-specific $CD8^+$ T cells at the indicated time points. Each histogram is representative of two to six individual samples.

are already low at this time point even in the absence of peptide stimulation (Fig. 1 and see Refs. 4, 15, and 37). This demonstrates that CD8 expression on virus-specific T cells is transiently reduced during the course of an acute viral infection, but returns to high levels during the memory phase of the immune response.

LFA-1 and CTLA-4 levels on activated and memory $CD8^+$ T cells

Above we describe differences in cytokine production which provide evidence indicating that activated $CD8^+$ T cells can be distinguished from memory cells. Although we also found that CD8 expression levels vary during the course of virus infection, this parameter alone is insufficient to distinguish activated T cells from memory T cells. To determine whether a combination of phenotypic markers could be used to discriminate between these two $CD8^+$ T cell populations, we characterized LFA-1 and CTLA-4 expression in virus-specific (IFN-γ^+) $CD8^+$ T cells at each stage of the antiviral immune response (Fig. 6). LFA-1 was expressed at a high level on >95% of antiviral $CD8^+$ T cells at every time point examined, confirming previous studies that describe this as an excellent marker for distinguishing activated/memory T cells from naive T cells (11). There were no substantial differences in LFA-1 expression on virus-specific $CD8^+$ T cells examined between 5 and 60 days p.i. (Fig. 6 and Table I), indicating that this marker alone is insufficient for segregating activated T cell populations from memory T cell populations. In striking contrast, CTLA-4 expression was high in activated virus-specific T cells at the early stages of the immune response but was sharply down-regulated as T cells entered the memory phase. At 4 days p.i., only 1–2% of splenic $CD8^+$ T cells were virus specific, but they already expressed high levels of CTLA-4 (MFI, 880; data not shown). As shown in Fig. 6, >90% of virus-specific $CD8^+$ T cells at 5 and 6 days p.i. expressed CTLA-4 at high levels (MFI, 570–590) compared with naive T cells (MFI, 30). At 8 days p.i., only ~50% of LCMV-specific T cells expressed CTLA-4. However, >80% of the blast-size $CD8^+$IFN-γ^+ cells at this time point expressed CTLA-4, albeit at intermediate levels (data not shown). This suggests again that the most highly activated cells continued to express CTLA-4. By 15 days p.i., CTLA-4 expression had declined further and was indistinguishable from naive T cell expression levels. Similar kinetics of CTLA-4 expression was observed in virus-specific $CD4^+$ T cells in C57BL/6 mice after LCMV infection (data not shown), indicating that early high level expression of CTLA-4 is not limited to the $CD8^+$ T cell subset. A summary of CD8, LFA-1, and CTLA-4 expression on Ag-specific T cells during the course of acute viral infection is given in Table I. Together, these results show that activated/memory T cells can be discriminated from naive T cells on the basis of LFA-1 expression and demonstrate for the first time that CD8 and CTLA-4 expression levels can be used in combination to distinguish activated T cells from memory T cells. In addition, these results suggest that primary $CD8^+$ T cells may be further subdivided into two groups, early activated ($CD8^{high}CTLA\text{-}4^{high}$) and late activated ($CD8^{low}CTLA\text{-}4^{int/low}$), both of which can be clearly distinguished from memory T cells ($CD8^{high}CTLA\text{-}4^{low}$).

Table I. *Summary of marker expression on virus-specific $CD8^+$ T cells*[a]

Day p.i.	CD8	LFA-1	CTLA-4	Immune Status
0	High	Low	Low	Naive
4	High	High	High	Activated
5	High	High	High	Activated
6	High	High	High	Activated
8	Low	High	Intermediate	Activated
15	Low	High	Low	Activated
60	High	High	Low	Memory

[a] Adult BALB/c mice were infected with LCMV, and splenic $CD8^+$ T cells were analyzed directly ex vivo at the indicated times p.i. Virus-specific T cells were identified by their production of IFN-γ after 6 h of in vitro stimulation with $NP_{118–126}$ peptide. Cells were surface stained for CD8 and LFA-1 and stained intracellularly for IFN-γ and CTLA-4. Day 0 indicates the relative level of each marker on naive (IFN-γ^-) T cells from uninfected mice. Similar results were obtained following LCMV infection of C57BL/6 mice or after infection with recombinant vaccinia virus (data not shown).

