Precursor Frequency, Nonlinear Proliferation, and Functional Maturation of Virus-Specific CD4\(^+\) T Cells

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Precursor Frequency, Nonlinear Proliferation, and Functional Maturation of Virus-Specific CD4\(^+\) T Cells

Jason K. Whitmire, Nicola Benning, and J. Lindsay Whitton

The early events regulating antiviral CD4 responses were tracked using an adoptive transfer model. CD4\(^+\) T cell expansion was nonlinear, with a lengthy lag phase followed by 2 days of explosive proliferation. A small number of naive Ag-specific CD4\(^+\) T cells were found in nonlymphoid tissues and, in the 8 days following infection, the number of activated cells increased in all tissues analyzed, and their effector functions matured. Finally, we show that a naive mouse contains \(\sim 100\) naive CD4\(^+\) precursor cells specific for a single epitope, a precursor frequency of \(\sim 10^{-5}\), similar to that of naive CD8\(^+\) T cells, indicating that the \(\sim 50\)-fold difference in size of the two responses to virus infection is determined by something other than the number of precursor cells. The Journal of Immunology, 2006, 176: 3028–3036.

The CD4\(^+\) T cell plays a cardinal role in regulating the immune response, but our understanding of how CD4\(^+\) T cells change in quantity and quality over the course of virus infection remains incomplete. Studies in many model systems have shown that CD8\(^+\) T cells outnumber CD4\(^+\) cells by \(\sim 50:1\) at \(\sim 8–10\) days postinfection (1–4), and several hypotheses have been advanced to explain this difference. These include: different requirements for recurrent Ag contact; different sensitivities to other extrinsic factors such as cytokines or cosstimulatory molecules; and intrinsic differences in the proliferative potential of the cells. However, the simplest explanation—that CD4\(^+\) and CD8\(^+\) T cells may differ in the frequency of epitope-specific precursors—has not been rigorously tested. The measurement of precursor frequency is complicated by the difficulty in detecting the extremely low numbers of naive Ag-specific cells that exist in vivo; indeed, functional virus-specific CD4\(^+\) T cells may differ in frequency of epitope-specific precursors—has not been rigorously tested. The measurement of precursor frequency is complicated by the difficulty in detecting the extremely low numbers of naive Ag-specific cells that exist in vivo; indeed, functional virus-specific CD4\(^+\) T cells may differ in frequency of epitope-specific precursors—has not been rigorously tested. The measurement of precursor frequency is complicated by the difficulty in detecting the extremely low numbers of naive Ag-specific cells that exist in vivo; indeed, functional virus-specific CD4\(^+\) T cells may differ in frequency of epitope-specific precursors—has not been rigorously tested. The measurement of precursor frequency is complicated by the difficulty in detecting the extremely low numbers of naive Ag-specific cells that exist in vivo; indeed, functional virus-specific CD4\(^+\) T cells may differ in frequency of epitope-specific precursors—has not been rigorously tested. The measurement of precursor frequency is complicated by the difficulty in detecting the extremely low numbers of naive Ag-specific cells that exist in vivo; indeed, functional virus-specific CD4\(^+\) T cells may differ in frequency of epitope-specific precursors—has not been rigorously tested.

Our knowledge of how CD4\(^+\) T cell effector functions are regulated—and, perhaps, change—over the course of infection also lags behind that of CD8\(^+\) T cells. As viral load and tissue distribution evolve, there will be shifting demands for CD4 effector activities. It is, therefore, important to determine whether CD4\(^+\) T cells become effector cells very early, and simply increase in number thereafter, or if their differentiation continues throughout the expansion phase; an analysis of cytokine production at different times during the expansion phase may illuminate this issue. Another fundamental question concerns the tissue localization of Th cell responses. Most studies of CD4\(^+\) T cell responses have focused on lymphoid tissues, and much experimental evidence indicates that dendritic cells bearing foreign Ag migrate to these organs, where they prime naive CD4\(^+\) T cells; locally activated CD4 T cells then act on other resident cell types. However, MHC class II\(^+\) cells also are present in peripheral tissues. Do antiviral T cells move to these sites after lymphophytic choriomeningitis virus (LCMV)\(^3\) infection, and if they do, is this concurrent with the sequence of events in the lymphoid organs? Moreover, CD4 T cells maximize early CD8 responses in some models, and new evidence indicates that CD4\(^+\) T cells may be required to maintain the proliferative potential of CD8\(^+\) memory T cells. These recent observations raise other interesting questions related to where and when these T cell subsets communicate. Answers to these questions would hint at the cellular and molecular interactions that regulate CD4\(^+\) and CD8\(^+\) T cell responses.

