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Differential T cell responses to residual viral antigen prolongs CD4+ T cell contraction following the resolution of infection [1]

Ichiro Misumi[*], **Mehrdad Alirezaei**[§], **Boreth Eam**[§], **Maureen A. Su**[†,‡], **J. Lindsay Whitton**[§], and **Jason K. Whitmire**[*,†,2]

[*]Department of Genetics, UNC School of Medicine, Chapel Hill, NC 27599

[†]Department of Microbiology & Immunology, UNC School of Medicine, Chapel Hill, NC 27599

[‡]Department of Pediatrics, UNC School of Medicine, Chapel Hill, NC 27599

[§]Department of Immunology & Microbial Sciences, The Scripps Research Institute, La Jolla, CA 92037

Abstract

The contraction phase of the T cell response is a poorly understood period after the resolution of infection when virus-specific effector cells decline in number and memory cells emerge with increased frequencies. CD8+ T cells plummet in number and quickly reach stable levels of memory following acute lymphocytic choriomeningitis virus (LCMV) infection in mice. In contrast, virus-specific CD4+ T cells gradually decrease in number and reach homeostatic levels only after many weeks. Herein, we provide evidence that MHCII-restricted viral antigen persists during the contraction phase following this prototypical acute virus infection. We evaluated whether the residual antigen affected the cell division and number of virus-specific naïve and memory CD4+ T cells and CD8+ T cells. We found that naïve CD4+ T cells underwent cell division and accumulated in response to residual viral antigen for more than two months after the eradication of infectious virus. Surprisingly, memory CD4+ T cells did not undergo cell division in response to the lingering antigen, despite their heightened capacity to recognize antigen and make cytokine. In contrast to CD4+ T cells, CD8+ T cells did not undergo cell division in response to the residual antigen. Thus, CD8+ T cells ceased division within days after the infection was resolved, indicating that CD8+ T cell responses are tightly linked to endogenous processing of *de novo* synthesized virus protein. Our data suggest that residual viral antigen delays the contraction of CD4+ T cell responses by recruiting new populations of CD4+ T cells.

Introduction

Following acute LCMV infection, virus-specific T cells undergo a process of cell division and differentiation that increases their number several-thousand-fold and results in functional changes in these cells that include improved sensitivity to low amounts of antigen, changes in migratory properties, increased secretion of cytokine, and the simultaneous expression of multiple cytokines (1). The T cell response peaks around one week after infection and, soon thereafter, the virus is completely eliminated by virus-specific T cells. During the subsequent 1–2 weeks, there is a rapid decline in antiviral CD8+ T cell

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[2]Address correspondence to Jason K. Whitmire, Department of Genetics, Department of Microbiology & Immunology, UNC-Chapel Hill School of Medicine, 5062 Genetic Medicine Research Building, 120 Mason Farm Road, Chapel Hill, NC 27599-7264; Phone: 919-843-7081; Fax: 919-843-4682; jwhitmir@email.unc.edu.

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number. However, antiviral CD4+ T cells show a gradual decline in number until they reach a homeostatic level 1–2 months post infection (2–7). It is not known what accounts for the differential kinetics of the contraction phase.

Recent analyses of several acute infection models (influenza, vesicular stomatitis virus) have shown that long after the infection is resolved to levels below detection, viral material – perhaps from low-level persistent infection – stimulates T cells (8–12). For influenza infection, both CD4+ T cells (8) and CD8+ T cells (10, 11) continued to divide several weeks after acute infection, and the cell-division was restricted to virus-specific T cells. Although infectious influenza virus was undetectable by plaque assay and viral RNA was not detected by RT-PCR, a residual population of activated and memory CD8+ and CD4+ T cells were found in the lung and had undergone cell-division (8, 11, 13). The selective recruitment of virus-specific cells to divide and localize to the lung is consistent with the presence of low-level antigen long after the acute phase of infection. There is evidence that the antigen reservoir in the lung is captured and transported by respiratory dendritic cells to the draining lymph node to stimulate T cells (14). Memory CD8+ T cells that were primed in the lung draining lymph nodes are more sensitive to this antigen than cells that were primed elsewhere (15). Similarly, CD8+ T cells continued to undergo rapid cell division weeks after the resolution of acute vesicular stomatitis virus infection (9), but CD8+ T cell cell-division was not seen following *Listeria monocytogenes* infection (9), implying that the phenomenon varies according to the infection. Thus, some acute infections may result in low-grade persistent infection that cannot be detected by standard techniques. LCMV-Armstrong induces an acute infection in immune-competent mice and is resolved within 8 days by cytolytic CTL. Numerous reports show that infectious virus and viral RNA are undetectable after this time. Based on the above reports and the finding that primary CD4+ T cell responses and memory are tightly linked to antigen (16–18), we considered the possibility that the duration of the CD4+ T cell contraction phase following acute infection may be related to the persistence of viral antigen that lingers long after the resolution of the infection.

Because LCMV-specific CD4+ and CD8+ T cells differ in their rates of contraction (2), we hypothesized that the two lineages of cells recognize antigen for different lengths of time after infectious virus has been eliminated. Here, we report that antiviral CD8+ T cells do not undergo antigen-dependent cell division during the contraction or memory phases, consistent with earlier data showing that wildtype mice completely eliminate LCMV-Armstrong infection, and that long-term CD8 memory does not require antigen (19). We also show that naive virus-specific CD4+ T cells undergo limited cell division that is somewhat faster than cytokine-driven homeostatic cell division, but slower than that seen during the acute phase of infection. This persisting CD4+ T cell division occurred throughout the contraction phase but subsided soon thereafter. These data are consistent with the notion that MHCII-restricted antigen is present long after infectious virus and MHCI-restricted viral antigens are lost. This MHCII-restricted antigen selectively signals into CD4+ T cells and inflates their number over time, thus delaying the establishment of antigen-independent memory homeostasis. Interestingly, memory CD4+ T cells failed to undergo cell-division. Thus, the proliferative response of CD4+ T cells during the contraction phase is restricted to naïve cells, which may serve to diversify the pool of virus-reactive cells. The viral antigen also does not spur CD8+ T cells to undergo cell division over the same time period, which implies that the cross-presentation of viral antigen to $CD8^+$ T cells is inefficient and/or short-lived and does not drive residual $CD8^+$ T cell turnover during the contraction phase. These data show there are different *in vivo* requirements for antigenic stimulation of naïve $CD4^+$ T cells, memory $CD4^+$ T cells, and $CD8^+$ T cells.

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Materials and Methods

Mice, virus & bacteria

C57BL/6 mice were purchased from The Scripps Research Institute (TSRI) breeding facility. C57BL/6 mice congenic for Thy1.1 (B6.PL-*Thy1*^{a}/CyJ) or congenic for Ly5a (B6.SJL-*Ptprc*^{a}*Pep3*^{b}*/BoyJ*) were purchased from The Jackson Laboratory. SMARTA TCR-transgenic mice specific for the I-A^{b} LCMV epitope GP_{61-80} (20) were originally made by Dr. H. Hengartner (U. Hospital Zürich) were crossed to B6.PL-*Thy1*^{a}*/CyJ* mice to generate SMARTA/Thy1.1+ mice or to B6.SJL-*Ptprc*^{a}*Pep3*^{b}*/BoyJ* to generate SMARTA/Ly5a+ mice. P14 TCR-transgenic mice specific for the LCMV epitope GP^{33-41} (21) on the H-2^{b} background were crossed to B6.SJL-*Ptprc*^{a}*Pep3*^{b}*/BoyJ* mice to generate the P14/Ly5a+ strain or to B6.PL-*Thy1*^{a}/CyJ to generate P14/Thy1.1+ mice. OTII TCR-transgenic mice (22) specific for the I-A^{b}-restricted epitope, $ovalbumin_{323-339}$, were originally made by Dr. F. Carbone's group (U. Melbourne) and kindly provided by Dr. Beutler (TSRI) or were purchased from The Jackson Laboratory. Mice were infected by i.p. administration of 2×10^{5} plaque forming units of LCMV (Armstrong strain). Quantitation of virus in the tissues was done by plaque assay on Vero cell monolayers. To assay for virus RNA, harvested tissues were homogenized in the presence of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA), and total RNA was isolated from the aqueous phase after chloroform and isopropanol extractions. The presence of viral RNA was measured by RT-PCR using primers specific for sequences within the glycoprotein. Some mice received recombinant-*Listeria monocytogenes* that express LCMV-GP_{61-80} or OVA. These recombinant bacteria were originally made by Dr. Hao Shen (U. Pennsylvania) (23). The bacteria were grown to log-phase before titration and injection into mice. Colony forming units were determined by plating dilutions of tissue homogenates or bacterial stocks onto BHI/agar plates. All experiments were approved by the UNC Animal Care and Use Committee.

Adoptive transfers

Flow cytometry was used to determine the frequency of transgenic $CD4^{+}$ T cells ($V\alpha2^{+}V\beta8.3^{+}$) among all spleen cells in SMARTA mice or the frequency of transgenic $CD8^{+}$ T cells ($V\alpha2^{+}V\beta8.1/2^{+}$) among all spleen cells in P14 mice. OTII cells (CD4+Vα2+Vβ5.1+) were identified in recipient by flow cytometry. In the experiments that followed cell division by CFSE dilution in immune or naïve mice, $5–10\times10^{5}$ Tg cells were labeled with 5μM CFSE before transfer into the recipient mice. In some experiments, $1–3\times10^{4}$ Tg+ T cells were injected intravenously into naïve mice that were infected 1–2 days later.