Discussion

In this study, several criteria were used to compare and contrast the differences between activated T cells and memory T cells. Directly ex vivo, both T cell populations were similar in their ability to turn on and turn off cytokine production in an Ag-specific manner, although memory T cells were less capable of Ag-specific reinitiation of cytokine production. However, IFN-γ and TNF-α levels differed significantly between these T cell populations, and the activated T cell pool contained both IFN-γ^+TNF-α^- and IFN-γ^+TNF-α^+ cells, whereas memory T cells almost exclusively expressed the IFN-γ^+TNF-α^+ phenotype. Furthermore, analyses of CD8, LFA-1, and CTLA-4 levels in virus-specific $CD8^+$ T cells revealed a pattern of expression that distinguished activated T cell populations from memory T cell populations.

Cytolytic activity is a defining feature of activated $CD8^+$ T cells, and, since this activity is typically lost by ~30 days p.i. (9), it is often used to distinguish effector T cells from memory T cells (18). An exception to this rule is that some studies using TCR transgenic $CD8^+$ T cells find relatively strong cytolytic activity for months in the apparent absence of antigenic stimulation (38, 39). Although nontransgenic memory T cells may demonstrate some

low-level CTL activity (40), most memory $CD8^+$ T cells in immune hosts require restimulation for 15 h or more before high-level cytolytic activity is observed (41, 42). We show here that, in contrast to lytic functions, there is little difference in the on-rate kinetics of cytokine production by activated and memory T cell populations (Figs. 1 and 2). We find that activated and memory T cells initiate IFN-γ and TNF-α production within 30–60 min of Ag contact, and the rates at which virus-specific cells are recruited into cytokine production are almost superimposable. The maximum number of responding cells was attained by 6 h poststimulation, but the resulting levels of cytokine production differed significantly; activated cells produced more IFN-γ, whereas memory cells produced more TNF-α. This suggests that there may be a preset maximum production level for each cytokine, based on the activation state of the T cell upon Ag contact. Our results confirm and extend a recent study which showed rapid onset of IFN-γ production but delayed perforin-mediated lytic activity in transgenic T cells (42). Why does a dichotomy exist between cytokine production and cytolytic activity? Perhaps the lag period observed before the acquisition of perforin-mediated lytic activity is beneficial to the host. Direct lysis of a target cell is irreversible, and, if this is the first response to Ag contact, then low-level cross-reactivity or bystander activation may lead to the inappropriate destruction of uninfected cells. However, by first secreting antiviral cytokines, the activated memory T cell may impede viral growth while preparing to mount a cytolytic response. The perforin lag period may be assured by simple biochemical constraints; it takes up to 24–48 h for perforin, granzymes, and other constituent granule proteins to be synthesized and assembled (43).

We and others have found that, even at the peak of the antiviral immune response, cytokine-producing $CD8^+$ T cells are not detectable ex vivo in the absence of peptide stimulation (4, 26, 44). The biological implication of this observation, that most virus-specific $CD8^+$ T cells may not be actively producing cytokines in vivo, has not been previously appreciated. We have shown that activated (day 8) T cells can rapidly terminate cytokine production upon disruption of Ag contact (26), but down-regulation of cytokine responses in memory $CD8^+$ T cells has not been previously described. Remarkably, when peptide-coated APCs were removed, LCMV-specific primary and memory T cells terminated IFN-γ production very rapidly at a rate nearly identical to that observed in cultures treated with cycloheximide (Fig. 4, *A* and *B*). Nevertheless, IFN-γ^+ cells remained detectable for 1–2 h after removal of peptide or addition of cycloheximide, presumably reflecting the kinetics of IFN-γ release, degradation, and so forth. Therefore, if $CD8^+$ T cells had been actively producing cytokines in vivo at the time of cell harvest, then they would have remained detectable, even in the absence of peptide stimulation. Since no such cells were detected, we suggest that the vast majority of virus-specific T cells are not actively producing cytokines in vivo and instead rapidly initiate cytokine production in response to Ag contact. What are the biological implications of such rapid down-regulation of cytokine synthesis? It is critical that cytokine secretion be tightly regulated, because these molecules can be toxic and are responsible for many symptoms of disease (45–49). Therefore, by limiting cytokine production to periods of direct contact with infected targets, the release of inflammatory and antiviral cytokines is confined specifically to sites of infection.

