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Differential T Cell Responses to Residual Viral Antigen Prolong CD4⁺ T Cell Contraction following the Resolution of Infection

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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 infection in mice. In contrast, virus-specific CD4⁺ T cells gradually decrease in number and reach homeostatic levels only after many weeks. In this study, we provide evidence that MHCII-restricted viral Ag persists during the contraction phase following this prototypical acute virus infection. We evaluated whether the residual Ag affected the cell division and number of virus-specific naive and memory CD4⁺ T cells and CD8⁺ T cells. We found that naive CD4⁺ T cells underwent cell division and accumulated in response to residual viral Ag for >2 mo after the eradication of infectious virus. Surprisingly, memory CD4⁺ T cells did not undergo cell division in response to the lingering Ag, despite their heightened capacity to recognize Ag and make cytokine. In contrast to CD4⁺ T cells, CD8⁺ T cells did not undergo cell division in response to the residual Ag. 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 Ag delays the contraction of CD4⁺ T cell responses by recruiting new populations of CD4⁺ T cells. The Journal of Immunology, 2013, 191: 5655–5668.

Follow acute lymphocytic choriomeningitis virus (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 Ag, changes in migratory properties, increased secretion of cytokine, and the simultaneous expression of multiple cytokines (1). The T cell response peaks ~1 wk postinfection and, soon thereafter, the virus is completely eliminated by virus-specific T cells. During the subsequent 1–2 wk, there is a rapid decline in antiviral CD8⁺ T cell number. However, antiviral CD4⁺ T cells show a gradual decline in number until they reach a homeostatic level 1–2 mo postinfection (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 was 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 Ag long after the acute phase of infection. There is evidence that the Ag 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 Ag 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 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 d 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 Ag (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 Ag 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 Ag for different lengths of time after infectious virus has been eliminated. In this study, we report that antiviral CD8+ T cells do not undergo Ag-dependent cell division during the contraction or memory phases, consistent with earlier data showing that wild-type (WT) mice completely eliminate LCMV-Armstrong infection, and that long-term CD8 memory does not require Ag (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 Ag is present long after infectious virus and MHC-I-restricted viral Ags are lost. This MHCII-restricted Ag selectively signals into CD4+ T cells and inflates their number over time, thus delaying the establishment of Ag-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 naive cells, which may serve to diversify the pool of virus-reactive cells. The viral Ag also does not spur CD8+ T cells to undergo cell division over the same time period, which implies that the cross-presentation of viral Ag 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 naive CD4+ T cells, memory CD4+ T cells, and CD8+ T cells.

Materials and Methods

Mice, virus, and bacteria

C57BL/6 mice were purchased from the Scripps Research Institute breeding facility. C57BL/6 mice congenic for Thy1.1 (B6-PL-Thy1+/CyJ) or congenic for Ly5a (B6.SJL-PtprcaPep3b/BoyJ) were purchased from The Jackson Laboratory. SMARTA TCR-transgenic mice specific for the I-Ab LCMV epitope gp33-41 (20) were originally made by H. Hengartner (University Hospital Zürich, Zürich, Switzerland) and were crossed to B6-PL-Thy1+/CyJ mice to generate SMARTA/Try1.1+ mice or to B6.SJL-PtprcaPep3b/BoyJ to generate SMARTA/Ly5a+ mice. P14 TCR-transgenic mice specific for the LCMV epitope gp12–28 (21) on the H-2b background were crossed to B6.SJL-PtprcaPep3b/BoyJ mice to generate the P14/Ly5a+ strain or to B6-PL-Thy1+/CyJ to generate the P14/Try1.1+ mice. OTII TCR-transgenic mice (22) specific for the I-Aα-restricted epitope, OVA257–264, were originally made by F. Carbone’s group (University of Melbourne, Parkville, VIC, Australia) and provided by B. Beutler (Scripps Research Institute). OTII mice under the control of the MHC class Ii promoter were infected by i.p. administration of 10⁵ PFU 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 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 L. monocytogenes (gLM) that express LCMV-gp126–140 or OVA. These recombinant bacteria were originally made by Dr. H. Shen (University of Pennsylvania) (23). The bacteria were grown to log phase before titration and injection into mice. CFUs were determined by plating dilutions of tissue homogenates or bacterial stocks onto brain heart infusion/agar plates. All experiments were approved by the University of North Carolina Animal Care and Use Committee.

