

Long-lived virus-reactive memory T cells generated from purified cytokine-secreting T helper type 1 and type 2 effectors

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Many vaccination strategies and immune cell therapies aim at increasing the numbers of memory T cells reactive to protective antigens. However, the differentiation lineage and therefore the optimal generation conditions of CD4 memory cells remain controversial. Linear and divergent differentiation models have been proposed, suggesting CD4 memory T cell development from naive precursors either with or without an effector-stage intermediate, respectively. Here, we address this question by using newly available techniques for the identification and isolation of effector T cells secreting effector cytokines. In adoptive cell transfers into normal, nonlymphopenic mice, we show that long-lived virus-specific memory T cells can efficiently be generated from purified interferon γ -secreting T helper (Th) type 1 and interleukin (IL)-4- or IL-10-secreting Th2 effectors primed *in vitro* or *in vivo*. Importantly, such effector-derived memory T cells were functional in viral challenge infections. They proliferated vigorously, rapidly modulated IL-7 receptor expression, exhibited partial stability and flexibility of their cytokine patterns, and exerted differential effects on virus-induced immunopathology. Thus, cytokine-secreting effectors can evade activation-induced cell death and develop into long-lived functional memory cells. These findings demonstrate the efficiency of linear memory T cell differentiation and encourage the design of vaccines and immune cell therapies based on differentiated effector T cells.

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Immunological memory, defined as faster and more vigorous responses of antigen-primed hosts, is a key characteristic of the adaptive immune system in vertebrates (1). Activation of naive CD4 T cells by antigen induces proliferation and the formation of large numbers of effectors and smaller numbers of memory cells (1–5). However, the lineage relationship of CD4 effector and memory T cells remains controversial (1, 5–8). It has long been debated whether CD4 memory cells can arise from naive precursors either with or without an effector-stage intermediate (9, 10). The corresponding models are termed linear or divergent differentiation models, respectively (1, 5, 6). In the latter case, the effector stage is assumed to be a terminal differentiation stage with the effector cells programmed to die (1, 4–6, 10).

The lineage relationship of CD4 effector and memory T cells has remained difficult to analyze, mostly due to the lack of reliable cell surface markers discriminating between effector and memory stages. Yet, the secretion of effector cytokines constitutes a functional hallmark of effectors (1). Upon antigen activation, naive CD4 cells can differentiate into distinct functional subsets characterized by differential cytokine patterns. Th1 populations produce IFN- γ , whereas Th2 populations secrete IL-4, IL-5, IL-13, and IL-10. Importantly, within short-term *in vitro*-differentiated or *in vivo*-generated Th1 and Th2 populations, considerable heterogeneity exists with regard to the production of the respective effector cytokines at the single-cell level (11). This characteristic prevented a clear answer to the question of whether cytokine-secreting effectors can survive the effector stage and develop into

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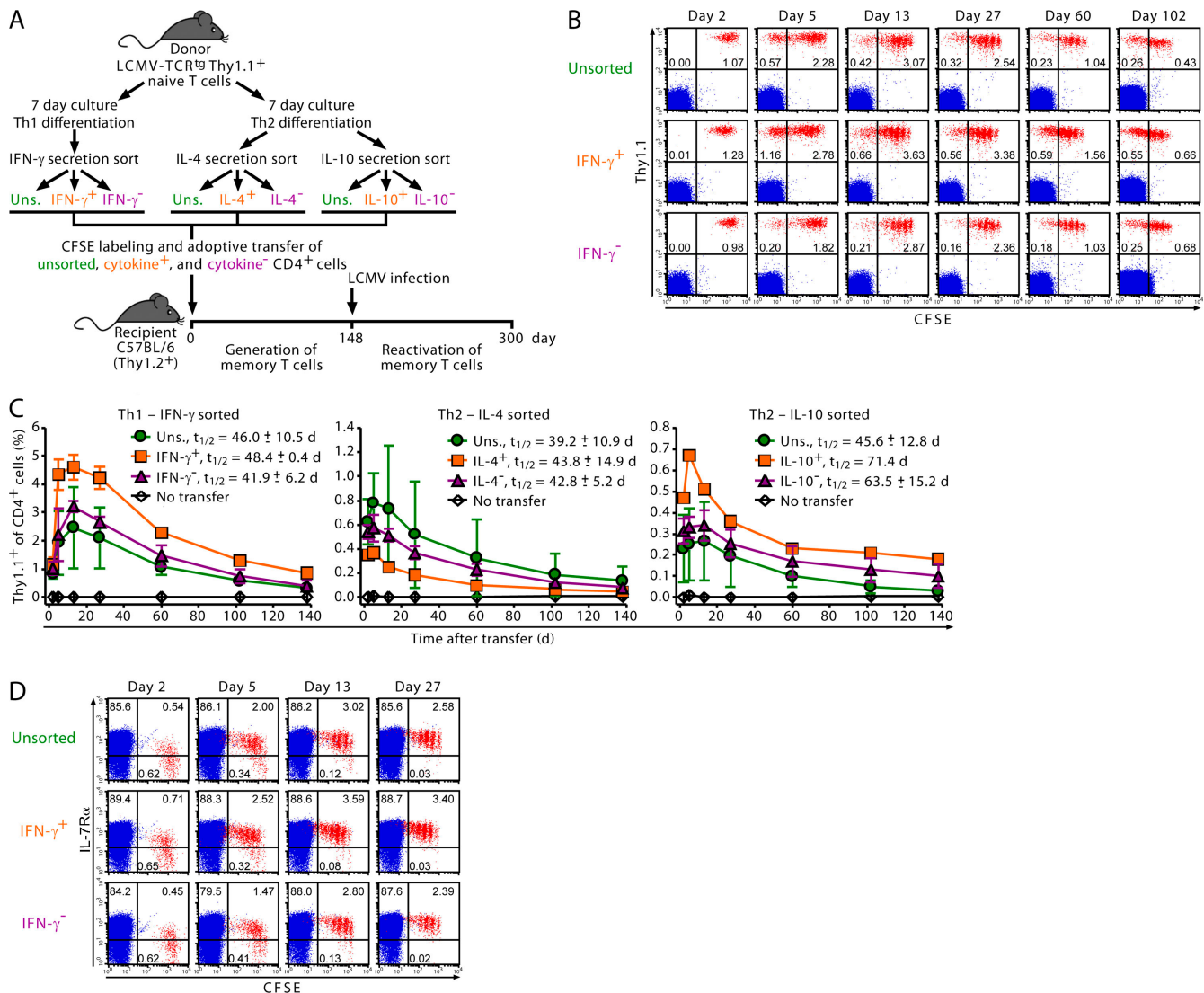