Flow cytometry

Single cell suspensions of spleen cells were stained directly *ex vivo* with fluorochrome-conjugated anti-CD4 (clone RM4–5), anti-CD8 (clone 53–6.7), anti-Thy1.2 (clone 53–2.1), anti-Thy1.1 (clone HIS51), anti-CD44 (clone IM7), CD62L (clone MEL-14), anti-Ly5a (clone A20), anti-Vα2 (clone B20.1), or anti-Vβ8.3 (clone 1B3.3) that were purchased from eBioscience.com (La Jolla, CA), Biolegend (La Jolla, CA), or BD-Pharmingen (La Jolla, CA). The staining reaction was done in the presence of unlabeled antibodies against Fc-receptors to block flurochrome-conjugated antibodies from binding to FcR+ cells; "FcBlock" was purchased from BD-Pharmingen. Intracellular staining for Ki67 was performed using a kit from BD-Pharmingen. For BrdU incorporation experiments, mice were given an initial ip injection of 0.8mg BrdU and fed 0.8mg/ml BrdU in drinking water for 5–6 days. Incorporated BrdU in T cells was detected using a "BrdU flow kit" from BDbiosciences followed by flow cytometry analysis. Cells were acquired by four-color flow cytometry using a BD-FACSCalibur, and small, non-viable cells were excluded from

analysis based on forward and side scatter profile. The cytometry data were analyzed with FlowJo software Tree Star (Treestar, San Francisco).

Statistics

Statistical analyses and graphing were done with Prism software (www.graphpad.com). An unpaired two-tailed Student's t-test was employed to evaluate the significance of differences between groups.

Results

Virus-specific CD8+ T cells do not undergo cell division once the infection is resolved

When given LCMV-Armstrong, wildtype mice generate a robust cytotoxic T cell response and eliminate all infectious virus and viral RNA within approximately one week. We wished to investigate whether low-level viral antigens might persist on into the contraction phase to stimulate T cells. To determine whether LCMV-specific T cells continue to undergo rapid cell division following the resolution of this infection, naïve, LCMV-specific, TCR-transgenic CD8+ T cells from P14 mice and CD4+ T cells from TCR-transgenic SMARTA mice were mixed, CFSE-labeled, and co-transferred into 3 groups of mice: 1) naïve mice, 2) naïve mice that were subsequently given acute LCMV infection for 6 days, or 3) LCMV-immune mice at various times during the contraction and memory phases (Figure 1A). The frequency of the donor T cells in the spleen and their loss of CFSE were measured by flow cytometry 8–9 days after transfer. This methodology employs the natural ability of T cells to circulate and react to minute quantities of antigen and undergo cell division *in vivo*. This sensitive assay can identify remnants of viral material and, because the donor T cells are present in the mice for several days, there is a lengthy period for the cells to encounter and respond to antigen that is rare or transiently expressed.

Donor P14+ CD8+ T cells were identified by their expression of the congenic marker, Ly5a (Figure 1B). The donor CD8+ T cells were gated and their level of CFSE fluorescence is shown in the histograms (Figure 1B). As expected, the donor CD8+ T cells did not divide in uninfected recipients and divided extensively in mice given acute infection (d6), which had a high virus and antigen burden. No donor CD8+ T cell division was observed in immune mice that received these indicator cells at 12 days post-infection or later, and the fraction of donor cells among CD8+ T cells in these immune mice resembled that observed in the uninfected recipients (Figure 1C). These findings contrast with those seen in influenza-immunized mice (8, 11); our data indicate that continued CD8 T cell division is not a general phenomenon seen during the contraction phase or memory phase after acute virus infection. The data confirm that wildtype (B6) mice fully resolve LCMV-Armstrong: no virus is detected at these times by plaque assay and no viral RNA is detected by RT-PCR.

The above data indicate that there is minimal MHCI-restricted viral antigen from day 12 onwards that is recognizable by naïve CD8+ T cells. However, under normal circumstances, antigen-experienced cells with increased sensitivity to low quantities of antigen would be present during these times (1). Perhaps trace amounts of antigen are present during the contraction phase that stimulate antigen-experienced cells to divide but not naïve CD8+ T cells. To examine this possibility, naïve mice containing a small number of naïve P14+ CD8+ T cells were infected with LCMV-Armstrong, and the proportion of dividing donor cells was determined by intracellular staining against the nuclear antigen, Ki67 (Figure 1D). Without infection (day 0), few of the P14 CD8+ T cells were Ki67+, but 41–95% of them became Ki67+ during the virus-induced expansion phase at day 6, consistent with the rapid cell division that is known to occur during this time. At the peak of the response, only 12% of the cells had recently divided and this number dropped by day 13 (3–10%). We also used

BrdU incorporation into DNA as a measure of P14 cell proliferation. Naïve mice were engrafted with P14 cells and infected. The mice were exposed to BrdU for 6 days during the expansion phase or one week later (Figure 1E). A large proportion of cells (~60%) underwent cell division during the acute stage of infection and incorporated BrdU (day 2–8); in contrast few P14 cells were BrdU+ at day 16–21 (~10%), which is comparable to the amount of cell division in uninfected P14 mice. So like their naive counterparts, the vast majority of antigen-experienced CD8+ T cells halt cell-division within a few days after the infection has been eliminated, and from day 12 onwards, MHC1-restricted viral antigen is unavailable or insufficient to stimulate CD8+ T cell division.

Virus antigen persists for at least 40 days post-infection, in a form that can trigger division of naïve virus-specific CD4+ T cells

Some studies with influenza virus (10, 11) suggest that virus protein synthesis may continue for some time in the absence of detectable infectious virus. Thus, the CD4+ T cell proliferation observed in those models (8) could be attributed to newly synthesized secreted protein, or protein from apoptotic cells that is taken up, processed, and presented on MHCII. Our finding that $CD8^+$ T cell proliferation ends by D12 p.i. (Figure 1) indicates that LCMV protein synthesis terminates before this time. Hence, the division of CD4+ T cells at day 12 and later can be analyzed in the complete absence of infectious virus or *de novo* synthesized viral proteins.

The naïve SMARTA CD4+ T cells in the same recipient mice that contained the P14 cells described in Figure 1B & 1C were also analyzed to determine their frequency and cell division status. The donor SMARTA CD4+ T cells were characterized after being in either 1) uninfected mice for 8 days (d0), 2) acutely infected mice for 6 days, or 3) immune mice for 8 days. The donor SMARTA CD4+ T cells could be identified due to their expression of the congenic marker, Thy1.1 (Figure 2A). The responses of the CFSE-labeled SMARTA CD4+ T cells were similar to that of P14 CD8+ T cells in the uninfected mice (no division) and in the in the acutely infected day 6 mice (extensive division). However, there was a dramatic difference in their division following transfer to mice at times later than 12 days p.i. As shown in the histograms of Figure 2A, a major portion of the SMARTA CD4+ T cells underwent one or more cell divisions in mice that were in the contraction phase (day 12–20) and this ongoing capacity to drive $CD4^+$ T cell proliferation was maintained in early memory (day 20–30; day 30–40) mice. Long-term immune mice (day 515) appear to be devoid of such antigen. The cell division observed in the early-immune mice was much faster than the homeostatic cell division reported for quiescent memory T cells in immune-competent mice (24–28).

The frequency of the donor CD4+ T cells diminished with time post-infection, and this contraction of SMARTA cells occurred over a prolonged period (at least 30–40 days), mirroring the findings of several other labs for the SMARTA or endogenous CD4+ T cell responses (2–4, 29), and most easily interpreted as “delayed contraction” (Figure 2B). However, this phenomenon may better be understood as prolonged antigen-driven proliferation, because a high proportion of donor SMARTA CD4+ T cells in the early immune mice underwent cell division, and this proportion declined with time (Figure 2C). Note that the proportion of divided P14+ CD8+ T cells in the same mice did not change over time, confirming that the antigen-specific stimulatory effect is limited to CD4+ T cells. The stimulus appears to have a *functional* half-life of approximately 5–6 weeks and it stimulates some naïve CD4+ T cells up to 8 weeks. The finding that the stimulus has a 5–6-week half-life argues against homeostatic mechanisms, as IL-7 and IL-15 production are thought to be fairly constant over time.

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There was a strong correlation between the proportion of SMARTA CD4+ T cells that underwent cell division at any given time point post-infection and the overall frequency of SMARTA CD4+ T cells at that time (Figure 2D). Thus, mice containing SMARTA CD4+ T cells that underwent extensive cell division tended to have more SMARTA CD4+ T cells than mice whose donor cells underwent minimal cell-division. The early immune mice contained highly divided SMARTA CD4+ T cells that were more abundant, and this implies that the dividing cells do not undergo apoptosis but add to the overall number of cells. These data show a direct relationship between the presence of stimulatory antigen (measured by SMARTA CD4+ T cell division) and $CD4^+$ T cell numbers; as the quantity of stimulatory antigen diminishes, antiviral CD4+ T cells also decay in number until reaching a baseline of memory that does not require antigen.

CD4+ T cell division is driven by antigen

Most of the CD4+ T cell division was seen in early immune mice, during the contraction phase. This is a time when the lymphoid organs purge many virus-reactive cells, so we considered the possibility that unusual amounts of cytokines during this time selectively affect CD4+ T cells and not CD8+ T cells. If the turnover of the cells is a product of non-specific global changes, then other CD4+ T cell populations should also divide when transferred into these mice. On the other hand, if the observed changes are antigen-driven, then CD4+ T cells that are not specific for LCMV should not divide in these mice. Therefore, the previous experiments were repeated, but now with a group of non-LCMV-specific CD4+ T cells: OTII CD4+ T cells are TCR-transgenic and recognize an epitope within ovalbumin (22) and do not react to LCMV infection. Sets of mice were immunized with LCMV or left un-immunized. 12 days later, the mice received CFSE-labeled OTII CD4+ T cells, SMARTA CD4+ T cells, or P14+ CD8+ T cells. 8 days after transfer, the donor cells were identified in the spleens of the recipient mice and their level of CFSE fluorescence was quantified by flow cytometry (Figure 3A). As expected, the SMARTA CD4+ T cells underwent cell division in the immune recipients but not in the naïve hosts, and the P14+ CD8+ T cells showed minimal cell division in both recipients. OTII+ CD4+ T cells also showed minimal cell division in both the LCMV-immune and naïve recipients, yet they were capable of undergoing cell division when exposed to acute rLM-OVA infection. A large fraction of the SMARTA CD4+ T cells underwent division in the LCMV-immune hosts; whereas few OTII cells underwent cell division in LCMV-immune or naive mice (Figure 3B).