One intriguing difference between day 8 and memory T cells was the ability to resume cytokine production following restimulation with peptide-coated APCs. Essentially all of the day 8 T cells that responded to the primary stimulation were able to reinitiate cytokine production after secondary stimulation with peptide-coated targets (Fig. 4*A*). In contrast, only about 30% of memory T cells were able to resume cytokine production after restimulation (Fig. 4*B*). It is possible that some T cells were lost when the APCs were depleted, but this is unlikely because day 8 cells and memory T cells were assayed in parallel; no loss of day 8 T cells was observed even though these cells may be more likely to be removed from the cultures when the APCs were depleted, as they have a slightly higher avidity for peptide/MHC than do memory T cells (our unpublished results). It is possible that memory T cells may have become anergic or that the shut-down of cytokine production may have occurred to begin cell division or the production of cytolytic proteins. These questions may be best addressed by using peptide-tetramer staining to identify Ag-specific T cells, independent of their effector functions. This strategy has been used successfully to identify nonresponding (IFN-γ^-) LCMV-specific $CD8^+$ T cells in chronically infected mice (50) and to identify dysfunctional virus-specific T cells circulating in the liver (35).

Long-term T cell memory was maintained by T lymphocytes that, upon peptide stimulation, produced both IFN-γ and TNF-α simultaneously (Fig. 5). This was in contrast to day 8 $CD8^+$ T cells, which comprised both IFN-γ^+TNF-α^- and IFN-γ^+TNF-α^+ populations. The mechanisms that regulate these differing patterns of cytokine production in the two $CD8^+$ T cell populations remain obscure and are under investigation. Direct ex vivo analysis of cytokine expression patterns from 4 to 300 days p.i. demonstrated that differentiation from an activated phenotype to a memory phenotype was completed within 30–60 days after viral challenge. Following reinfection of LCMV-immune mice with virus, memory T cells and their progeny dominate the antiviral immune response (9). Interestingly, our study shows that within 5 days after secondary infection, virus-specific cytokine profiles changed from a memory phenotype to an activated phenotype similar to that observed during the primary response (Fig. 5). This demonstrates that memory T cells (or their progeny) can alter their preset Ag-specific cytokine expression patterns. The induction of IFN-γ^+TNF-α^- T cells may protect the host against TNF-α-mediated toxicity during the early stages of the secondary infection. Alternatively, the IFN-γ^+TNF-α^- subpopulation may represent T cells that are in the process of being purged from the system and are simply losing the ability to produce both cytokines as they undergo activation-induced cell death. Experiments are under way to distinguish between these possibilities.

The identification of surface markers that discriminate between naive T cells and activated/memory T cells has been instrumental in the characterization of T cell-mediated immune responses. A recent study has used transgenic technology to mark memory T cell precursors (51) but, until now, no markers had been identified which specifically distinguished activated T cells from memory T cells in normal mice. Our analysis of activation markers on virus-specific T cells revealed that both day 8 and memory T cells were uniformly LFA-1^{high}, $CD2^{high}$, and VLA-4^{high} (data not shown). We chose LFA-1 for further analysis because it is a commonly used and well-characterized marker of T cell activation/memory (11). At all time points studied (day 4–300 p.i.), virus-specific T cells expressed high levels of LFA-1. In contrast to constitutively high LFA-1 expression, we found that CD8 expression levels varied considerably during the course of infection; high CD8 levels were observed at 4–6 days p.i. followed by greatly decreased CD8 levels at 8–15 days p.i. (Fig. 5). By 60 days p.i., CD8 levels on the majority of virus-specific memory T cells reverted to high levels (Fig. 5). Furthermore, we demonstrated that CD8 expression was greatly reduced at 5–8 days following secondary infection, indicating that a transient reduction in CD8 levels occurs regardless of whether the responding cells were derived from a naive T cell