Adoptive transfers

Flow cytometry was used to determine the frequency of transgenic CD4+ T cells (Vα2-Vβ8.3+) among all spleen cells in SMARTA mice or the frequency of transgenic CD8+ T cells (Vα2-Vβ8.12+) among all spleen cells in P14 mice. OTII cells (CD4+Vα2-Vβ5.1+) were identified in recipient mice by flow cytometry. In the experiments that followed cell division by CFSE dilution in infected or naive mice, mice were mixed, CFSE labeled, and cotransferred into three groups of mice. In naive mice, 2/3 of the donor T cells were adoptively transferred and the recipient mice were infected with approximately 10⁵ PFU LCMV-Armstrong per mouse. The third group received no donor cells. All mice were monitored for signs of disease and were sacrificed at the end of the experiment. The frequency of transgenic cells in the recipient mice was determined 8–9 d after transfer. Flow cytometry was used to determine the frequency of transgenic CD4+ T cells in SMARTA mice or the frequency of transgenic CD8+ T cells in P14 mice.

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-Thy-1.2 (clone 53-2.1), anti-Thy-1.1 (clone HS51), and anti-CD44 (clone IM7), CD62L (clone MEL-14), anti-Ly5a (clone A20), anti-Vα2 (clone BV20.1), or anti-Vβ8.3 (clone 1B3.3) that were purchased from eBioscience (La Jolla, CA), BioLegend (La Jolla, CA), or BD Pharmingen (La Jolla, CA). The staining reaction was done in the presence of unlabeled Abs against FcRs to block flurochrome-conjugated Abs from binding to FeR+ cells; FeBlock was purchased from BD Pharmingen. Intracellular staining for Ki67 was performed using a kit from BD Pharmingen. Cells were acquired by four-color flow cytometry using a BD FACS Calibur, and small, nonviable cells were excluded from analysis based on forward and side scatter profile. The cytometry data were analyzed with FlowJo software (Tree Star).

Statistics

Statistical analyses and graphing were done with Prism software. An unpaired two-tailed Student 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, WT mice generate a robust cytotoxic T cell response and eliminate all infectious virus and viral RNA within ~1 wk. We wished to investigate whether low-level viral Ags 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, naive, LCMV-specific, TCR-transgenic CD8+ T cells from P14 mice and CD4+ T cells from TCR-transgenic SMARTA mice were mixed, CFSE labeled, and cotransferred into three groups of mice, as follows: 1) naive mice, 2) naive mice that were subsequently given acute LCMV infection for 6 d, or 3) LCMV-immune mice at various times during the contraction and memory phases (Fig. 1A). The frequency of the donor T cells in the spleen and their loss of CFSE were measured by flow cytometry 8–9 d after transfer. This methodology empowers the natural ability of T cells to circulate and react to minute quantities of Ag 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 Ag that is rare or transiently expressed.

Donor P14+ CD8+ T cells were identified by their expression of the congenic marker, Ly5a (Fig. 1B). The donor CD8+ T cells were gated, and their level of CFSE fluorescence is shown in the histograms (Fig. 1B). As expected, the donor CD8+ T cells did not divide in uninfected recipients and divided extensively in mice given acute infection (day 6), which had a high virus and Ag burden. No donor CD8+ T cell division was observed in immune mice that received these indicator cells at 12 d postinfection or later, and the fraction of donor cells among CD8+ T cells in these immune mice resembled that observed in the uninfected recipients (Fig. 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 WT (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 MHCII-restricted viral Ag from day 12 onward that is recognizable by naïve CD8+
T cells. However, under normal circumstances, Ag-experienced cells with increased sensitivity to low quantities of Ag would be present during these times (1). Perhaps trace amounts of Ag are present during the contraction phase that stimulate Ag-experienced cells to divide, but not naive CD8+ T cells. To examine this possibility, naive mice containing a small number of naive P14+ CD8+ T cells were infected with LCMV-Armstrong, and the proportion of dividing donor cells was determined by intracellular staining against the nuclear Ag, Ki67 (Fig. 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 in-
corporation into DNA as a measure of P14 cell proliferation. Naive mice were engrafted with P14 cells and infected. The mice were exposed to BrdU for 6 d during the expansion phase or 1 wk later (Fig. 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 Ag-experienced CD8+ T cells halt cell division within a few days after the infection has been eliminated, and from day 12 onward, MHC1-restricted viral Ag is unavailable or insufficient to stimulate CD8+ T cell division.