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The division status of the donor cells correlated with their overall frequency in the recipient mice: while SMARTA CD4+ T cells increased in abundance in the LCMV-immune hosts, there was no increase in the frequency of OTII CD4+ T cells or P14+ CD8+ T cells in LCMV-immune hosts compared to the non-immune hosts (Figure 3C). The OTII cells accumulated when confronted with rLM-OVA infection, which indicates that they are capable of proliferation and accumulation in response to cognate antigen. These data show that the observed cell division is restricted to antigen-specific CD4+ T cells and is not a consequence of cytokine overabundance or some other general non-specific effect of the contracting spleen.

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We followed polyclonal populations of T cells to evaluate whether they would undergo cell division in the contracting spleen. In uninfected mice, there are approximately 100 cells specific for GP_{61-80} and comparable frequencies have been noted for other specificities. Thus, the vast majority of endogenous T cells are not specific for LCMV-derived epitopes and should not respond to the residual antigen. However, if the cytokine environment in the contracting spleen non-specifically induces cell-division, then these other populations of cells should divide. Therefore, a large population of CFSE-labeled naïve splenocytes were adoptively transferred to the same 3 groups of recipients: uninfected mice, mice given acute

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LCMV infection, and LCMV-immune mice (Figure 3D). After 8 days, the loss of CFSE by the donor cells was measured. As expected, few donor cells underwent cell division in the uninfected recipient mice. A large percentage of the transferred CD4+ T cells and CD8+ T cells lost CFSE in the acutely infected mice, which reflects the proliferative response of a very small number of transferred precursor cells during the infection. However, the vast majority of donor cells are not specific for LCMV antigens and very few of them underwent cell-division in the LCMV-immune mice during the day 12–20 period. Thus, the cytokine milieu in the contracting spleen does not induce appreciable amounts of cell division among cells that are not specific for LCMV.

The functional avidity of SMARTA CD4+ T cells and endogenous CD4+ T cell populations is similar

The above data indicate that transferred naïve SMARTA CD4+ T cells undergo cell-division when in LCMV-immune mice due to persistence of viral protein antigen. Because SMARTA cells are TCR transgenic, we next evaluated whether SMARTA T cells respond like endogenous T cells. SMARTA CD4+ T cells and endogenous virus-specific CD4+ T cells increase their functional avidity and increase their capacity to produce IFNγ during primary infection with LCMV (1, 3). We evaluated whether these changes are sustained into the memory phase and impacted by residual antigen.

The SMARTA CD4+ T cells and endogenous populations of GP_{61}-specific CD4+ T cells were identified at days 8, 20, and 45 by direct staining or by intracellular cytokine staining (ICCS) (Figure 4A). When given saturating amounts of peptide (10^{-5}M), 66% of SMARTA CD4+ T cells made IFN γ at day 8 and a slightly lower percentage (55%) made IFN γ at day 45. The IFNγ+ endogenous population was identified by ICCS in the same mice; at day 8, 3.6% of CD4+ T cells were GP_{61-80}-specific, and following contraction, they accounted for 0.4% of CD4+ T cells.

As CD4+ T cells mature during the expansion phase, they increase their capacity to make cytokines on a per cell basis (1). To determine whether T cells continue to improve their ability to make cytokines after day 8, the amount of IFNγ and IL-2 made per cell was quantified by ICCS at days 8, 20, & 45 (Figure 4B). The SMARTA and endogenous CD4+ T cells made high amounts of IFN γ at day 8 (Figure 4B), and cells at day 20 and 45 made slightly lower amounts. The IL-2 production by endogenous cells was fully overlapping between days 8, 20, and 45 (Figure 4B, right) and a similar pattern was apparent for the SMARTA CD4+ T cells, indicating that there was minimal further change in the ability of CD4+ T cells to make IL-2 after day 8. Interestingly, the SMARTA CD4+ T cells tended to make more IFNγ and IL-2 at each time than did endogenous CD4+ T cells.

To determine whether T cells continue to improve their sensitivity to trace amounts of antigen, we quantified their cytokine responses to differing amounts of peptide (Figure 4C). Spleen cells from these mice were exposed to different amounts of GP_{61-80} peptide and their IFNγ production was measured by ICCS (Figure 4C, top row). SMARTA and endogenous CD4+ T cells showed overlapping sensitivity to the peptide at day 8 (Figure 4C), similar to our earlier report (1). The functional avidity of the cells remained at this level at days 20 and 45 (Figure 4D, top), implying no additional evolution in the functional avidity of CD4+ T cells after day 8. Similar analyses of T cell production of IL-2 revealed that endogenous and SMARTA CD4+ T cells showed a similar pattern: memory and day 8 cells made IL-2 in response to the same range of peptide concentrations (Figure 4C, middle row & Figure 4D, bottom), implying no further functional avidity maturation in IL-2 output, and the trend was observed when evaluating only cells that were double-positive for IFNγ and IL-2. At all times, the SMARTA cells showed slightly higher sensitivity to peptide compared to endogenous CD4+ T cells (Figure 4D), although the effect was minor.

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These data indicate that transgenic and non-transgenic polyclonal CD4+ T cells closely resemble each other in their functional maturation after LCMV: they are largely functionally mature by day 8 and these characteristics are sustained over time into memory. Thus, SMARTA CD4+ T cells can be used to accurately reflect how endogenous populations respond during the memory phase after LCMV (3). Because the functional maturation of the CD4+ T cells did not change with time after day 8 (Figure 4D), we infer that the lingering antigen does not affect these parameters. Instead, the residual antigen functions to sustain virus-specific T cell numbers.

Lingering viral antigen stimulates naïve CD4+ T cells but not memory CD4+ T cells

Others and we have shown that populations of CD4+ T cells with identical specificity compete for access to antigen and cytokine, and this competition influences how well T cells develop into memory (30–36). Competition for antigen also affects CD8+ T cell memory number and quality (37–40). Because effector and memory CD4+ T cells display increased sensitivity to antigen compared to naïve CD4+ T cells ((1) and Figure 4D), one might expect that differentiated T cells would better compete for antigen and prevent the cell-division of naïve T cells. Moreover, the effect might be greater for CD8+ T cells, since the magnitude of the endogenous CD8+ T cell response is 10-fold greater than the endogenous CD4+ T cell response. The data in Figures 1 & 2 show that transferred naïve SMARTA cells, but not naïve P14 cells, undergo cell division when in early immune mice, where there might be significant competition by inflated populations resident differentiated T cells. We next assessed whether differentiated CD4+ T cells divide during the contraction phase. Naïve SMARTA CD4+ T cells were engrafted into mice that were subsequently infected. At day 6 after infection, the vast majority of responding SMARTA cells were Ki67+ (Figure 5A); however, by day 14, few of these antigen-experienced donor cells were Ki67+. In another approach, a cohort of uninfected or LCMV-infected mice were exposed to BrdU (Figure 5B). While many SMARTA cells divided during the day 3–8 period after infection, few of the SMARTA cells incorporated BrdU during the contraction phase. By these measures, antigen-experienced SMARTA CD4+ T cells minimally divide during the contraction phase.

To directly test the effects of T cell competition for lingering antigen, we compared the cell-division of antigen-experienced CD4+ T cells and naïve T cell in the same host using a dual adoptive transfer approach (Figure 5C). To generate antigen-experienced CD4+ T cells, a small population of naïve SMARTA/Ly5a+ CD4+ T cells was transferred into mice that were subsequently infected with LCMV, and at day 12, these cells were harvested. The antigen-experienced day 12 cells were then mixed in equal proportion with naïve SMARTA/Thy1.1+ CD4+ T cells, labeled with CFSE and transferred into either naïve B6 mice or day 12 B6 mice; after 8 days the spleens of the recipient mice were harvested and the two populations were identified by FACS and analyzed for CFSE-dilution.

The representative dot plots in Figure 5D identify the naïve donor SMARTA CD4+ T cells (ovals, left dot plots) and day 12 donor SMARTA CD4+ T cells (ovals, right dot plots) in the uninfected recipients (top) and early immune mice (bottom) 8 days after transfer. The histograms are gated on the corresponding donor cells and show their level of CFSE fluorescence; the cells under the bar have undergone at least 1 round of cell-division. As expected, neither naïve SMARTA CD4+ T cells nor antigen-experienced CD4+ T cells underwent cell-division when in the same uninfected mice (top histograms), as there was no cognate antigen present to stimulate their division. Consistent with the data in Figures 2 & 3, the naïve SMARTA CD4+ T cells underwent significant cell-division in early-immune mice (bottom left histogram), most likely due to the presence of residual viral antigen. In striking contrast, the antigen-experienced cells did not undergo cell division in these same hosts (bottom right histogram). An analysis of several mice showed that ~74% of naïve SMARTA

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cells divided at least once during this period whereas only 1% of antigen-experienced SMARTA CD4+ T cells divided (Figure 5E).

ICCS was used to evaluate whether exposure to lingering antigen induced the naive SMARTA CD4+ T cells to become IFNγ producers. The naïve CD4+ T cells continued to not make IFNγ despite undergoing cell division (Figures 5F & 5G). In contrast, the day 12 CD4+ T cells vigorously made IFNγ upon ex-vivo peptide stimulation, whether they were engrafted in naïve hosts or immune hosts (Figures 5F & 5G). Thus, the residual antigen recruits naïve CD4+ T cells to undergo cell-division and accumulate but not sufficiently so to induce them to make IFNγ, although it is plausible that the naïve CD4+ T cells made another cytokine instead.