In this study, we use an adoptive transfer model to measure the precursor frequency of naive CD4\(^+\) T cells. This approach also has allowed us to evaluate their early proliferation, as well as their functional maturation. We find that CD4 and CD8 T cells have a similar precursor frequency, of \(\sim 10^{-5}\). After virus infection, CD4\(^+\) T cell proliferation shows a striking lag phase, during which cell division is extremely limited. This is followed by an explosive 2-day multiplication, during which a new cell is generated, on average, every \(\sim 80\) ms. Our studies also allow us to evaluate the distribution of naive CD4\(^+\) T cells in nonlymphoid tissues, and to document the increases in virus-specific cells that occur in these sites after infection, concurrent with the period of rapid cell division. Finally, we show that, during the first few days of infection, the effector functions of CD4\(^+\) T cells undergo functional maturation, as previously reported for their CD8\(^+\) counterparts.

Materials and Methods

Mice and virus

C57BL/6 mice were purchased from The Scripps Research Institute (TSRI) breeding facility and used when 6–8 wk of age. SMARTA transgenic mice (9) produce CD4\(^+\) T cells expressing V\(\alpha\)2 and V\(\beta\)8.3 TCR specific for the LCMV epitope \(\text{g}_{681–690}\) and were provided to us by Dr. C.

\(^3\)Abbreviations used in this paper: LCMV, lymphophytic choriomeningitis virus; ICCS, intracellular staining; IEL, intraepithelial lymphocyte.
Surt (TSRI, La Jolla, CA). These mice were backcrossed to B6.Ly5a (also provided by Dr. C. Surt) to produce Ly5a+ SMARTA cells. In some cases they were backcrossed to the B6.PL background to produce Thy1.1-positive cells. P14 mice express the TCR for the LCMV gp33 MHC class I epitope (10). Mice were infected by i.p. administration of 2 × 10^9 PFU of LCMV, Armstrong strain. All experiments involving mice received prior approval from the institutional animal care and use committee.

**Flow cytometry**

Single-cell preparations of splenocytes were prepared without collagenase pretreatment (which has been reported to improve T cell recovery; Ref. 11) and were surface-stained directly ex vivo with combinations of anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti-Ly5.1 (Ly5a, clone A20), anti-Ly5.2 (Ly5b, clone 104), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-Thy1.1 (clone HIS51), and anti-Thy1.2 (clone 53-2.1) (all purchased from eBioscience). Anti-Via2 TCR (clone B20.1) and anti-V8.3 TCR (clone B1B3.3) were purchased from BD Pharmingen. The intracellular staining (IFCS) assay was performed as described previously (12). Cells were acquired by four-color flow cytometry using BD FACScalibur at the TSRI core facility and the data was analyzed with FlowJo software (Tree Star).

**Adoptive transfer**

Single-cell suspensions of splenocytes from SMARTA mice were counted, and defined numbers of Vα2+ and Vβ8.3+ donor cells were injected i.v. in a volume of 0.5–0.9 ml. The number of donor cells found in the spleen between 1 and 10 days posttransfer (the “take”) is ~10% of that transferred into the mice. Therefore, the number of SMARTA cells referred to in the text is 10% of the number injected into the mice. Unless otherwise indicated, mice were infected 1–2 days after cell transfer.