population or from a memory T cell pool. The observed down-regulation of CD8 expression is not limited to LCMV infection; we found a similar degree of CD8 down-regulation following recombinant vaccinia virus infection in which ~50% of $NP_{118-126}$-specific $CD8^+$ T cells have reduced levels of CD8 expression at 8 days p.i. (data not shown). Down-regulated CD8 expression also occurs on a substantial population of splenic T cells at 8 days after infection with either LCMV, influenza, vaccinia, or vesicular stomatitis virus (4, 5, 15, 37, 44), although these findings were not discussed. Recent evidence indicates that, if mature $CD8^+$ T cells do not simultaneously engage their TCR and CD8 coreceptor, the cells down-regulate CD8 expression and eventually die by apoptosis (52). Thus, CD8 down-regulation may be a mechanism for purging the immune system of excess $CD8^+$ T cells after resolving acute infection. This hypothesis corresponds well with our data showing that CD8 down-regulation occurs before progression into the memory phase, after which virus-specific T cells expressing high levels of CD8 are maintained at nearly steady-state levels (4, 9).

When first discovered, CTLA-4 was considered an activation Ag since it shared homology with CD28, and anti-CTLA-4 Abs were found to act synergistically with anti-CD28 Abs in promoting T cell activation (53). However, more recent studies suggest that CTLA-4 expression has an inhibitory role in T cell activation (54) and may abrogate cell cycle progression (55, 56). It is thought that CTLA-4 may exert its inhibitory effect by interfering with TCR signal transduction (57) or by binding to the B7 ligand, thus preventing CD28-mediated T cell activation. Mice deficient in CTLA-4 succumb within 2–4 wk of birth to a massive lymphoid expansion (58, 59), which appears to be driven by endogenous Ag, since $CTLA\text{-}4^-$ cells bearing transgenic TCRs do not expand to the same extent (60). The role of CTLA-4 expression during acute viral infection is unclear. Adoptive transfer/challenge experiments using TCR transgenic CTLA-4-deficient $CD8^+$ T cells have shown that these cells respond normally to viral infection and do not demonstrate unrestrained lymphoproliferation upon Ag stimulation, but the interpretation of these results is complicated by the rejection of the transgenic cells at ~2 wk after transfer due to mismatched minor histocompatibility loci (61). In our studies, we found CTLA-4 expression to be highest during the early stages of the immune response (days 4–6 p.i.), at which time the LCMV-specific $CD8^+$ T cells are rapidly expanding and have an approximate division rate of 6–8 h (4). From 5 to 8 days p.i., most of the infectious virus has been cleared and the doubling time correspondingly decreases to 24–30 h. Although CTLA-4 levels were declining by 8 days p.i. (Fig. 6), expression remained highest in the blasting cells (data not shown), consistent with the hypothesis that CTLA-4 is important during T cell expansion. We are now conducting experiments to determine whether CTLA-4 expression is playing a positive (62, 63) or negative (54) role in virus-specific T cell expansion in vivo. In either case, our study provides convincing evidence that CTLA-4 expression, in combination with CD8 and LFA-1 levels, is a useful phenotypic marker for distinguishing between activated T cells and memory T cells.

In this study, we analyzed virus-specific T cell responses directly ex vivo without extensive in vitro manipulation and in a nontransgenic model system. This allowed the function and phenotype of activated and memory T cells to be directly compared during both primary and secondary virus infections. In addition to demonstrating similar on/off regulation of cytokine production, these analyses show that CD8, LFA-1, and CTLA-4 expression levels can be used in combination to readily distinguish between naive, activated, and memory T cell populations.