Figure 1. Generation of memory T cells from purified cytokine-secreting Th1 and Th2 effectors. (A) Experimental scheme. Naive LCMV-specific CD4⁺Thy1.1⁺ cells were differentiated toward Th1 or Th2 for 7 d. Cytokine-secreting and -nonsecreting cells were identified and separated by cytometric cytokine secretion assays. Unsorted Th1 and Th2 cells and sorted cytokine⁺ and cytokine⁻ cells were labeled with CFSE, and 2×10^6 cells were transferred into normal, previously untreated C57BL/6 mice. (B) At the indicated time points, the frequencies and CFSE dilution of transferred, Th1-derived Thy1.1⁺ cells within the peripheral CD4⁺ population were determined by FACS (CD4⁺Thy1.1⁺ donor cells in red, CD4⁺Thy1.1⁻ recipient cells in blue). (C) Mean frequencies $\pm$ SD of transferred Thy1.1⁺ cells within peripheral CD4⁺ cells are plotted, and $t_{1/2} \pm$ SDs are indicated. Control mice (no transfer) had received buffer only. Data are representative of two to three independent experiments, each with two to nine mice per group and time point (IL-10⁺ group, one mouse per experiment). (D) At the indicated time points after cell transfer, IL-7R α expression and CFSE dilution were analyzed within gated CD4⁺Thy1.1⁺ donor cells (red) and CD4⁺Thy1.1⁻ recipient cells (blue). Sorted IL-4⁺ or IL-10⁺ Th2 cells and their cytokine⁻ counterparts showed similar IL-7R α regulation (not depicted).

long-lived memory cells, or whether memory cells rather arise from the cytokine-nonsecreting cells within the heterogeneous populations (9, 10). The question is of particular importance because novel immune cell therapies of malignant and infectious diseases in humans focus on the isolation and adoptive transfer of antigen-specific CD4 and CD8 effector T cells identified by antigen-induced secretion of effector cytokines (12). To clarify this issue, here we have purified cytokine-secreting Th1 and Th2 effectors and their nonsecreting counterparts for adoptive transfer into normal mice, followed by the analysis of memory cell formation in vivo (13).

Previous studies in T cell-deficient mice had shown that antigen and MHC class II molecules were not required to sustain adoptively transferred Th1 and Th2 populations (9, 14–16). However, the recipients' T cell deficiency could have favored the generation of long-lived cells in unphysiological ways, including the lack of competition with endogenous T cells for survival signals such as cytokines (7, 8). Moreover, the functional properties of memory T cells maintained in the absence of MHC class II interactions were partly impaired upon antigen reencounter (17). Therefore, we have used normal, nonlymphopenic mice as recipients and tested the functional

potential of effector-derived Th1 and Th2 memory cells by exposure to cognate antigen in lymphocytic choriomeningitis virus (LCMV) infections. Furthermore, we have analyzed the stability and flexibility of memory Th1 and Th2 cytokine patterns in viral challenge infections and assessed the impact of memory T cell reactivation on LCMV-induced immunopathology. Our results in normal mice show that purified cytokine-secreting Th1 and Th2 effectors can develop into long-lived memory cells with distinct functional properties *in vivo*, providing direct evidence for the concept of linear memory T cell differentiation.

RESULTS AND DISCUSSION

Generation of memory T cells from purified cytokine-secreting Th1 and Th2 effectors

To investigate whether CD4 effector T cells can survive the effector stage, we purified CD4 effectors secreting effector cytokines for adoptive transfer and analysis of memory cell formation *in vivo* (Fig. 1 A) (13). Control experiments demonstrated that the reagents used for the purification of cytokine-secreting cells did not affect the survival and maintenance of the cells after adoptive transfer (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20071855/DC1>). To generate T cell populations containing cytokine-producing effectors, naive LCMV-specific CD4⁺Thy1.1⁺ cells were stimulated under Th1- or Th2-differentiating conditions. Within the resulting populations, production of the respective effector cytokines is heterogeneous at the single-cell level (11). Therefore, IFN- γ -secreting Th1 and IL-4- or IL-10-secreting Th2 effectors and their nonsecreting counterparts were identified and sorted by cytometric cytokine secretion assays (Fig. S2) (18). Sorted populations were labeled with CFSE to assess cell divisions after adoptive transfer into previously untreated normal mice. In all recipients, the transferred Thy1.1⁺ cells and/or their progeny were clearly detectable during the next 138 d (Fig. 1, B and C). Sorted cytokine-secreting and -nonsecreting cells showed similar proliferation, half-lives, and persistence over time (Fig. 1 C). Thus, cytokine-secreting Th1 and Th2 effectors gave rise to long-lived memory cells as efficiently as did their respective cytokine-nonsecreting counterparts.

Survival and homeostatic proliferation of CD4 memory cells depend on IL-7R signals (7, 8). At day 2 after transfer, a large proportion of the transferred CD4⁺Thy1.1⁺ cells expressed only low levels of IL-7R α chain (IL-7R α), the activation-regulated subunit of the IL-7R complex (Fig. 1 D). By day 5, however, the vast majority of the transferred cells were characterized by high IL-7R α expression. Notably, IL-7R α levels correlated positively with the number of divisions that the cells had undergone, which is in line with a role for IL-7 in homeostatic proliferation (7, 8). Cytokine-secreting and -nonsecreting T cells showed a virtually indistinguishable pattern of IL-7R α expression over time. Thus, IL-7 signals could provide a molecular mechanism for how cytokine-secreting Th1 and Th2 effectors became long-lived and entered the memory pool.