**Precursor frequency determination**

This method was adapted from Blattman et al. (5). Individual C57BL/6 mice were given different numbers of gp61–80-specific SMARTA spleen cells i.v. in 0.8 ml of RPMI 1640. The donor cells were either Ly5a or Thy1.1, allowing them to be distinguished from the host cells (Ly5b/Thy1.2). One to 2 days after transfer, the recipient mice were infected with LCMV and 8 days later, the gp61–80-specific CD4+ T cell responses of the host and donor populations were determined by intracellular IFN-γ staining. As described in the text, in one series of experiments the total number of injected cells was equalized by including 2 × 10^5 C57BL/6 spleen cells along with the different (but much smaller) numbers of SMARTA cells.

**Isolation of lymphocytes**

Lymphocytes were isolated from the spleen and lymph nodes (axillary,inguinal, brachial) using standard procedures. Cell isolation from nonlymphoid tissues was done as described previously (13). In brief, blood was drawn for preparation of PBMC, then the inferior vena cava was severed, and saline was perfused through the right ventricle of the heart using a syringe. The lobes of the lung were removed, diced, digested for 1 h with collagenase, filtered, and centrifuged through a Percoll gradient, from which cells were isolated. The liver was directly perfused with saline through the hepatic artery, mashed across a metal screen, filtered through a cell strainer, and cells were isolated from a Percoll gradient. The small intestine was dissected, and the Peyer’s patches were removed. The remaining material was diced into small pieces and, after vortexing, intraepithelial lymphocytes (IEL) were isolated from a Percoll gradient.

**Results**

**Regulation of marker expression, and of Ag-driven effector functions, is comparable in adoptively transferred SMARTA cells and wild-type gp61–80-specific CD4+ T cells**

One of the difficulties in studying antiviral CD4+ T cell responses is that Ag-specific cells are below detectable levels until ~5 days postinfection in wild-type mice; it is, therefore, difficult to quantify Ag-specific CD4+ T cell expansion before this time point. In this study, we circumvented this limitation by using an adoptive transfer model, in which TCR-transgenic CD4+ T cells specific for the LCMV MHC class II epitope gp61–80 are injected i.v. into wild-type C57BL/6 recipient mice. The donor cells were obtained from “SMARTA” mice (9) that had been bred to express the Ly5.1 (Ly5a) allele, rendering the transferred CD4+ T cells readily distinguishable from those of the host. Because our main goal, in this study, was to use SMARTA cells as an indicator of CD4+ T cell function and maturation following virus infection, it was important first to ensure that the responses of adoptively transferred SMARTA cells to virus infection were comparable to those of wild-type gp61–80-specific CD4+ T cells. A naive mouse with ~3 × 10^5 transferred SMARTA cells was infected with LCMV and, 8 days later, the progeny of these cells constituted the majority of CD4+ T cells in its spleen (Fig. 1A; ~5–10 × 10^5 SMARTA cells, 78% of the total CD4+ population), confirming the ability of these transferred cells to rapidly expand in vivo (14). The SMARTA cells were highly activated, as indicated by phenotypic markers (CD44high, CD62Llow, CD43high, and CD122high (Fig. 1B), and IL-7Rαlow (data not shown)); and they responded to peptide contact by making IFN-γ, TNF, and IL-2 (Fig. 1C), similar to wild-type virus-specific CD4+ T cells (3, 15–17). Furthermore, host cells and SMARTA cells displayed almost identical Ag responsiveness over a 10^4-fold range of peptide concentration; for both populations, the half-maximal response was achieved at a peptide concentration of ~5 × 10^-8 M (Fig. 1D). Hence, these TCR-transgenic cells resemble the endogenous CD4+ response in expansion, activation markers, cytokine expression, and Ag responsiveness. Finally, because CD4+ T cells have been reported, in some circumstances, to suppress CD8+ T cell responses (18), we wished to ensure that the transfer of SMARTA cells had no detrimental effect on the LCMV-specific CD8+ T cell response. This is especially important because CD8+ T cells play a key role in controlling LCMV infection, and any interference in this response might alter virus clearance and, consequently, change Ag load. As shown in Fig. 1E, CD8+ T cell responses at 8 days postinfection were essentially identical in the absence of SMARTA cells, and when 10^5 SMARTA cells were present at the time of infection.