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References

1. Webster, A. D. 1994. Virus infections in primary immunodeficiency. *J. Clin. Pathol. 47:965.*
2. Klavinskis, L. S., M. B. A. Oldstone, and J. L. Whitton. 1989. Designing vaccines to induce cytotoxic T lymphocytes: protection from lethal viral infection. In *Vaccines 89: Modern Approaches to New Vaccines Including Prevention of AIDS.* F. Brown, R. Chanock, H. Ginsberg, and R. Lerner, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, p. 485.
3. Whitton, J. L., and M. B. A. Oldstone. 1996. The immune response to viruses. In *Fields' Virology,* 3rd Ed. B. N. Fields, D. M. Knipe, and P. M. Howley, eds. Raven Press, New York, p. 345.
4. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity 8:177.*
5. Butz, E. A., and M. J. Bevan. 1998. Massive expansion of antigen-specific $CD8^+$ T cells during an acute virus infection. *Immunity 8:167.*
6. Zarozinski, C. C., and R. M. Welsh. 1997. Minimal bystander activation of CD8 T cells during the virus-induced polyclonal T cell response. *J. Exp. Med. 185: 1629.*
7. Green, D. R., and D. W. Scott. 1994. Activation-induced apoptosis in lymphocytes. *Curr. Opin. Immunol. 6:476.*
8. Russell, J. H. 1995. Activation-induced death of mature T cells in the regulation of immune responses. *Curr. Opin. Immunol. 7:382.*
9. Lau, L. L., B. D. Jamieson, T. Somasundaram, and R. Ahmed. 1994. Cytotoxic T-cell memory without antigen. *Nature 369:648.*
10. Demkowicz, W. E. J., R. A. Littaua, J. Wang, and F. A. Ennis. 1996. Human cytotoxic T-cell memory: long-lived responses to vaccinia virus. *J. Virol. 70: 2627.*
11. Andersson, E. C., J. P. Christensen, A. Scheynius, O. Marker, and A. R. Thomsen. 1995. Lymphocytic choriomeningitis virus infection is associated with long-standing perturbation of LFA-1 expression on $CD8^+$ T cells. *Scand. J. Immunol. 42:110.*
12. McFarland, H. I., S. R. Nahill, J. W. Maciaszek, and R. M. Welsh. 1992. CD11b (Mac-1): a marker for $CD8^+$ cytotoxic T cell activation and memory in virus infection. *J. Immunol. 149:1326.*
13. Budd, R. C., J. C. Cerottini, C. Horvath, C. Bron, T. Pedrazzini, R. C. Howe, and H. R. Macdonald. 1987. Distinction of virgin and memory T lymphocytes: stable acquisition of the Pgp-1 glycoprotein concomitant with antigenic stimulation. *J. Immunol. 138:3120.*
14. Tripp, R. A., S. Hou, and P. C. Doherty. 1995. Temporal loss of the activated L-selectin-low phenotype for virus-specific $CD8^+$ memory T cells. *J. Immunol. 154:5870.*
15. Galvan, M., K. Murali-Krishna, L. L. Ming, L. Baum, and R. Ahmed. 1998. Alterations in cell surface carbohydrates on T cells from virally infected mice can distinguish effector/memory $CD8^+$ T cells from naive cells. *J. Immunol. 161:641.*
16. Sprent, J. 1994. T and B memory cells. *Cell 76:315.*
17. Ahmed, R., and D. Gray. 1996. Immunological memory and protective immunity: understanding their relation. *Science 272:54.*
18. Dutton, R. W., S. L. Swain, and L. M. Bradley. 1999. The generation and maintenance of memory T and B cells. *Immunol. Today 20:291.*
19. Zinkernagel, R. M., and P. C. Doherty. 1979. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. *Adv. Immunol. 27:51.*
20. Oldstone, M. B. A., R. Ahmed, J. Byrne, M. J. Buchmeier, Y. Riviere, and P. Southern. 1985. Virus and immune responses: lymphocytic choriomeningitis virus as a prototype model of viral pathogenesis. *Br. Med. Bull. 41:70.*
21. Whitton, J. L. 1990. Lymphocytic choriomeningitis virus CTL. *Semin. Virol. 1:257.*
22. Campbell, I. L., M. V. Hobbs, P. Kemper, and M. B. A. Oldstone. 1994. Cerebral expression of multiple cytokine genes in mice with lymphocytic choriomeningitis. *J. Immunol. 152:716.*
23. Su, H. C., L. P. Cousens, L. D. Fast, M. K. Slifka, R. D. Bungiro, R. Ahmed, and C. A. Biron. 1998. $CD4^+$ and $CD8^+$ T cell interactions in IFN-γ and IL-4 responses to viral infections: requirements for IL-2. *J. Immunol. 160:5007.*
24. Varga, S. M., and R. M. Welsh. 1998. Detection of a high frequency of virus-specific $CD4^+$ T cells during acute infection with lymphocytic choriomeningitis virus. *J. Immunol. 161:3215.*
25. Whitmire, J. K., M. S. Asano, K. Murali-Krishna, M. Suresh, and R. Ahmed. 1998. Long-term CD4 Th1 and Th2 memory following acute lymphocytic choriomeningitis virus infection. *J. Virol. 72:8281.*
26. Slifka, M. K., F. Rodriguez, and J. L. Whitton. 1999. Rapid on/off cycling of cytokine production by virus-specific $CD8^+$ T cells. *Nature 401:76.*
27. Ahmed, R., A. Salmi, L. D. Butler, J. M. Chiller, and M. B. A. Oldstone. 1984. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice: role in suppression of cytotoxic T lymphocyte response and viral persistence. *J. Exp. Med. 160:521.*
28. Byrne, J. A., and M. B. A. Oldstone. 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus in vivo. *J. Virol. 51:682.*