Generation of memory T cells from long-term differentiated Th1 and Th2 effectors

To control for a potential influence of effector cell sorting on the formation of memory cells, we aimed at generating Th1 and Th2 populations consisting virtually completely of cytokine-secreting effectors. To this end, naive LCMV-specific CD4 cells were differentiated under Th1- or Th2-polarizing conditions for 3 wk. This resulted in an $\sim 98\%$ pure IFN- γ ⁺ Th1 population, within which virtually all cells also coexpressed TNF- α (Fig. 2 A). In the corresponding Th2 population, ~ 77 and 92% of the cells produced IL-4 and/or IL-10, respectively. These effector populations were adoptively transferred into normal mice. During the next 216 d, the transferred Th1 and Th2 cells persisted with very similar half-lives of 82 and 73 d, respectively, demonstrating the capacity also of long-term differentiated, highly polarized Th1 and Th2 effectors to develop into long-lived memory cells (Fig. 2 B).

Generation of effector memory T cells from purified IFN- γ -secreting effectors primed *in vivo*

To address the possibility that effectors primed *in vivo* or *in vitro* could differ in their capacity to give rise to memory cells, we adoptively transferred naive LCMV-specific CD4

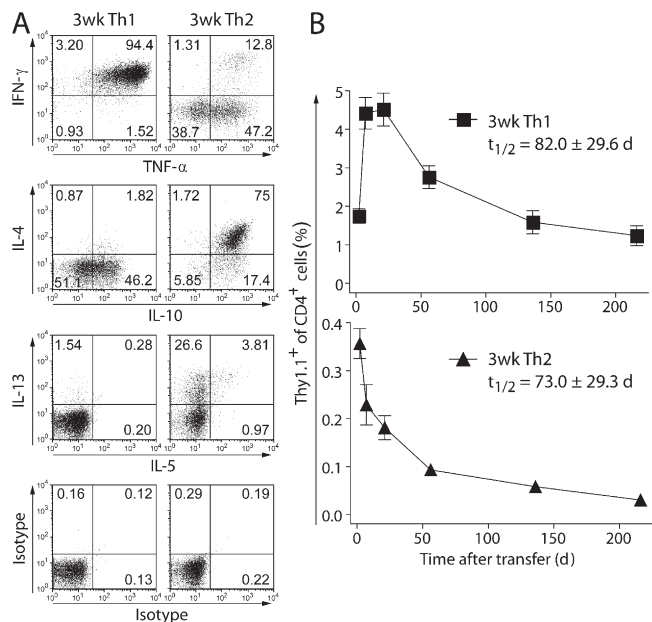


Figure 2. Generation of memory T cells from long-term differentiated Th1 and Th2 effectors. (A) Naive LCMV-specific CD4⁺Thy1.1⁺ cells were differentiated toward Th1 or Th2 for 3 wk with weekly reactivations. Cells were then restimulated with PMA/ionomycin and stained for CD4 and intracellular cytokines. The frequencies of cytokine⁺ cells within CD4⁺ cells are indicated. (B) 3-wk-differentiated Th1 cells (5×10^6) and Th2 cells (2×10^6) were transferred into normal C57BL/6 mice. At the indicated time points, the frequencies of transferred Thy1.1⁺ cells within the peripheral CD4⁺ population were determined by FACS, and the respective $t_{1/2} \pm$ SDs were calculated. Data represent the mean $\pm$ SEM of 10 mice per group and time point. Similar results were obtained in two independent experiments.

cells into normal mice and infected the recipients with LCMV. In the course of the infection, virtually all transferred Thy1.1^+ cells proliferated extensively (Fig. 3 A). Many of these cells differentiated toward Th1 and produced $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, and/or IL-2, whereas the numbers of cells producing IL-4,

IL-5, IL-13, IL-10, or IL-17 were low (Fig. 3 B). At day 10 of infection, we separated LCMV-specific $\text{IFN-}\gamma$ -secreting effectors and their nonsecreting counterparts from recipients' splenocytes for further transfer into uninfected normal mice (Fig. 3 C). During the next 101 d, sorted $\text{IFN-}\gamma$ -secreting