Virus Ag persists for at least 40 d postinfection, in a form that can trigger division of naive 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 day 12 p.i. (Fig. 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.

![Graph](image)

**FIGURE 2.** Naive SMARTA CD4+ T cells divide in early immune mice, but not in late immune mice. The same mice described in Fig. 1 received CFSE-labeled SMARTA CD4+ T cells, and, 8 d 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 postinfection 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-d interval is plotted against the immune status of the recipient mice. The data points depict the average (with SEM) for two to four 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 two independent series of experiments. The data are representative of two independent experiments with two to three mice per time point.
and it stimulates some naive CD4+ T cells up to 8 wk. The finding that the stimulus has a 5–6 wk postinfection and the overall frequency of SMARTA CD4+ T cells at that time (Fig. 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 Ag (measured by SMARTA CD4+ T cell division) and CD4+ T cell numbers; as the quantity of stimulatory Ag diminishes, antiviral CD4+ T cells also decay in number until reaching a baseline of memory that does not require Ag.

**CD4+ T cell division is driven by Ag**

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 turn-over of the cells is a product of nonspecific global changes, then other CD4+ T cell populations should also divide when transferred into these mice. In contrast, if the observed changes are Ag 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 OVA (22) and do not react to LCMV infection. Sets of mice were immunized with LCMV or left unimmunized. Twelve days later, the mice received CFSE-labeled OTII CD4+ T cells, SMARTA CD4+ T cells, or P14 CD8+ T cells. Eight 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 (Fig. 3A). As expected, the SMARTA CD4+ T cells underwent cell division in the immune recipients but not in the naive hosts, and the

**FIGURE 3.** The CD4+ T cell division during the contraction phase is Ag 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 naive mice that were given rLM-OVA 2 d 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-d interval. (C) The bar graph shows the average percentage of donor transgenic T cells among all Vα2+ T cells. The data are from two to three 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. A total of 6 × 10^6 CFSE-labeled spleen cells from naive Thy1.1+ mice (B6.PL) was transferred to three groups of recipients, as follows: 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 d 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 d in the indicated recipients. The data represent one experiment with two uninfected recipients, one acutely infected recipient, and four LCMV-immune recipients.
P14\(^+\) CD8\(^+\) T cells showed minimal cell division in both recipients. OTI\(^I\) CD4\(^+\) T cells also showed minimal cell division in both the LCMV-immune and naive recipients, yet they were capable of undergoing cell division when exposed to acute rLM-OVA (rLM) OVA infection. A large fraction of the SMARTA CD4\(^+\) T cells underwent division in the LCMV-immune hosts, whereas few OTI\(^I\) cells underwent cell division in LCMV-immune or naive mice (Fig. 3B).

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

We followed polyclonal populations of T cells to evaluate whether they would undergo cell division in the contracting spleen. In uninfected mice, there are ~100 cells specific for gp61-80, 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 Ag. However, if the cytokine environment in the contracting spleen nonspecifically induces cell division, then these other populations of cells should divide. Therefore, a large population of CFSE-labeled naive splenocytes was adoptively transferred to the same three groups of recipients: uninfected mice, mice given acute LCMV infection, and LCMV-immune mice (Fig. 3D). After 8 d, 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 Ags, 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 naive SMARTA CD4\(^+\) T cells undergo cell division when in LCMV-immune mice due to persistence of viral protein Ag. 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 IFN-\(\gamma\) during primary infection with LCMV (1, 3). We evaluated whether these changes are sustained into the memory phase and impacted by residual Ag.

The SMARTA CD4\(^+\) T cells and endogenous populations of gp61-specific CD4\(^+\) T cells were identified at days 8, 20, and 45 by direct staining or by intracellular cytokine staining (ICCS) (Fig. 4A). When given saturating amounts of peptide (10\(^{-3}\) M), 66% of SMARTA CD4\(^+\) T cells made IFN-\(\gamma\) at day 8, and a slightly lower percentage (55%) made IFN-\(\gamma\)-\(\gamma\) at day 45. The IFN-\(\gamma\)-\(\gamma\) endogenous population was identified by ICCS in the same mice; at day 8, 3.6% of CD4\(^+\) T cells were gp61-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 IFN-\(\gamma\)-\(\gamma\) and IL-2 made per cell was quantified by ICCS at days 8, 20, and 45 (Fig. 4B).