29. Moskophidis, D., S. P. Cobbold, H. Waldmann, and F. Lehmann-Grube. 1987. Mechanism of recovery from acute virus infection: treatment of lymphocytic choriomeningitis virus-infected mice with monoclonal Abs reveals that Lyt-2$^+$ T lymphocytes mediate clearance of virus and regulate the antiviral Ab response. *J. Virol. 61:1867.*
30. Fung-Leung, W. P., T. M. Kundig, R. M. Zinkernagel, and T. W. Mak. 1991. Immune response against lymphocytic choriomeningitis virus infection in mice without CD8 expression. *J. Exp. Med. 174:1425.*
31. Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature 369:31.*
32. Walsh, C. M., M. Matloubian, C. C. Liu, R. Ueda, C. G. Kurahara, J. L. Christensen, M. T. Huang, J. D. Young, R. Ahmed, and W. R. Clark. 1994. Immune function in mice lacking the perforin gene. *Proc. Natl. Acad. Sci. USA 91:10854.*
33. Whitton, J. L., A. Tishon, H. Lewicki, J. R. Gebhard, T. Cook, M. S. Salvato, E. Joly, and M. B. A. Oldstone. 1989. Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity. *J. Virol. 63:4303.*
34. Sourdive, D. J., K. Murali-Krishna, J. D. Altman, A. J. Zajac, J. K. Whitmire, C. Pannetier, P. Kourilsky, B. Evavold, A. Sette, and R. Ahmed. 1998. Conserved T cell receptor repertoire in primary and memory CD8 T cell responses to an acute viral infection. *J. Exp. Med. 188:71.*
35. Belz, G. T., J. D. Altman, and P. C. Doherty. 1998. Characteristics of virus-specific CD8$^+$ T cells in the liver during the control and resolution phases of influenza pneumonia. *Proc. Natl. Acad. Sci. USA 95:13812.*
36. Lalvani, A., R. Brookes, S. Hambleton, W. J. Britton, A. V. Hill, and A. J. McMichael. 1997. Rapid effector function in CD8$^+$ memory T cells. *J. Exp. Med. 186:859.*
37. Slifka, M. K., J. K. Whitmire, and R. Ahmed. 1997. Bone marrow contains virus-specific cytotoxic T lymphocytes. *Blood 90:2103.*
38. Opferman, J. T., B. T. Ober, and P. G. Ashton-Rickardt. 1999. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science 283:1745.*
39. Cho, B. K., C. Wang, S. Sugawa, H. N. Eisen, and J. Chen. 1999. Functional differences between memory and naive CD8 T cells. *Proc. Natl. Acad. Sci. USA 96:2976.*
40. Selin, L. K., and R. M. Welsh. 1997. Cytolytically active memory CTL present in lymphocytic choriomeningitis virus-immune mice after clearance of virus infection. *J. Immunol. 158:5366.*
41. Ehl, S., P. Klenerman, P. Aichele, H. Hengartner, and R. M. Zinkernagel. 1997. A functional and kinetic comparison of antiviral effector and memory cytotoxic T lymphocyte populations in vivo and in vitro. *Eur. J. Immunol. 27:3404.*
42. Bachmann, M. F., M. Barner, A. Viola, and M. Kopf. 1999. Distinct kinetics of cytokine production and cytolysis in effector and memory T cells after viral infection. *Eur. J. Immunol. 29:291.*
43. Griffiths, G. M. 1997. Protein sorting and secretion during CTL killing. *Semin. Immunol. 9:109.*
44. Flynn, K. J., G. T. Belz, J. D. Altman, R. Ahmed, D. L. Woodland, and P. C. Doherty. 1998. Virus-specific CD8$^+$ T cells in primary and secondary influenza pneumonia. *Immunity 8:683.*