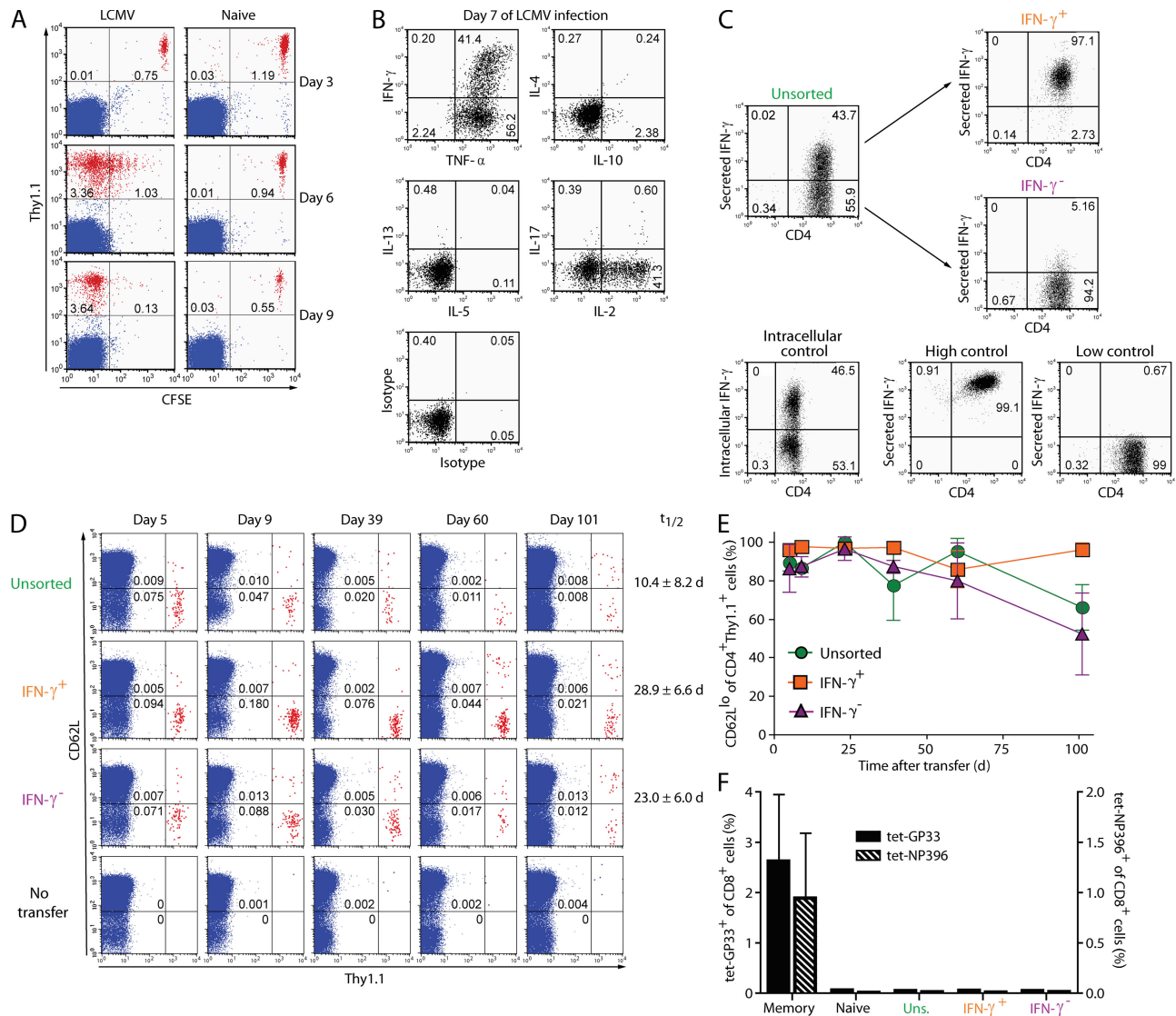


Figure 3. Generation of effector memory T cells from purified $\text{IFN-}\gamma$ -secreting effectors primed in an LCMV infection. Naive LCMV-specific $\text{CD4}^+\text{Thy1.1}^+$ cells were labeled with CFSE, and 5×10^5 cells were transferred into normal C57BL/6 mice, followed by infection of the recipients with LCMV-Armstrong. (A) At the indicated time points after infection, the frequencies and CFSE dilution of transferred Thy1.1^+ cells (red) within the peripheral CD4^+ population (blue) were determined. Control recipient mice were left uninfected (naive). (B) On day 7 of infection, splenocytes were stimulated with PMA/ionomycin, and the frequencies of cytokine⁺ cells within gated $\text{CD4}^+\text{Thy1.1}^+$ donor cells were analyzed by intracellular cytokine staining. (C) On day 10 of infection, LCMV-specific $\text{IFN-}\gamma$ -secreting effectors and their nonsecreting counterparts were identified within splenocytes of the recipients by $\text{IFN-}\gamma$ secretion assay after stimulation with GP_{64-80} peptide and were sorted by MACS. The frequencies of $\text{IFN-}\gamma$ ⁺ cells within Thy1.1^+ donor cells were measured in parallel by intracellular $\text{IFN-}\gamma$ staining (intracellular control). (D) Identical numbers of sorted $\text{IFN-}\gamma$ ⁺ and $\text{IFN-}\gamma$ ⁻ Thy1.1^+ cells as well as $\text{IFN-}\gamma$ -unsorted Thy1.1^+ cells (2×10^5) were transferred again into naive C57BL/6 mice ($n = 3-6$ mice per group). At the indicated time points, the frequencies and CD62L expression levels of transferred Thy1.1^+ cells within peripheral CD4^+ cells were determined. Mean $t_{1/2} \pm \text{SD}$ s of $\text{CD4}^+\text{Thy1.1}^+$ cells are indicated. Control mice (no transfer) had received buffer only. (E) Mean frequencies $\pm \text{SD}$ of $\text{CD62L}^{\text{low}}$ cells within $\text{CD4}^+\text{Thy1.1}^+$ donor cells were calculated after subtraction of the respective mean Thy1.1 staining background in control mice. (F) 11 d after transfer, LCMV-specific CD8 T cell responses were assessed by tetramer staining and FACS. Memory mice that had been infected with LCMV 21 d earlier and naive C57BL/6 mice served as positive and negative staining controls, respectively.

and -nonsecreting cells were maintained with very similar half-lives of 23–28.9 d without indication for a potential survival disadvantage of IFN- γ -secreting effectors (Fig. 3 D). The half-lives of IFN- γ^+ and IFN- γ^- cells primed in vivo were ~ 20 d shorter than the half-lives of sorted IFN- γ^+ and IFN- γ^- Th1 cells primed in vitro (41.9–48.4 d; Fig. 1 C), possibly reflecting enhanced cell turnover and apoptosis due to more vigorous activation by LCMV in vivo (2, 4, 19, 20). At the time of effector cell sorting, the LCMV-specific CD4 cells displayed an activated CD62L^{low}, CD44^{high}, CD45RB^{low}, and IL-7R α ^{low} phenotype (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20071855/DC1>). Remarkably, upon adoptive transfer, in all groups the vast majority of the

transferred T cells stably retained their CD62L^{low} phenotype over time (Fig. 3, D and E), revealing the preferential generation of effector memory T cells (4). To exclude at best that transfer of sorted CD4 cells had resulted in inadvertent co-transfer of LCMV infectivity, we measured the endogenous LCMV-specific CD8 T cell compartment of the recipients as the most sensitive readout of past LCMV infection. MHC class I tetramer staining failed to reveal LCMV-specific CD8 cells over naive background, showing that viral infection had not been cotransferred (Fig. 3 F). Thus, IFN- γ -secreting effectors primed in vivo in an LCMV infection efficiently gave rise to effector memory T cells maintained in an antigen-independent fashion.