A particular concern with the above results was whether the antigen-experienced T cells that might have divided were selectively rejected by the recipients, perhaps as an unlikely artifact related to the choice of congenic markers in the experiment. Therefore, we reversed the congenic markers in another experiment, so that the naïve SMARTA cells were Ly5a+ and the antigen-experienced SMARTA cells were Thy1.1+ (outlined in Figure S1A). Equal numbers of these 2 populations were CFSE-labeled and transferred into naïve or early-immune B6 mice. After 8 days in vivo, the 2 donor populations were identified by flow cytometry (Figure S1B, dot plots) and their division status was evaluated (Figure S1B, histogram). Consistent with the data in Figure 5, the SMARTA cells did not divide in the naïve hosts. However, the naïve SMARTA cells, but not the day 12 SMARTA cells, divided in the early immune mice (Figure S1C). Despite their ability to divide in the immune recipients, the naïve SMARTA cells did not vigorously make IFNγ ex-vivo (Figure S1D), thus the naïve cells divide and accumulate but across 8 days do not fully differentiate into effector or memory cells, although longer periods of in vivo stimulation might induce such differentiation. In total, the data in Figures 5 and S1 show that naïve and antigen-experienced CD4+ T cells differ intrinsically in their response to lingering antigen.

It has been suggested that CD8+ T cells are programmed early after infection to undergo contraction through cellular apoptosis (41, 42). We considered that the day 12 antigen-experienced CD4+ T cells in the above experiments might not undergo cell division if they were destined to eventually undergo cell death. We also considered that the block in their cell division might represent a transient phase in the CD4+ T cell response. Therefore, we examined whether long-term memory CD4+ T cells that have survived through the contraction phase would undergo cell division when exposed to the residual antigen. Quiescent memory CD4+ T cells were isolated from mice immunized 45–68 days earlier. The memory CD4+ T cells and naïve CD4+ T cells were mixed and labeled with CFSE and adoptively transferred into uninfected mice, acutely infected mice, or LCMV-immune mice (Figure 6A). As expected, the donor cells did not undergo cell division in uninfected/non-immune mice and vigorously divided in response to live infection. However, the memory CD4+ T cells did not undergo cell division in the LCMV-immune mice, whereas the naïve CD4+ T cells in the same immune hosts underwent multiple rounds of cell division (Figure 6B). Thus, memory CD4+ T cells do not proliferate in response to the residual antigen.

Antigen-experienced CD8+ T cells fail to undergo rapid cell division in early-immune mice

Like CD4+ T cells, CD8+ T cells also undergo functional maturation as they differentiate after virus infection. Using the same approach as in Figure 5, we compared the ability of naïve and antigen-experienced day 12 CD8+ P14+ T cell to undergo cell division when in the same naïve or early-immune mice (Figure 7A). Consistent with the findings in Figure 1, naïve CD8+ T cells did not undergo cell division when transferred to naïve or early immune mice (Figures 7B & 7C). Even antigen-experienced CD8+ T cells that are highly sensitive to low amounts of peptide did not undergo cell division after residing in naïve or early-immune

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mice for 8 days (Figures 7B & 7C). To determine whether the CD8+ T cells underwent functional changes while in the early-immune mice, spleen cells from the recipient mice were exposed to GP_{33-41} peptide (Figure 7D). Upon ex-vivo stimulation, >90% of the antigen-experienced CD8+ T cells produced robust amounts of IFNγ, whether they spent 8 days in naïve mice or early immune mice (Figure 7D & 7E). In contrast, few naïve P14 cells (~1%) made IFNγ after 8 days in the uninfected or LCMV-immune recipients, and the amount of IFNγ produced among the cytokine positive cells was very low. Thus, if MHC1-restricted antigen was presented in vivo, then it was ineffective over one week to stimulate naïve CD8+ T cells to differentiate into cells capable of making a key antiviral cytokine and did not induce CD8+ T cells to undergo a single round of cell division.

Following re-challenge infection, memory CD8+ T cells immediately express effector functions and proliferate after an initial delay (43). To determine how the naïve and antigen-experienced P14 cells would respond when confronted with live virus infection, both CFSE-labeled populations were transferred to the same naïve host that was subsequently challenged with LCMV. At day 6, both naïve and antigen-experienced P14 CD8+ T cells responded strongly and accumulated to represent 17–27% of CD8+ T cells (Figure 7F). The cells underwent significant cell division during this time and had diluted CFSE (Figure 7F, middle dot plots); cumulatively, >95% of the cells had divided and >80% were able to make IFNγ (Figure 7F, bar graphs). Thus, naïve and antigen-experienced P14+ CD8+ T cells were vigorous responders to live infection where direct antigen presentation occurs efficiently along with inflammatory signals.

Cumulatively, the data in Figures 5–7 indicate that lingering antigen during the early contraction phase preferentially stimulates the cell-division of naïve CD4+ T cells but not differentiated CD4+ T cells or MHC1-restricted CD8+ T cells. Remarkably, this effect occurred despite the enhanced sensitivity of differentiated CD4+ T cells to trace amounts of antigen and their vigorous multi-cytokine output. The lingering antigen did not stimulate naïve or antigen-experienced P14 cells, highlighting a fundamental difference between CD8+ T cells and CD4+ T cells.

Discussion

The period immediately following the peak response to acute virus infection is poorly understood; yet critical events unfold when the overall immune response contracts to homeostasis that impact T cell survival and differentiation into memory (44). Our analyses of this period reveal several interesting features. We discovered that viral material persists into the contraction/early memory phase long after replicating LCMV is eliminated, and it is immunologically relevant: CD4+ T cells – but not CD8+ T cells – are stimulated by this antigen. Surprisingly, the antigen selectively stimulated naïve CD4+ T cells and not antigen-experienced CD4+ T cells to undergo cell-division and increase numerically. As discussed below, the finding that antigen remains long after infection has several implications with regard to the relevance of cross-presentation of antigen, the effect of antigen on T cell contraction, and the delayed selection of virus-reactive T cells.

The lingering antigen is probably extracellular protein, but we do not know how it might persist. It is unlikely to be peptide-MHCII complexes, because these complexes survive only 2–3 days (45) and antigen-bearing DC only survive 3–12 days (46), which is not long enough to support the effects that we observe several weeks after infection (Figure 2). Immune complex deposition in the kidneys has been documented following neonatal LCMV infection of mice (47) or following infection with variants of LCMV that disseminate and persist over time. Artificial immune complexes formed with model antigens and monoclonal antibodies persist for 3–10 weeks (48, 49), and can be physically identified by immuno-

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staining or by following the decay rate of radiolabeled IC. Subcutaneous vaccination with protein in WT mice also results in long-lived (>10 weeks) depots of peptide antigen that stimulate the cell division of naïve pigeon cytochrome C-specific CD4+ T cells that are located near antigen-specific memory B cells (49). A recent study showed that there is a sustained population of LCMV-specific CD8+ T cells in the mediastinal lymph nodes after acute infection (50). However, the maintenance of that population of cells was independent of antigen (50). Interestingly, we found that the proportion of naïve SMARTA CD4+ T cells that underwent cell-division was similar in the spleen and inguinal lymph nodes but slightly higher in the mediastinal lymph nodes, where prolonged T cell reactivity has been observed following influenza and vesicular stomatitis virus infections (8–11). For example at day 12–20, ~86% were divided in the spleen & inguinal lymph node but 86% in the mediastinal lymph node (n=3 mice; data not shown). At day 32–40, 15–30% were divided in the spleen and inguinal lymph node but 43–55% in the mediastinal lymph node (n=4 mice; data not shown). It would be interesting to learn whether the mediastinal lymph node acts an antigen depot or is a destination for divided cells. Our analyses indicate the lingering LCMV antigen has a *functional* half-life of ~40 days (Figure 2C). We considered that the residual antigen might be in the form of immune-complexes that survive over time in the spleen or bone marrow in such a way that it is accessible to MHCII+ APC and CD4+ T cells. However, we found that naïve SMARTA CD4+ T cells divided in LCMV-immune B cell-deficient and FcγRII-deficient mice (Figure S2). This implies that antibody-dependent complex formation is not required to retain the antigen. Perhaps non-specific protein deposition is sufficient to stimulate CD4+ T cells across time, so long as the material is engulfed by MHCII+ APCs. Interestingly, the CD4+ T cell turnover in the immune B cell-deficient mice appeared greater than in the immune WT mice, which is consistent with the notion that B cell-dependent presentation of antigen without inflammation induces hyporesponsive CD4+ T cells (51).

The effects we see are clearly dependent on residual antigen. CD4+ T cells that are will unlikely be stimulated through the contraction phase in other infection models that deposit lower amounts of antigen. For example, mice that were infected with recombinant Listeria monocytogenes expressing LCMV-GP_{61-80} did not have sufficient residual antigen to stimulate SMARTA CD4+ T cells (Figure S3A). Likewise, OTII CD4+ T cells did not undergo cell division when transferred a few day after rLM-OVA was cleared (Figure S3B). Listeria and LCMV differ in many ways beyond antigen abundance (eg tropism, inflammation), but informative patterns may emerge from similar analyses using other infection models, particularly whether the sustained antigen-stimulation correlates with improved memory.

Virus-specific CD8+ T cells and CD4+ T cells differ in the rate of contraction following acute LCMV infection (2). The observed gradual loss of virus-specific CD4+ T cells has been interpreted as evidence that CD4+ T cell memory is inherently unstable as compared to CD8+ T cell memory. Our results suggest the longevity of the antigen governs the duration of the T cell contraction phase: for CD4+ T cells after LCMV, the contraction phase is protracted. We propose that CD4+ T cell contraction is not, in fact, a gradual process. Rather, the well-recognized prolongation of the antiviral CD4+ response reflects an overlap of two distinct biological processes: rapid contraction combined with ongoing recruitment. Thus, we speculate that the kinetics of CD4+ T cell contraction, and the establishment of CD4 memory, may be similar to that of CD8+ T cells, but these events are largely obscured by the continuing antigen-driven emergence of new populations of naïve CD4+ T cells; no such masking occurs for CD8+ T cell contraction, because the residual antigen is unable to recruit naïve CD8+ precursors. Eventually, the antigenic stimulus decays to levels that no longer stimulate CD4+ T cells, which implies that subsequent memory cell survival continues in the absence of viral antigen. Memory CD8+ T cells and memory CD4+ T cells

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survive over time in the complete absence of antigen and selecting MHC molecules (19, 52, 53). Our results are consistent with those findings: the pool of LCMV-specific CD4+ memory T cells present 1–2 years after infection has survived without antigen for many months, and the data in Figure 5 indicate that early memory cells do not divide or accumulate to antigen, even when it is present. Nevertheless, it is plausible that the residual antigen stimulates memory CD4+ T cells to express pro-survival factors to protract the contraction phase.