45. Vassalli, P. 1992. The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol. 10:411.*
46. Hayden, F. G., R. Fritz, M. C. Lobo, W. Alvord, W. Strober, and S. E. Straus. 1998. Local and systemic cytokine responses during experimental human influenza A virus infection: relation to symptom formation and host defense. *J Clin. Invest. 101*:643.
47. Masuda, N., S. Negoro, K. Takeda, N. Kurata, T. Kuwabara, S. Kobayashi, and M. Fukuoka. 1998. Interleukin 6 is a cause of flu-like symptoms in treatment with a deoxycytidine analogue. *Br. J Cancer 78:388.*
48. Selin, L. K., S. M. Varga, I. C. Wong, and R. M. Welsh. 1998. Protective heterologous antiviral immunity and enhanced immunopathogenesis mediated by memory T cell populations. *J. Exp. Med. 188:1705.*
49. Campbell, I. L. 1995. Neuropathogenic actions of cytokines assessed in transgenic mice. *Int. J. Dev. Neurosci. 13:275.*
50. Zajac, A. J., J. N. Blattman, K. Murali-Krishna, D. D. Sourdive, M. Suresh, J. D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J. Exp. Med. 188:2205.*
51. Jacob, J., and D. Baltimore. 1999. Modeling T-cell memory by genetic marking of memory T cells in vivo. *Nature 399:593.*
52. Pestano, G. A., Y. Zhou, L. A. Trimble, J. Daley, G. F. Weber, and H. Cantor. 1999. Inactivation of misselected CD8 T cells by *CD8* gene methylation and cell death. *Science 284:1187.*
53. Linsley, P. S., J. L. Greene, P. Tan, J. Bradshaw, J. A. Ledbetter, C. Anasetti, and N. K. Damle. 1992. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J. Exp. Med. 176:1595.*
54. Thompson, C. B., and J. P. Allison. 1997. The emerging role of CTLA-4 as an immune attenuator. *Immunity 7:445.*
55. Krummel, M. F., and J. P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J. Exp. Med. 183:2533.*
56. Walunas, T. L., C. Y. Bakker, and J. A. Bluestone. 1996. CTLA-4 ligation blocks CD28-dependent T cell activation. *J. Exp. Med. 183:2541.*
57. Lee, K. M., E. Chuang, M. Griffin, R. Khattri, D. K. Hong, W. Zhang, D. Straus, L. E. Samelson, C. B. Thompson, and J. A. Bluestone. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science 282:2263.*
58. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity 3:541.*
59. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. *Science 270: 985.*
60. Waterhouse, P., M. F. Bachmann, J. M. Penninger, P. S. Ohashi, and T. W. Mak. 1997. Normal thymic selection, normal viability and decreased lymphoproliferation in T cell receptor-transgenic CTLA-4-deficient mice. *Eur. J. Immunol. 27: 1887.*
61. Bachmann, M. F., P. Waterhouse, D. E. Speiser, K. McKall-Faienza, T. W. Mak, and P. S. Ohashi. 1998. Normal responsiveness of CTLA-4-deficient anti-viral cytotoxic T cells. *J. Immunol. 160:95.*
62. Liu, Y. 1997. Is CTLA-4 a negative regulator for T-cell activation? *Immunol. Today 18*:569.
63. Zheng, P., Y. Wu, Y. Guo, C. Lee, and Y. Liu. 1998. B7-CTLA4 interaction enhances both production of antitumor cytotoxic T lymphocytes and resistance to tumor challenge. *Proc. Natl. Acad. Sci. USA 95:6284.*