The SMARTA and endogenous CD4\(^+\) T cells made high amounts of IFN-\(\gamma\)-\(\gamma\) at day 8 (Fig. 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 (Fig. 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 IFN-\(\gamma\)-\(\gamma\) 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 Ag, we quantified their cytokine responses to differing amounts of peptide (Fig. 4C). Spleen cells from these mice were exposed to different amounts of gp61-80 peptide, and their IFN-\(\gamma\)-production was measured by ICCS (Fig. 4C, top row). SMARTA and endogenous CD4\(^+\) T cells showed overlapping sensitivity to the peptide at day 8 (Fig. 4C), similar to our earlier report (1). The functional avidity of the cells remained at this level at days 20 and 45 (Fig. 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 (Fig. 4C, middle row, Fig. 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 IFN-\(\gamma\)-\(\gamma\) and IL-2. At all times, the SMARTA cells showed slightly higher sensitivity to peptide compared with endogenous CD4\(^+\) T cells (Fig. 4D), although the effect was minor.

These data indicate that transgenic and nontransgenic 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 (Fig. 4D), we infer that the lingering Ag does not affect these parameters. Instead, the residual Ag functions to sustain virus-specific T cell numbers.

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

To directly test the effects of T cell competition for lingering Ag, we compared the cell division of Ag-experienced CD4+ T cells and naive T cells in the same host using a dual adoptive transfer approach (Fig. 5C). To generate Ag-experienced CD4+ T cells, a small population of naive SMARTA/Ly5a+ CD4+ T cells was transferred into mice that were subsequently infected with LCMV, and, at day 12, these cells were harvested. The Ag-experienced day 12 cells were then mixed in equal proportion with naive
SMARTA/Thy1.1+ CD4+ T cells, labeled with CFSE, and transferred into either naive B6 mice or day 12 B6 mice; after 8 d, 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 Fig. 5D identify the naive 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 d 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 one round of cell division. As expected, neither naive SMARTA CD4+ T cells nor Ag-experienced CD4+ T cells underwent cell division when in the same uninfected mice (top histograms), as there was no cognate Ag present to stimulate their division. Consistent with the data in Figs. 2 and 3, the naive SMARTA CD4+ T cells underwent significant cell division in early immune mice (bottom left histogram), most likely due to the presence of residual viral Ag. In striking contrast, the Ag-experienced cells did not undergo cell division in these same hosts (bottom right histogram). An analysis of several mice showed that ∼74% of naive SMARTA cells divided at least once during this period, whereas only 1% of Ag-experienced SMARTA CD4+ T cells divided (Fig. 5E).

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

A particular concern with the above results was whether the Ag-experienced T cells that might have divided were selectively rejected by the recipients, perhaps as an unlikely artifact related to the choice of congeneric markers in the experiment. Therefore, we reversed the congeneric markers in another experiment, so that the naive SMARTA cells were Ly5a+ and the Ag-experienced SMARTA cells were Thy1.1+ (outlined in Supplemental Fig. 1A). Equal numbers of these two populations were CFSE labeled and transferred into naive or early immune B6 mice. After 8 d in vivo, the two donor populations were identified by flow cytometry (Supplemental Fig. 1B, dot plots), and their division status was evaluated (Supplemental Fig. 1B, histogram). Consistent with the data in Fig. 5, the SMARTA cells did not divide in the naive hosts. However, the naive SMARTA cells, but not the day 12 SMARTA cells, divided in the early immune mice (Supplemental Fig. 1C). Despite their ability to divide in the immune recipients, the naive SMARTA cells did not vigorously make IFN-γ ex vivo (Supplemental Fig. 1D); thus, the naive cells divide and accumulate, but across 8 d 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 Fig. 5 and Supplemental Fig. 1 show that naive and Ag-experienced CD4+ T cells differ intrinsically in their response to lingering Ag.