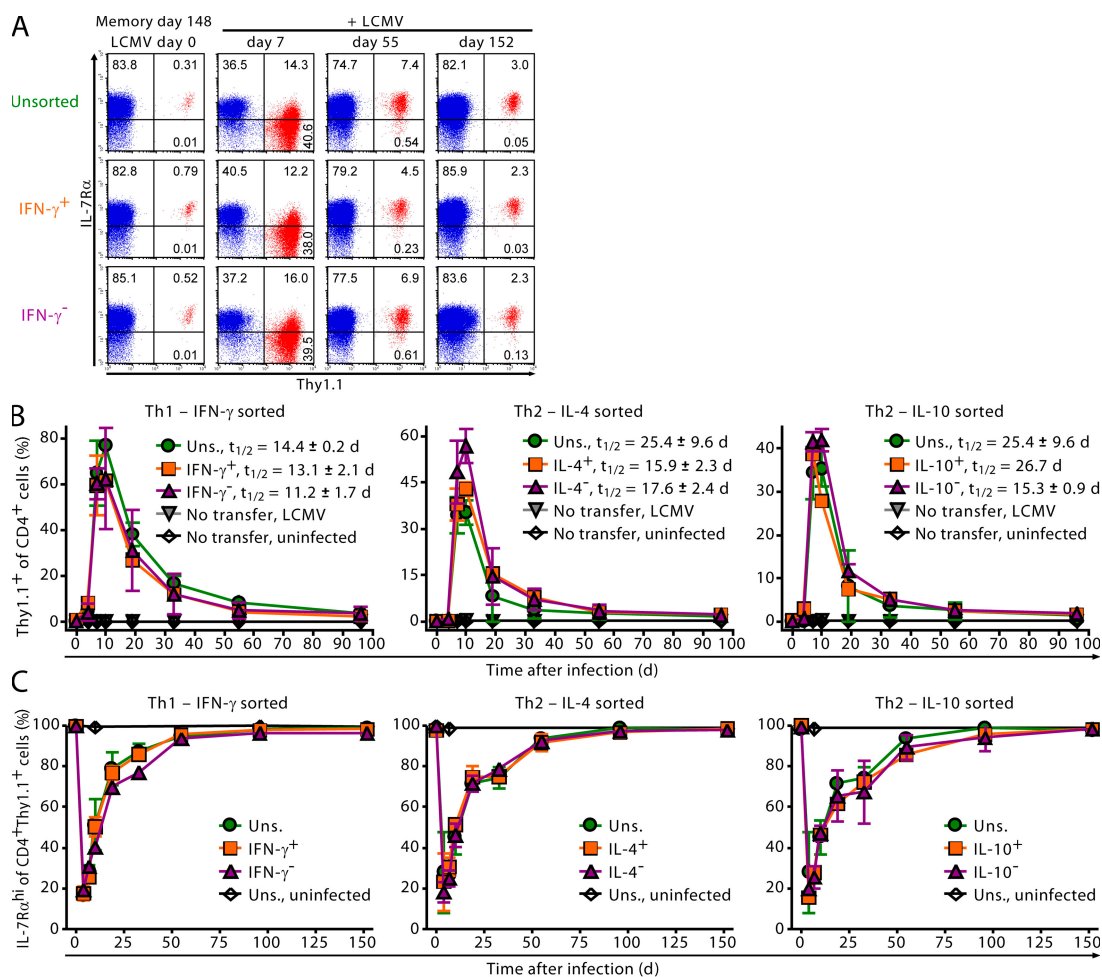
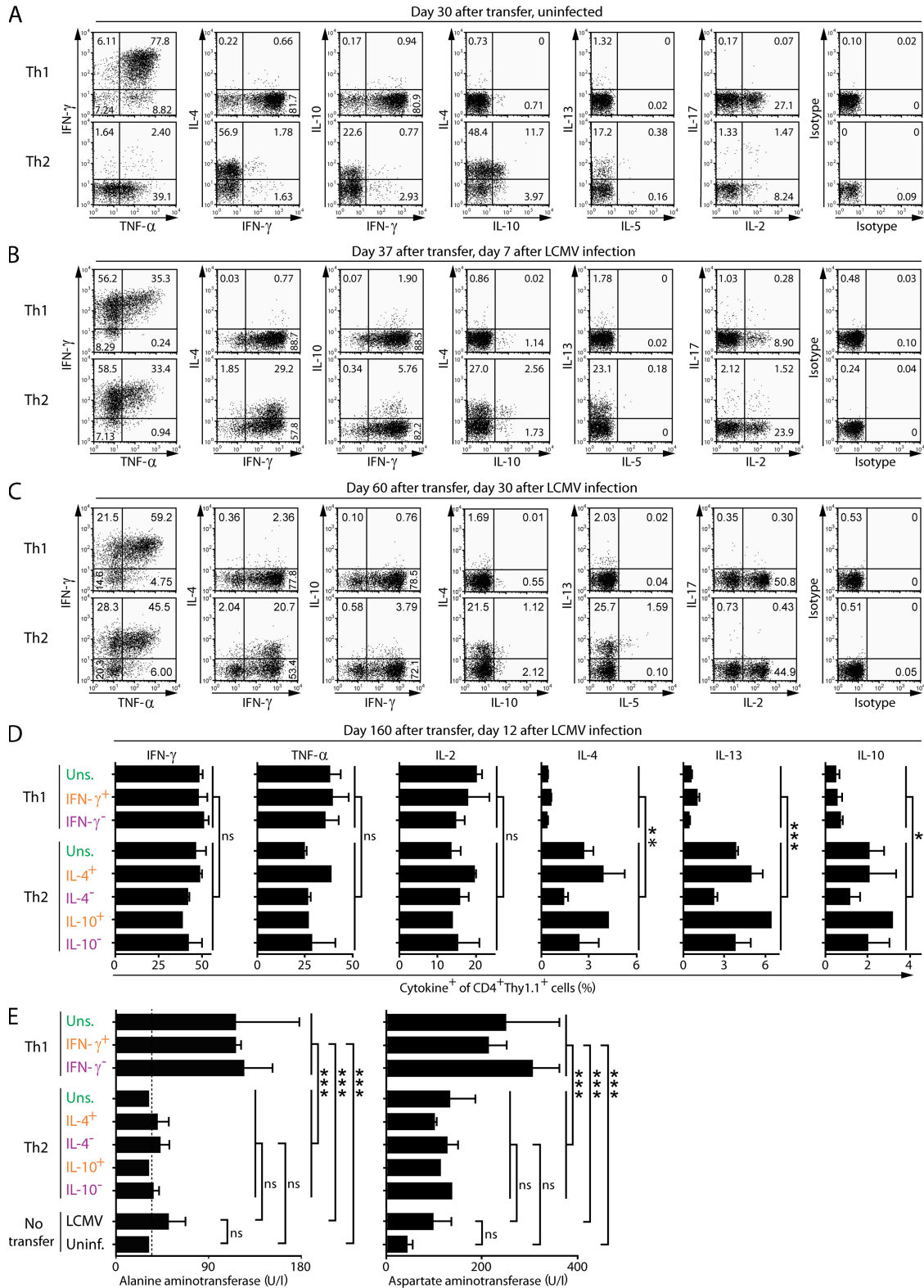