Since the stimulus is most likely reprocessed viral protein from a "depot", why are virus-specific CD8+ T cells not stimulated to divide? Cross-priming (54) is an alternative of "classical" antigen presentation; it is a process whereby exogenous antigen is redirected to the MHC-I pathway to stimulate CD8+ T cells. Numerous investigators have advanced the notion that cross-presentation of antigen by dendritic cells is a significant mechanism for inducing CD8+ T cells (55, 56). In the LCMV model, residual viral antigen is present for several weeks at sufficient levels to stimulate LCMV-specific CD4 T cells, but not LCMV-specific CD8 T cells in the same mice. This implies that the cross-presentation of antigen plays an insignificant role in the modulation of CD8 T cell responses during these times. Admittedly, our studies do not exclude the possibilities that cross-priming occurs, and that CD8+ T cells are unresponsive; or that the signaling threshold required to stimulate cell division is higher for naïve CD8+ T cells than for CD4+ T cells. However, these possibilities seem unlikely given that the TCR signaling threshold for CD4+ T cells and CD8+ T cells is similar. CD4+ T cells can recognize a single MHCII/agonist peptide complex and begin forming an immunological synapse when 10 agonist peptide/MHC complexes are present (57) with 200 MHCII/peptide complexes stimulating CD4+ T cells and 5000 complexes inducing a maximal response (58). Likewise, CD8+ T cells can detect a single foreign antigen, are cytolytic with 3 complexes, and require about 10 complexes to form a mature synapse and release calcium (59). Additionally, interactions between CD8+ T cells and peptide/MHC complexes should be more frequent and more stable than those between CD4+ T cells and peptide/MHC complexes: effector CD8+ T cells respond to lower concentrations of peptide than do CD4+ T cells (sub-nanomolar versus ~100 nanomolar) ((1, 60) & Figure 4); the affinity of GP_{33} peptide for Db (Immune Epitope Database; ANN IC_{50}=5nM) is greater than GP_{61} for I-Ab (IC_{50}=128nM)(61, 62); most cells express large amounts of MHC-I, whereas only a few cell types express MHC-II. Finally, we cannot exclude the possibility that CD4 T cells – but not CD8+ T cells – transit through an anatomical niche containing only MHCII-loaded antigen derived from long-lived immune complexes (49). Nevertheless, our data show clearly that virus-specific CD8+ T cells are unaffected by antigen that is sufficient to stimulate naïve CD4+ T cells in the same mice.

Given that CD4+ T cell memory persists in the absence of antigen and even in the absence of MHCII (53, 63), what is the benefit to the host to continue stimulating CD4+ T cells after the infection is resolved? Perhaps the protein/peptide material supports a selection process that enriches for CD4+ T cells that are best able to express particular and ideal combinations of memory cell cytokines. Ultimately, a small population of highly functional memory CD4+ T cells is selected that enhances protection against re-infection. We considered that the antigen might be retained in the follicle/germinal center and that the CD4+ T cells that are stimulated to divide become T_{FH} cells to enhance humoral immunity; however, the LCMV-specific cells were $CD44^{hi}$ but not distinctly CXCR5+. Sustained CD4+ T cell responses might impact CD8+ T cell memory, as CD8+ T cells show functional changes that continue through the contraction phase (64, 65), despite no evidence of MHC-I viral antigen (Figures 1 & 7). The quality and number of CD8+ T cell memory is impacted by the presence of CD4+ T cells during the memory phase (17, 66–70), so there may be a role for

virus-derived protein in these processes whereby locally stimulated CD4+ T cells deliver a cytokine that stimulates memory CD8+ T cell differentiation or affects their movement (71).

The first response of memory cells to infection is to carry out multiple effector functions, which does not require cell-division (43). However, we do not know whether lingering viral antigen stimulates memory T cells to express cytokine or carry out some other immediate function in vivo. In contrast, naïve cells minimally acquire the ability to make IFNγ despite 8 days of exposure to lingering viral antigen (Figures 5, SF1, 7). This finding is consistent with an earlier report that showed naïve CD8+ T cells made little IFNγ upon brief exposure to TCR stimulation or to virus-infected cells, although the cells robustly made TNF in these conditions (72). Thus, it is plausible that the lingering antigen may stimulate naïve T cells to make TNF, perhaps to affect APC functions (72).

Our finding that antigen-experienced CD4+ T cells do not divide, whereas naïve cells undergo cell-division and accumulate (Figures 5 & 6), suggests that there is a period after the acute phase of infection when there may be two populations of virus-specific CD4+ T cells. One major population is a product of memory cell differentiation that unfolds during infection. A second population of cells might arise from naïve T cells that are stimulated by lingering viral antigen without inflammation. Following the acute phase of influenza infection, late antigen presentation by certain DC subsets preferentially stimulates naïve CD8+ T cells (new recruits) over memory T cells (10, 73). Analyses in the influenza model are consistent with late arriving CD4+ T cells efficiently converting into memory cells with minimal division (8, 74). Other studies have shown that naïve CD4+ T cells can be recruited into the anti-gammaherpesvirus response during latency (12). Thus, naïve and memory T cells could respond similarly but differ in access to residual antigen in vivo or it may be that naïve and memory cell have equal access to antigen but differ intrinsically in how they respond to it. The recruitment of naïve cells but not antigen-experienced cells may diversify the memory pool, which would be advantageous to a host that is exposed to highly-mutable pathogens like RNA viruses. Given that immune mice already have large populations of Th1 memory cells, it may be beneficial to the host to expand a pool of virus-specific CD4+ T cells that are functionally uncommitted but ready to quickly differentiate into other lineages (eg, T_{FH} or T_{reg}) during re-challenge infection.

Memory cells undergo an explosive proliferative response 3 days after re-infection that leads to peak numbers by day 6 (43, 75). This apparent paradox – memory T cells do not proliferate when lingering antigen is present but do proliferate during re-challenge infection –may be explained by the significant inflammatory signals and very high antigen loads that are achieved during infection that induce memory cell proliferation and accumulation. The underlying processes that limit the cell proliferation of antigen-experienced cells and how memory cells regain their ability to proliferate in response to re-infection remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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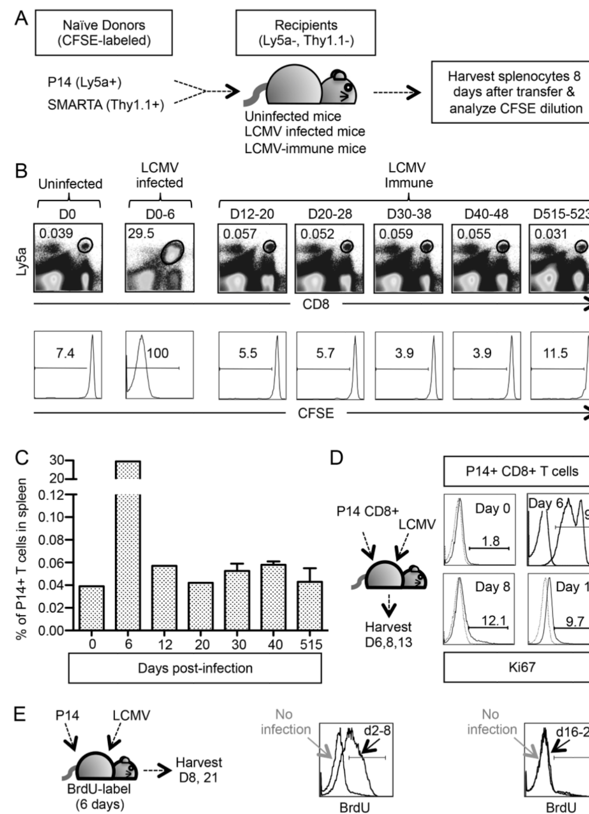

Figure 1. LCMV-specific CD8+ T cells do not rapidly divide during the contraction or memory phases

(**A**) The dual transfer approach used to evaluate CD8+ T cell and CD4+ T cell proliferation in uninfected, LCMV-infected, or LCMV-immune mice. A mix of naïve CFSE-labeled P14 and SMARTA TCR-transgenic splenocytes were given to 3 groups of mice: 1) uninfected B6 mice, 2) uninfected mice that were subsequently infected with LCMV 2 days later, or 3) LCMV-immune mice. 8 days after transfer, the splenocytes were isolated and costained with antibodies against CD8 and Ly5a to identify the donor P14+ cells. (**B**) The headings above the dot plots indicate the immune status of the recipient mice and the days post-infection when the donor cells were in the recipient mice. The ovals within the dot plots identify the donor P14+ CD8+ T cells and the numbers represent their frequency among all splenocytes. The histograms are gated on the cells within the ovals, and the numbers indicate the percentage of these cells that have undergone one or more cell divisions as indicated by the bar. (**C**) The bar graphs show the frequency of donor P14 CD8+ T cells among all splenocytes in each group. (**D**) A separate set of mice containing 3×10^3 P14+ CD8+ T cells was given LCMV infection and, at days 6, 8, and 13, the spleens of the recipient mice were surface stained for CD8 and Ly5a and for intracellular Ki67. The donor P14+ cells were gated and the histograms show their level of Ki67 expression. A control mouse received 8×10^5 P14+ CD8+ T cells and was left uninfected (day 0) for the duration of the experiment. The dark lines indicate Ki67 staining and the dotted lines show isotype control staining. The data are representative of 2 independent experiments with 2–3 mice per time point. (E) Mice that were engrafted with 3×10^4 P14 CD8+ T cells were given LCMV. The mice were pulsed with BrdU to label dividing cells at days 2–8 or days 16–21 after infection. The histograms are gated on the P14 cells and show their incorporation of BrdU. The data represent 1 uninfected P14 mouse, 1 acutely infected mouse, and 3 mice at day 21.