It has been suggested that CD8+ T cells are programmed early postinfection to undergo contraction through cellular apoptosis (41, 42). We considered that the day 12 Ag-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 Ag. Quiescent memory CD4+ T cells were isolated from mice immunized 45–68 d earlier. The memory CD4+ T cells and naive CD4+ T cells were mixed and labeled with CFSE and adoptively transferred into uninfected mice, acutely infected mice, or LCMV-immune (Fig. 6A). As expected, the donor cells did not undergo cell division in uninfected/nonimmune 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 naive CD4+ T cells in the same immune hosts underwent multiple rounds of cell division (Fig. 6B). Thus, memory CD4+ T cells do not proliferate in response to the residual Ag.

Ag-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 Fig. 5, we compared the ability of naive and Ag-experienced day 12 CD8+ P14+ T cell to undergo cell division when in the same naive or early immune mice (Fig. 7A). Consistent with the findings in Fig. 1, naive CD8+ T cells did not undergo cell division when transferred to naive or early immune mice (Fig. 7B, 7C). Even Ag-experienced CD8+ T cells that are highly sensitive to low amounts of peptide did not undergo cell division after residing in naive or early immune mice for 8 d (Fig. 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 gp33–41 peptide (Fig. 7D). Upon ex vivo stimulation, >90% of the Ag-experienced CD8+ T cells produced robust amounts of IFN-γ, whether they spent 8 d in naive mice or early immune mice (Fig. 7D, 7E). In contrast, few naive P14 cells (~1%) made IFN-γ after 8 d in the uninfected or LCMV-immune recipients, and the amount of IFN-γ produced...
among the cytokine-positive cells was very low. Thus, if MHC1-restricted Ag was presented in vivo, then it was ineffective over 1 wk to stimulate naive 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 rechallenge infection, memory CD8⁺ T cells immediately express effector functions and proliferate after an initial delay (43). To determine how the naive and Ag-experienced P14 cells would respond when confronted with live virus infection, both CFSE-labeled populations were transferred to the same naive host that was subsequently challenged with LCMV. At day 6, both naive and Ag-experienced P14 CD8⁺ T cells responded strongly and accumulated to represent 17–27% of CD8⁺ T cells (Fig. 7F). The cells underwent significant cell division during this time and had diluted CFSE (Fig. 7F, middle dot plots); cumulatively, >95% of the cells had divided and >80% were able to make IFN-γ (Fig. 7F, bar graphs). Thus, naive and Ag-experienced P14 CD8⁺ T cells were vigorous responders to live infection in which direct Ag presentation occurs efficiently along with inflammatory signals.

Cumulatively, the data in Figs. 5–7 indicate that lingering Ag during the early contraction phase preferentially stimulates the cell division of naive 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 Ag and their vigorous multicytokine output. The lingering Ag did not stimulate naive or Ag-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 impacts 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 Ag. Surprisingly, the Ag selectively stimulated naive CD4⁺ T cells and not Ag-experienced CD4⁺ T cells to undergo cell division and increase numerically. As discussed below, the finding that Ag remains long postinfection has several implications with regard to the relevance of cross-presentation of Ag, the effect of Ag on T cell contraction, and the delayed selection of virus-reactive T cells.

The lingering Ag 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 d (45) and Ag-bearing dendritic cells only survive 3–12 d (46), which is not long enough to support the effects that we observe several weeks postinfection (Fig. 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 (ICs) formed with model Ags and mAbs persist for 3–10 wk (48), and can be physically identified by immunostaining or by following the decay rate of radiolabeled IC. Subcutaneous vaccination with protein in WT mice also results in long-lived (>10-wk) depots of peptide Ag that stimulate the cell division of naive pigeon cytochrome C-specific CD4⁺ T cells that are located near Ag-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 Ag (50).

Interestingly, we found that the proportion of naive 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 and 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 as an Ag depot or is a destination for divided cells. Our analyses indicate the lingering LCMV Ag has a functional ½t of ~40 d (Fig. 2C). We considered that the residual Ag might be in the form of ICs 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 naive SMARTA CD4⁺ T cells divided in LCMV-immune B cell–deficient FcγRII-deficient mice (Supplemental Fig. 2). This implies that Ab-dependent complex formation is not required to retain the Ag. Perhaps nonspecific 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 Ag without inflammation induces hyporesponsive CD4⁺ T cells (51).