Figure 4. Unimpaired functional reactivity of Th1 and Th2 effector-derived memory cells upon virus challenge. Naive LCMV-specific CD4⁺Thy1.1⁺ cells were differentiated toward Th1 or Th2 for 7 d. Cytokine-secreting and -nonsecreting cells were sorted by cytometric cytokine secretion assays. Unsorted Th1 and Th2 cells and sorted cytokine⁺ and cytokine⁻ cells (2×10^6) were transferred into normal C57BL/6 mice and rested for 148 d to allow for their differentiation into memory cells. The recipients were then infected with LCMV-WE. (A) At the indicated time points after infection, the frequencies and IL-7R α expression levels of transferred, Th1-derived CD4⁺Thy1.1⁺ memory cells (red) within the peripheral CD4⁺ population (blue) were determined. (B) Mean frequencies $\pm$ SD of transferred Thy1.1⁺ cells within peripheral CD4⁺ cells are plotted, and $t_{1/2} \pm$ SDs are indicated. Control mice (no transfer) had received buffer only and were or were not infected with LCMV at day 148. (C) Mean frequencies $\pm$ SD of IL-7R α ^{hi} cells within CD4⁺Thy1.1⁺ donor cells were calculated. Control mice that had received unsorted Th1 or Th2 cells were left uninfected and monitored in parallel for IL-7R α expression levels. Data are representative of two to three independent experiments, each with two to six mice per group and time point (IL-10⁺ group, one mouse per experiment).



Antigen responsiveness of Th1 and Th2 effector-derived memory cells upon virus challenge

We tested the antigenic reactivity of effector-derived memory cells by LCMV challenge. When recipients were infected 148 d after adoptive transfer, LCMV-specific memory cells reacted with vigorous proliferation, followed by a protracted contraction phase after virus clearance (Fig. 4, A and B). In recipients of cytokine-secreting and -nonsecreting Th1 and Th2 cells, expansion and subsequent contraction occurred at a very similar rate, with similar kinetics and half-lives, not providing any evidence for defective *in vivo* expansion of cytokine-secreting effector T cell progeny. Notably, the frequencies of Thy1.1⁺ memory cells that persisted throughout day 152 after virus challenge ranged from 1.1 to 5.2% (mean 2.3%) of peripheral CD4 cells, which on average exceeded the frequencies before infection by a factor >10 (range 0.02–0.9%; mean 0.2%) (Fig. 4, A and B). Moreover, the kinetics and extent of IL-7R α regulation were virtually identical for all reactivated memory T cell populations (Fig. 4, A and C). Thus, the usage of IL-7 as a survival factor may provide cytokine-secreting and -nonsecreting T cell progeny with similar capabilities of entering and reentering the memory pool after repeated antigen encounter.

Functional characteristics of Th1 and Th2 effector-derived memory cells upon virus challenge

Little is known about the stability of Th1 and Th2 cytokine patterns in immune responses *in vivo*. It has been reported that *in vitro*-differentiated Th1 or Th2 populations largely maintained their respective cytokine profiles when “parked” under neutral conditions in T cell-deficient recipients (9, 14–16). Here, we extend these observations in normal, non-lymphopenic mice and at the single-cell level. Memory progeny of transferred Th1 cells continued to produce IFN- γ , whereas Th2 memory cells continued to produce IL-4 and other Th2 cytokines upon restimulation after a rest of 30 d in uninfected recipients (Fig. 5 A). However, upon LCMV infection, the Th2 cytokine production profile changed considerably at the population level. Similarly high frequencies of cells of both Th1 and Th2 origin were now found to produce IFN- γ , and this was regardless of whether the memory cells had been generated from sorted IFN- γ -, IL-4-, or IL-10-secreting or -nonsecreting cells (Fig. 5, B and D). Nevertheless, reminiscent of their original differentiation and selectively in cells from Th2 ancestors, a fraction of IFN- γ ⁺ cells coexpressed IL-4 and other Th2 cytokines. Remarkably, these partially differential cytokine patterns, including the substantial number of IL-4/IFN- γ -coexpressing cells in the Th2 cell recipients, were stably maintained even in the memory phase 30 and 60 d

after LCMV infection (Fig. 5 C and not depicted). Given the extensive proliferation of memory T cells upon LCMV infection and the associated potential for outgrowth of selected subpopulations, the pathways for the generation of such plastic IL-4⁺IFN- γ ⁺ cells cannot easily be delineated. However, together with previous studies (21–24), our findings raise the possibility that during viral challenge, some cells of Th2 origin still memorize Th2 cytokine expression while acquiring the additional capacity to produce IFN- γ .