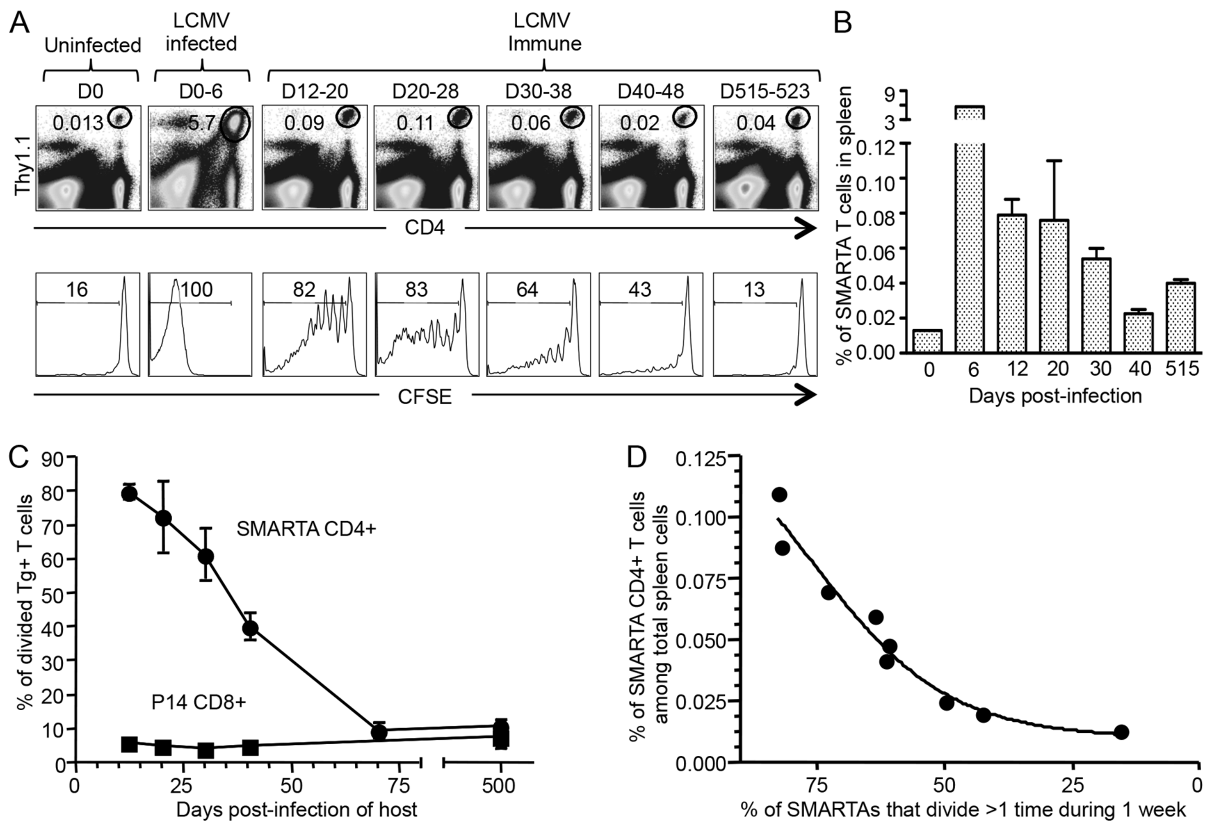

Figure 2. Naïve SMARTA CD4+ T cells divide in early immune mice, but not in late immune mice

The same mice described in Figure 1 received CFSE-labeled SMARTA CD4+ T cells and, 8 days after transfer, their splenocytes were costained with CD4 and Thy1.1 to identify the donor SMARTA cells. (**A**) The headings above the dot plots indicate the days post-infection when the donor cells were present in the mice. The ovals within the dot plots identify the donor SMARTA CD4+ T cells, and the numbers indicate the percentage of these cells among all spleen cells. The histograms are gated on the cells within the ovals, and the numbers represent the percentage of these cells that have undergone cell division as indicated by the bars. (**B**) The bar graphs show the average (+/− sem) percentage of SMARTA CD4+ T cells among splenocytes. (**C**) The percentage of transgenic T cells that underwent at least one round of cell division during the 8 day interval is plotted against the immune status of the recipient mice. The data points depict the average (with sem) for 2–4 mice per time point. (**D**) The line graph shows the percentage of engrafted donor SMARTA CD4+ T cells among spleen cells versus the fraction of those cells that have undergone cell division. Each data point represents an individual recipient mouse and the data are compiled from 2 independent series of experiments. The data are representative of 2 independent experiments with 2–3 mice per time point.

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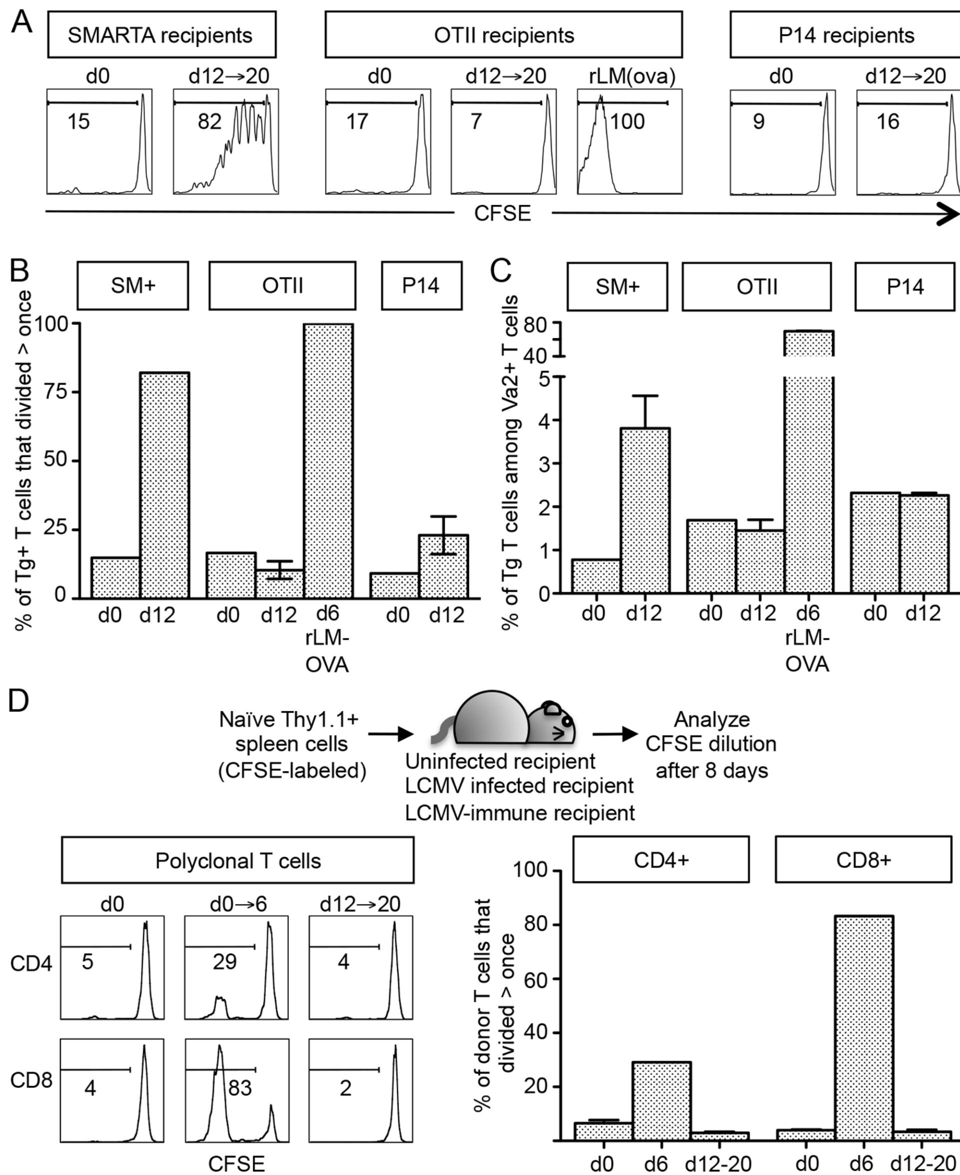

Figure 3. The CD4+ T cell cell-division during the contraction phase is antigen-dependent

(**A–C**) Spleen cells from OTII TCR-transgenic mice, SMARTA mice, and P14 mice were CFSE-labeled and transferred to Ly5a+ mice that were either uninfected (d0) or LCMV-immune (day 12), or a set of naïve mice that were given rLM-OVA two days after transfer. Eight days after the cell transfer, the spleens of the recipient mice were isolated and surface stained for CD4, CD8, Vα2, and Ly5b to identify the donor transgenic T cells. (**A**) The histograms are gated on the donor transgenic T cells and show their level of CFSE fluorescence; the numbers indicate the percentage of the donor cells that have undergone at least one cell division. (**B**) The bar graphs show the percentage of donor transgenic T cells that had undergone cell division at least once during the 8 day interval. (**C**) The bar graph shows the average percentage of donor transgenic T cells among all Vα2+ T cells. The data are from 2–3 mice per group per time point. (**D**) The illustration shows the experimental plan to measure the proliferative response of polyclonal T cell populations in LCMV immune mice. 6×10^6 CFSE-labeled spleen cells from naïve Thy1.1+ mice (B6.PL) were

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transferred to 3 groups of recipients: 1) uninfected mice that were left uninfected; 2) uninfected mice that were challenged with LCMV infection; 3) LCMV immune mice that had received LCMV 12 days before. Eight days after transfer, the donor cells were identified by flow cytometry due to their expression of Thy1.1. The histograms show the CFSE fluorescence of the indicated CD4+ or CD8+ donor cells. The bar graphs show the percentage of donor cells that had divided at least once over the course of 8 days in the indicated recipients. The data represent one experiment with 2 uninfected recipients, 1 acutely infected recipient, and 4 LCMV-immune recipients.