The effects we see are clearly dependent on residual Ag. CD4⁺ T cells will be poorly stimulated through the contraction phase in other infection models that deposit lower amounts of Ag. For example, mice that were infected with rLM expressing LCMV-gp100 do not have sufficient residual Ag to stimulate SMARTA CD4⁺ T cells (Supplemental Fig. 3A). Likewise, OTII CD4⁺ T cells did not undergo cell division when transferred a few days after rLM-OVA was cleared (Supplemental Fig. 3B). Listeria and LCMV differ in many ways beyond Ag abundance (e.g., tropism, inflammation), but informative patterns may emerge from similar analyses using other infection models, particularly whether the sustained Ag 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 with CD8⁺ T cell memory. Our results suggest the longevity of the Ag 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 Ag-driven emergence of new populations of naive CD8⁺ T cells; no such masking occurs for CD8⁺ T cell contraction, because the residual Ag is unable to recruit naive 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 Ag. Memory CD8⁺ T cells and memory CD4⁺ T cells survive over time in the complete absence of Ag 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 y post-infection has survived without Ag for many months, and the data in Fig. 5 indicate that early memory cells do not divide or...
accumulate to Ag, even when it is present. Nevertheless, it is plausible that the residual Ag stimulates memory CD4+ T cells to express prosurvival factors to protract the contraction phase.

Because 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 Ag presentation; it is a process whereby exogenous Ag is redirected to the MHC-I pathway to stimulate CD8+ T cells. Numerous investigators have advanced the notion that cross-presentation of Ag by dendritic cells is a significant mechanism for inducing CD8+ T cells (55, 56). In the LCMV model, residual viral Ag 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 Ag 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 naive CD8+ T cells than for CD4+ T cells. However, these possibilities seem unlikely given that the TCR expresses prosurvival factors to protract the contraction phase.

Division is higher for naive CD8+ T cells than for CD4+ T cells. Naive CD8+ 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 Ag, are cytolytic with 3 complexes, and require ~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 (subnanomolar versus ~100 nanomolar) (1, 60) (Fig. 4); the affinity of gp33 peptide for Db (Immune Epitope Database; IC50 = 5 nM) is greater than gp61 for I-Ab (IC50 = 128 nM) (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 divide, but not CD8 T cells, transit through an anatomical niche containing only MHCII-loaded Ag derived from long-lived ICs (49). Nevertheless, our data show clearly that virus-specific CD8+ T cells are unaffected by Ag that is sufficient to stimulate naive CD4+ T cells in the same mice.

Given that CD4+ T cell memory persists in the absence of Ag 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 reinfection. We considered that the Ag might be retained in the follicle/germinal center and that the CD4+ T cells that are stimulated to divide become T follicular helper cells to enhance humoral immunity; however, the LCMV-specific cells were CD44high but not distinctly CCR5$. 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 Ag (Figs. 1, 7). The quality and number of CD8+ T cell memory are impacted by the presence of memory 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 Ag stimulates memory T cells to express cytokine or carry out some other immediate function in vivo. In contrast, naive cells minimally acquire the ability to make IFN-$\gamma$ despite 8 d of exposure to lingering viral Ag (Fig. 5, Supplemental Figs. 1, 7). This finding is consistent with an earlier report that showed naive CD8+ T cells made little IFN-$\gamma$ 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 Ag may stimulate naive T cells to make TNF, perhaps to affect APC functions (72).

Our finding that Ag-experienced CD4+ T cells do not divide, whereas naive cells undergo cell division and accumulate (Figs. 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 naive T cells that are stimulated by lingering viral Ag without inflammation. Following the acute phase of influenza infection, late Ag presentation by certain dendritic cell subsets preferentially stimulates naive 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 naive CD4+ T cells can be recruited into the antigmammaherpesvirus response during latency (12). Thus, naive and memory T cells could respond similarly but differ in access to residual Ag in vivo, or it may be that naive and memory cells have equal access to Ag but differ intrinsically in how they respond to it. The recruitment of naive cells but not Ag-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 (e.g., T follicular helper or regulatory T) during rechallenge infection.

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

Disclosures

The authors have no financial conflicts of interest.

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