Notably, the Th1 or Th2 differentiation history of LCMV-specific memory populations exerted differential effects on T cell-dependent liver disease (25) upon LCMV challenge. All groups of recipient mice with Th1 memory cells developed clear signs of hepatitis, as reflected in elevated serum aminotransferase activities (Fig. 5 E). Yet, none of the groups with Th2-derived memory cells exhibited aminotransferase activities exceeding the levels found in LCMV-infected control mice that had not received LCMV-specific cells, although the reactivated Th2 memory cells expanded vigorously (Fig. 4 B). Virus clearance was not affected in a differential manner by Th1- or Th2-derived memory cells, as LCMV was not detectable in the blood or spleens of any of the recipients at days 7 or 12 after infection, respectively (not depicted). Thus, the partial maintenance of Th2 cell characteristics, such as Th2 cytokine production, may play a role in limiting LCMV-induced immunopathology. Both IL-4 and IL-10 can counteract IFN- γ - and TNF-driven inflammatory processes, possibly by limiting the stimulatory capacity of APCs (26, 27). This may have implications for the activation of endogenous LCMV-specific T cells, including CD8⁺ CTLs that are most potent in mediating LCMV-triggered liver pathology in mice (25). However, other mechanisms, such as direct cytokine effects, could also be envisaged and will be addressed in future work.

The findings here show that purified cytokine-secreting Th1 and Th2 effectors can efficiently develop into long-lived, functional memory cells, providing direct evidence for the concept of linear memory T cell differentiation. This highlights the similarity of CD4 memory differentiation to analogous processes in CD8 T cell populations (28). Our data, however, do not exclude the possibility of the divergent differentiation model because sorted cytokine-nonsecreting T cells gave rise to memory cells as well. This is compatible with a model in which both linear and divergent differentiation pathways may contribute to the formation of the CD4 memory compartment. Yet, the relative importance of each of the two pathways remains to be investigated. A previous study has reported that IFN- γ ⁺ Th1 effectors tended to be short-lived after adoptive transfer, and memory cells were generated preferentially from IFN- γ [−] T cells (10). This was interpreted to indicate that the

peptide yielded very similar cytokine profiles as in A, B, and C (not depicted). Data are representative of two independent experiments. (D) Unsorted and sorted cytokine-secreting and -nonsecreting Th1 or Th2 cells polarized for 1 wk were transferred into normal C57BL/6 mice and rested for 148 d. The recipients were then infected with LCMV-WE ($n = 2$ –6 mice per group; IL-10[−] group, one mouse per experiment). 12 d later, splenocytes were stimulated with GP_{64–80} peptide. Mean frequencies $\pm$ SD of cytokine⁺ cells within CD4⁺Thy1.1⁺ donor cells were determined in duplicate by FACS. (E) 7 d after infection, serum aminotransferase activities were measured (mean $\pm$ SD). Dashed line, detection limit; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

secretion of IFN- γ demarcates a terminal differentiation stage (10), a view that may appear contradictory to the present data and conclusions. We assume that differences in the experimental systems or procedures could have led to different functional properties of the transferred effector cells, e.g., differential co-production of IL-2 or other survival factors (7, 8, 29) affecting the effectors' capacity to become long-lived. Using several experimental strategies, our study demonstrates that activation-induced cell death, which can occur in highly activated T cells (19, 20), does not selectively eliminate cytokine-secreting cells.

Importantly, Th1 and Th2 effector-derived memory cells retained functional characteristics reminiscent of their original differentiation. Thus, selected differentiation subsets of antigen-specific effector T cells, adapted to the specific application and selected according to cytokine secretion profile, could be envisaged to optimally target infectious agents or tumor cells and/or to reduce immunopathological side effects of such treatments. Such cells could be provided by immune cell therapy, or they could be induced by appropriately tailored vaccine formulations and adjuvants. Therefore, our finding of efficient generation and maintenance of effector-derived memory T cell populations may open new perspectives in preventive and cell-therapeutic strategies against infectious or malignant diseases.

MATERIALS AND METHODS

Mice. Mice transgenic (tg) for the MHC class II-restricted Smarta1 TCR (30) specific for LCMV glycoprotein (GP)_{61–80} were maintained on the C57BL/6 background under specific pathogen-free conditions and were crossed with Thy1.1⁺ B6.PL mice. C57BL/6 mice were bred at the Institute of Laboratory Animal Research, University of Zurich, and were used as recipients for adoptive cell transfers at the age of 8–12 wk. All animal experiments were performed in accordance with the Swiss law for animal protection and with permission from the Kantonale Veterinärämter.

Viruses. The LCMV strains WE and Armstrong were grown and propagated as described previously (25), and mice were infected by i.v. injection of 200 PFU in 200 μ l.

T cell activation and differentiation. Cell cultures of naive LCMV-TCR^{tg} CD4⁺Thy1.1⁺ T cells and APCs from spleens and lymph nodes were set up in complete RPMI 1640 (cRPMI) as described previously (18). GP_{64–80} peptide (Neosystem) was used at 0.5 μ g/ml. For Th1 differentiation, 3 ng/ml IL-12 and 5 μ g/ml anti-IL-4 (11B11; BD Biosciences) were added. For Th2 differentiation, 30 ng/ml IL-4 (Sigma-Aldrich) plus 5 μ g/ml anti-IL-12 (C17.8) and 10 μ g/ml anti-IFN- γ (R4-6A2; BD Biosciences) were used. Cell cultures were split on days 2 and 4. On day 7, cells were reactivated with fresh Thy1.2-depleted C57BL/6 splenocytes plus GP_{64–80} peptide, 5 ng/ml IL-2 (R&D Systems), and cytokines and anti-cytokine mAbs as above.