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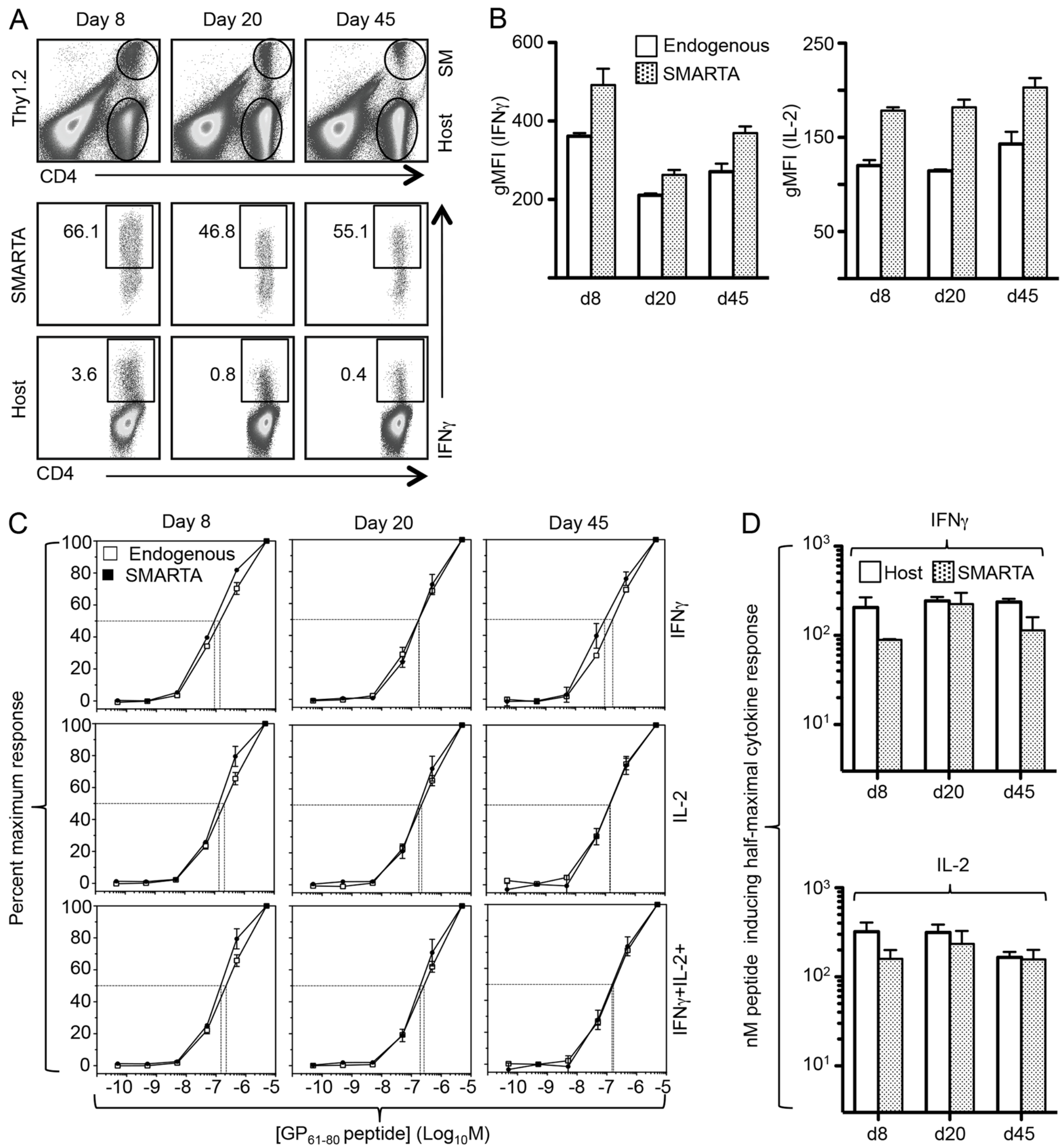

Figure 4. The functional avidity of memory CD4+ T cells is set during the expansion phase and is similar for SMARTA and endogenous CD4+ T cells

SMARTA CD4+ T cells were transferred into B6 mice followed by infection several days later. At days 8, 20, and 45 after infection, the SMARTA and endogenous CD4+ T cell responses were quantified by ICCS. (**A**) The representative dot plots show spleen cells at different times after infection, with the SMARTA and endogenous (host) CD4+ T cell populations indicated by ovals. The lower rows are gated on either SMARTA or host cells and show their production of IFNγ after GP_{61-80} stimulation; the numbers indicate the percentage that are IFNγ +ve. (**B**) The amount of IFNγ or IL-2 made on a per-cell basis was determined by gating on cytokine+ T cells as indicated in A, and then quantifying the geometric mean fluorescence intensity of the cells. (**C**) Spleen cells from mice at the indicated times were exposed to different concentrations of peptide, and the ability of the

host and donor CD4+ T cells to make IFNγ, IL-2, or co-express IFNγ with IL-2 was determined by ICCS. The dotted lines indicate the peptide concentration that gives a half-maximal response. (**D**) The bar graphs show the average (+ sem) concentration of peptide to induce the half-maximal response, as identified in C.

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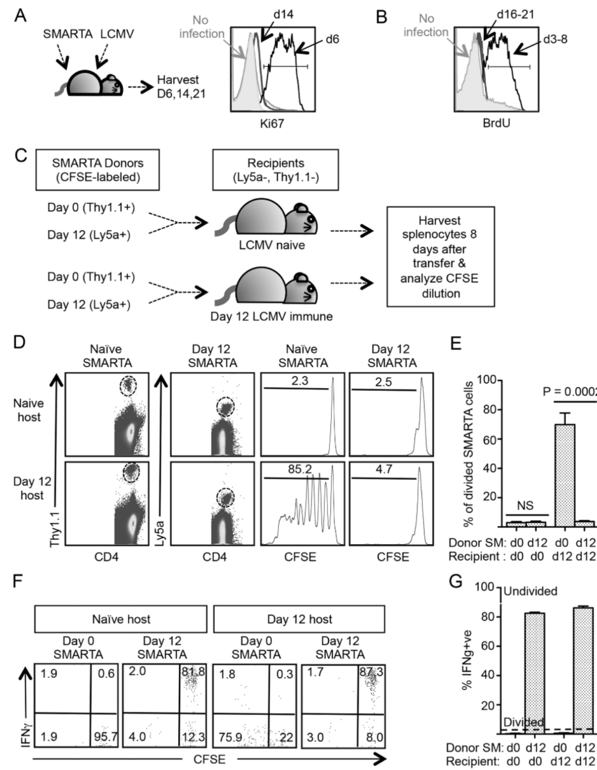

Figure 5. Lingering antigen does not stimulate the cell-division of antigen-experienced CD4+ T cells

(**A**) B6 mice were given 2×10^4 SMARTA/Thy1.1+ CD4+ T cells and subsequently given LCMV infection. The histogram shows the level of Ki67 expression in the responding SMARTA CD4+ T cells at day 6 (n=1) and day 14 (n=2) after infection and in CD4+ T cells from an uninfected SMARTA mouse (shaded, n=1). An isotype-control stain is shown as an unshaded gray line. (**B**) B6 mice were given 2×10^4 SMARTA/Thy1.1+ CD4+ T cells and then infected. During days 3–8 or days 16–21, the mice were given an initial ip injection of BrdU and fed drinking water containing BrdU. Uninfected SMARTA mice were also given BrdU for 6 days to determine the baseline amount of cell-division without infection. At the end of the labeling period, the spleen cells were surface stained to identify the donor cells and stained for intracellular BrdU. The histogram shows BrdU levels in SMARTA CD4+ T cells (n=1 for day 3–8; n=3 for day 16–21; n=1 for uninfected SMARTA mouse). The unshaded gray line represents an isotype-control stain. (**C**) The dual adoptive transfer approach used to compare the proliferation of naïve and antigen-experienced cells in the same host. To generate antigen-experienced SMARTA cells, 2×10^4 SMARTA/Ly5a+ CD4+ T cells were injected intravenously into a recipient mouse (B6), and the mouse was infected 3 days after the cell transfer. The antigen-experienced SMARTA cells were harvested at day 12 after infection and used for the dual adoptive transfer into B6 mice (Thy1.1-Ly5a-). For the dual adoptive transfer, the donor cells from naïve SMARTA/Thy1.1+ mice and the antigen experienced SMARTA/Ly5a+ cells were mixed in equal proportion, labeled with CFSE, and 10^6 total SMARTA CD4+ T cells (5×10^5 each kind) were given intravenously to either naïve mice or LCMV-immune mice (day 12 p.i.). T cell dilution of CFSE was measured in these mice 8 days later. (**D**) The dot plots show examples of CD4+ T cells from the indicated recipient mice 8 days after the cell transfer. The dashed ovals identify the donor naïve or day 12 SMARTA CD4+ T cells. The histograms are gated on the indicated SMARTA CD4+ T cell populations and show their level of CFSE fluorescence. The

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horizontal bar indicates cells that have undergone at least one cell division. (**E**) The bar graph indicates the percentage of SMARTA cells that have undergone at least one cell division. Note that naïve but not day 12 SMARTA cells underwent cell division in the immune (d12–20) recipient mice. (**F–G**) Spleen cells from the recipient mice were harvested and exposed to GP_{61-80} peptide in an ICCS assay. The dot plots are gated on the indicated donor cells and show their division status (CFSE) and expression of IFNγ. (**G**) The bar graphs show the percentage of undivided SMARTA CD4+ T cells that made IFNγ; the dashed line indicates the percentage of SMARTA CD4+ T cells that diluted CFSE and made IFNγ. Note that naïve (day 0) CD4+ T cells did not make cytokine despite undergoing cell-division and that antigen-experienced cells (day 12) CD4+ T cells made IFNγ but did not divide. The data were independently measured from 5 immunized mice; an un-paired Student's t-test was used to determine significance with the P-values indicated above bars.