Identification of live IFN- γ -, IL-4-, or IL-10-secreting cells with cytometric cytokine secretion assays. Thy1.1⁺ LCMV-TCR^{tg} CD4 cells were activated either with GP_{64–80} peptide plus Thy1.2-depleted C57BL/6 splenocytes or with 5 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich). Neutralizing mAbs to IL-4, IL-12, and IFN- γ were added simultaneously at concentrations as above. After 3 h, cells were labeled with an IFN- γ -, IL-4-, or IL-10-specific high-affinity capture matrix (Miltenyi Biotec) as described previously (18). Control samples were either incubated with the respective recombinant cytokine to control the homogeneous labeling of all cells with the cytokine capture matrix (high control), or they were put immediately in ice-cold, instead of 37°C warm, medium to prevent cytokine secretion (low

control). The remaining capture matrix-labeled cells were placed in 37°C warm cRPMI. After 20 (IFN- γ assay) or 30 min (IL-4 and IL-10 assays), matrix-captured cytokine was stained on the cell surfaces with PE-coupled mAb to IFN- γ , IL-4, or IL-10 (Miltenyi Biotec).

Sorting and adoptive transfer of live IFN- γ -, IL-4-, or IL-10-secreting and –nonsecreting cells. After labeling of secreted cytokine with PE-coupled mAb, cells were incubated with anti-PE microbeads (Miltenyi Biotec). Samples of unsorted Th1 and Th2 cells were then taken aside. Cytokine⁺ and cytokine[–] cells were separated by magnetic cell sorting (MACS) as described previously (18). In cases of GP_{64–80} peptide stimulations, APCs were depleted either by using the CD4 T cell isolation kit (Miltenyi Biotec) or by using a cocktail of biotinylated mAbs to CD8, CD11b, CD11c, CD19, and NK1.1 (all from BD Biosciences), followed by anti-biotin microbeads (Miltenyi Biotec). Sort purity was controlled on a FACSCalibur (BD Biosciences). Analysis gates were set on live lymphocytes by forward and side scatter and exclusion of propidium iodide-binding particles (0.3 μ g/ml; Sigma-Aldrich). Sorted T cells (10⁷/ml) were labeled with 5 μ M CFSE (Invitrogen) in PBS for 5 min at room temperature. Cells were washed once with cRPMI and then twice with BSS. Identical numbers of Thy1.1⁺CD4⁺ cells of each sorted population were adoptively transferred into previously untreated normal C57BL/6 mice by injection into the tail vein.

FACS analysis of transferred T cells and intracellular cytokines. Transferred T cells were stained with mAbs to Thy1.1 (OX-7), CD4 (RM4-5), CD62L (MEL-14), CD44 (IM7), CD45RB (16A; all from BD Biosciences), and IL-7R α (A7R34; eBioscience). To prevent unspecific binding of mAbs, all samples were preincubated with 30 μ g/ml of blocking anti-Fc γ R2/III (2.4G2; American Type Culture Collection) and 100 μ g/ml of purified rat IgG (Biotrend). Before FACS analysis, erythrocytes were lysed and cells were fixed with FACS lysing solution (BD Biosciences). For analysis of intracellular cytokines, spleen or lymph node cells were stimulated with GP_{64–80} peptide or with PMA/ionomycin as described above. 5 μ g/ml brefeldin A (Sigma-Aldrich) was added at 2 h. After 4–5 h, cells were fixed with 2% formaldehyde (Merck) and stained with anti-CD4 and anti-Thy1.1, and with the following rat anti-mouse cytokine mAbs or isotype control mAbs in permeabilization buffer containing 0.05% saponin (Sigma-Aldrich) as described previously (18): anti-IFN- γ (XMG1.2), anti-TNF- α (MP6-XT22), anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), anti-IL-5 (TRFK5), anti-IL-10 (JES5-16E3), anti-IL-17 (TC11-18H10.1; all from BD Biosciences), and anti-IL-13 (38213.11; R&D Systems). Rat IgG1 (R3-34) and rat IgG2a (R35-95) isotype control mAbs (BD Biosciences) were used at the same concentrations as the respective anti-cytokine mAbs.

Tetramer staining. LCMV-specific CD8 T cell responses to the dominant GP- and nucleoprotein-derived epitopes (GP33 and NP396) were assessed by MHC class I tetramer staining and FACS analysis (30).

Measurement of aminotransferase activity in serum. Aminotransferase activities were determined on a Roche/Hitachi Modular Analytics according to the recommendations of the International Federation of Clinical Chemistry.

Calculation of the half-life. The half-lives ($t_{1/2}$) of memory T cells after transfer and after LCMV infection were obtained by fitting the exponentially decaying function

$$n(t) = n_0 e^{-k(t-t_0)}$$

to the declining parts of the measured time series. This function results from the assumption that the number of CD4⁺ T cells of interest declines with a constant net rate k , so that the cells will disappear eventually (2). $n(t)$ denotes the cell number at time t , for which data have been measured at distinct time points. The data have been taken into account from time point t_0 , after which the cell number declined exponentially as determined by logarithmic data plotting. The decay rate k and the initial cell number n_0 have been chosen

to obtain the best fit to the data (minimization of the squared differences as implemented in the program package Mathematica). The $t_{1/2}$ is then

$$t_{1/2} = \ln 2 / k.$$

The fits were performed separately for the time series from individual mice. Mean value and SD of $t_{1/2}$ are shown for the numbers of replicate experiments indicated (first averaging the data of replicate experiments and calculating $t_{1/2}$ from the averaged data gave very similar results).

Statistical analysis. Two groups were compared using an unpaired, two-tailed *t* test. More than two groups were compared using one-way ANOVA with Bonferroni's multiple comparison test. Data from recipients of unsorted and cytokine-sorted T cell subpopulations of either Th1 or Th2 origin were not significantly different ($P > 0.05$) and were combined for statistical analysis.

Online supplemental material. Fig. S1 shows that the cytometric cytokine secretion assay reagents do not affect the survival and long-term maintenance of the labeled cells after adoptive transfer. Fig. S2 shows the sorting of IFN- γ -secreting Th1 and IL-4- or IL-10-secreting Th2 effectors and their nonsecreting counterparts identified by cytometric cytokine secretion assays. Fig. S3 shows that LCMV-specific CD4 T cells primed by LCMV *in vivo* acquire an activated CD62L^{low}, CD44^{high}, CD45RB^{low}, and IL-7R α ^{low} phenotype. Figs. S1–S3 are available at <http://www.jem.org/cgi/content/full/jem.20071855/DC1>.

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