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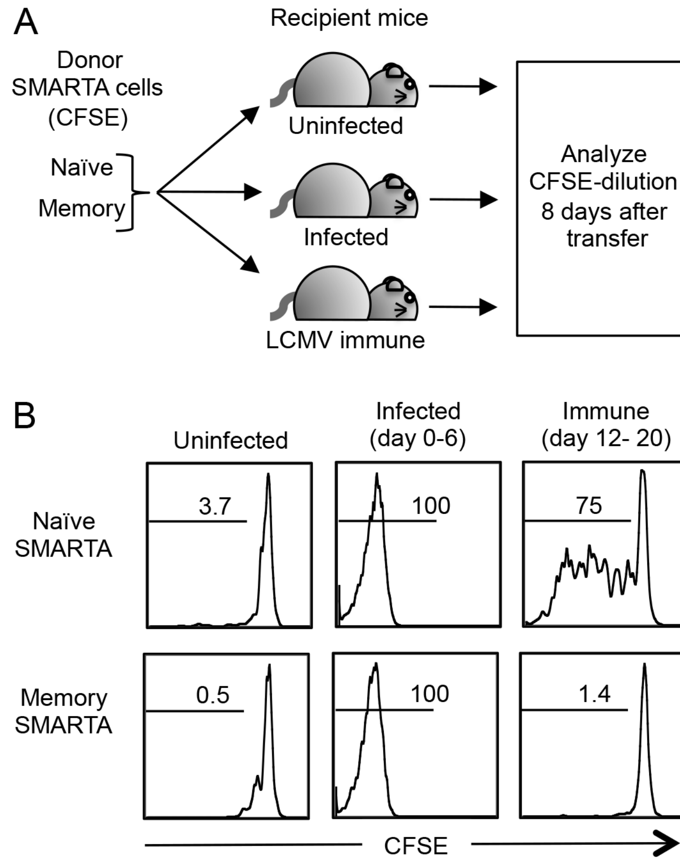

Figure 6. Memory CD4+ T cells do not divide in response to residual viral antigen
(**A**) The experimental plan to measure the effect of residual viral antigen on memory CD4+ T cell proliferation. Memory SMARTA cells were generated by giving B6 mice ~3×10^3 SMARTA/Thy1.1+ CD4+ T cells followed by LCMV infection. At days 45 or 68 post infection, splenocytes were isolated, mixed with naïve SMARTA CD4+ T cells (Ly5a+), and labeled with CFSE. The mixture was given to 3 groups of mice that were uninfected, acutely infected 2 days after transfer, or LCMV-immune (12 days post-LCMV). Each recipient received approximately 5×10^5 naïve and memory SMARTA cells. After 8 days, spleen cells from the recipients were isolated and the donor cells were identified and analyzed for CFSE-dilution. (**B**) The histograms show the level of CFSE fluorescence by the naïve or memory donor cells in the 3 hosts. The data are representative of findings from two independent experiments with 2 uninfected recipients, 2 infected recipients, and 5 immune recipients.

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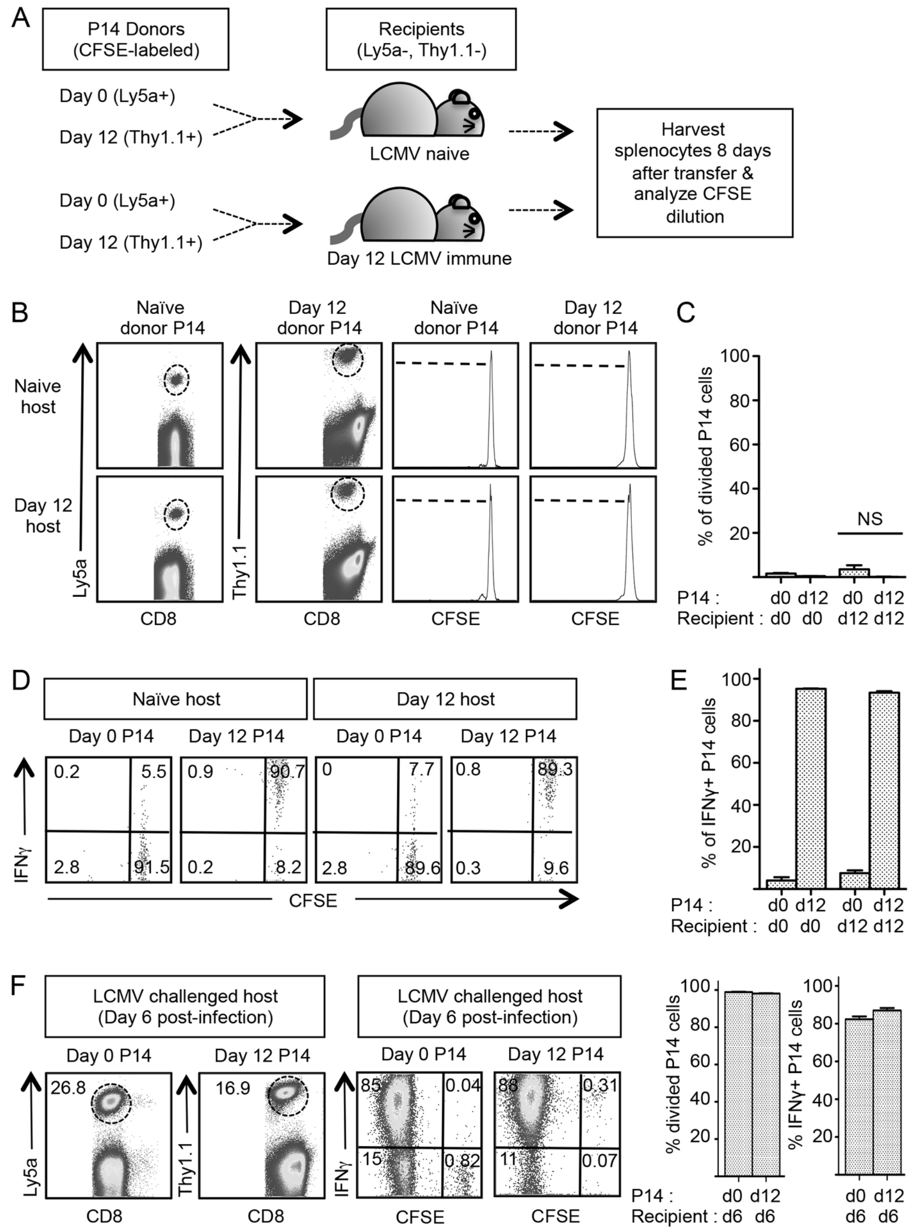

Figure 7. Naïve and antigen-experienced CD8+ T cells fail to undergo cell-division in early immune mice but respond to live infection

(**A**) The dual adoptive transfer approach used to compare the proliferation of naïve and antigen-experienced CD8+ T cells in the same host. To generate antigen-experienced P14 cells, $2{\times}10^4$ P14/Thy1.1+ CD8+ T cells were injected intravenously into a recipient mouse (B6), and the mouse was infected 3 days after the cell transfer. The antigen-experienced P14 cells were harvested at day 12 after infection and used for the dual adoptive transfer into B6 mice (Thy1.1-Ly5a-). For the dual adoptive transfer, the donor cells from naïve P14/Ly5a+ mice and the antigen experienced P14/Thy1.1+ cells were mixed in equal proportion, labeled with CFSE, and 10^6 total P14 CD8+ T cells (5×10^5 each kind) were given intravenously to either naïve mice or LCMV-immune mice (day 12 p.i.). T cell dilution of CFSE was measured in these mice 8 days later. (**B**) The dot plots show examples of CD8+ T

cells from the indicated recipient mice 8 days after the cell transfer. The dashed ovals identify the donor naïve or day 12 P14 CD8+ T cells. The histograms are gated on the indicated P14 CD8+ T cell populations and show their level of CFSE fluorescence. The dashed bar indicates cells that have undergone at least one cell division. (**C**) The bar graph indicates the percentage of P14 cells that have undergone at least one cell division. (**D–E**). Spleen cells from the recipient mice were harvested and exposed to $GP_{33–41}$ peptide in an ICCS assay. (**D**) The representative dot plots are gated on the indicated donor P14 cells and show their division status (CFSE) and expression of IFNγ. (**E**) The bar graphs show the percentage P14 cells that made IFNγ in the indicated recipient mice. Note that naïve (day 0) CD8+ T cells did not make cytokine and antigen-experienced (day 12) CD8+ T cells made IFNγ, but neither underwent cell division. The data were independently measured from 2 naïve recipient mice and 4 immunized recipient mice. (**F**) Naïve P14/Ly5a+ cells and day 12 P14/Thy1.1+ cells were mixed in equal proportion, labeled with CFSE, and 10^6 total P14 cells (5×10^5 each kind) were given intravenously to naïve mice; the recipient mice were given LCMV 2 days later and their spleens were harvested at day 6 post-infection and analyzed by ICCS. The circles within the left dot plots identify the donor cells within the day 6 mice, with the percentage of each donor among CD8+ T cells indicated. The middle dot plots are gated on the indicated donor cells and show their production IFNγ and CFSE-dilution 6 days after infection. The left bar graphs depict the percentage of each P14 population that underwent >1 cell division during the infection; the right bar graphs show the percentage of each P14 population that produced IFNγ. The bar graphs represent the average (+ sem) of 4 challenged recipient mice.

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