**CD4+ T cell expansion in vivo is nonlinear; a prolonged lag phase precedes explosive proliferation**

Next, we wished to determine the proliferation rate of CD4+ T cells. This cannot be accurately estimated by merely enumerating the T cells at various times after virus infection, because cell abundance reflects the balance between cell proliferation and cell death; the proliferation rate is better achieved by measuring dilution of CFSE in the cell membrane. To evaluate CD4+ T cell expansion at the earliest times postinfection, CFSE-labeled SMARTA cells were adoptively transferred. Cell division rapidly dilutes the CFSE signal from progeny cells, so that to ensure that the progeny cells in the various generations were sufficiently numerous to be counted, mice with a large number of CFSE+ SMARTA cells (3 × 10^5 cells) were infected with LCMV, and cell expansion and CFSE content were measured at 2, 4, and 6 days postinfection. As shown in Fig. 2A, this method revealed a pronounced lag phase preceding explosive proliferation.
clonotypically identical cells can dramatically modify their capacity to proliferate (22). Thus, we considered it important to determine the range over which the expansion of SMARTA cells might be linearly related to the number initially transferred. Therefore, increasing numbers of SMARTA cells were transferred into C57BL/6 mice which were subsequently infected with LCMV and, 8 days later, SMARTA cells and gp61-specific host cells were enumerated. As shown in Fig. 2B, when between 1 and 10^3 SMARTA cells were present before infection, there was a linear increase in the number present at 8 days postinfection. The total number of gp61-specific cells (host plus SMARTA) was additive over this 1000-fold range of SMARTA cells, implying that the host and SMARTA cells proliferate equivalently, thus providing additional support for using transferred SMARTA cells as an indicator population. However, a plateau effect was observed when a very large number of cells was adoptively transferred; the number of SMARTA cells detected at 8 days postinfection was similar in mice that had contained either 10^4 or 10^5 SMARTA cells before infection, consistent with there being an inhibitory effect when a very large number of clonotypic cells is present. Therefore, we repeated our evaluation of the early kinetics of CD4^+ T cell expansion in response to virus infection, this time using groups of C57BL/6 mice that contained ~3 x 10^3 SMARTA cells. These mice were infected with LCMV, and the numbers of SMARTA cells were enumerated at 4, 6, 8, and 12 days postinfection (Fig. 2C and D). Once again, CD4^+ T cell expansion was nonlinear, this time with an even more prolonged lag phase of ~4 days. This was again followed by a dramatic 2-day burst of proliferation, during which there was a ~150-fold increase in cell number (approximately seven rounds of cell division), generating ~2 x 10^6 progeny cells—on average, a doubling time of 6–7 h during this 2-day period.

Naive virus-specific CD4^+ T cells found in nonlymphoid tissues show delayed expansion after infection

Previous studies have shown that, under normal circumstances, naive T cells are tightly restricted to lymphoid tissues, and it is in these tissues that they usually first encounter their cognate Ag, which has been captured by APCs in peripheral sites, and carried into draining lymph nodes. Although polyclonal virus-specific CD4^+ T cells have been detected in certain peripheral tissues (for example, the lung (23)) late in acute infection, when cell numbers are sufficiently high to permit their detection, the kinetics with which activated virus-specific CD4^+ T cells, and their naive precursors, enter many peripheral tissues is uncertain. We have used the adoptive transfer approach to provide a better understanding of the tissue distribution of naive virus-specific T cells, and to determine the abundance of virus-specific CD4^+ T cells in various lymphoid and nonlymphoid tissues at early stages of infection. The tissue distribution of naive virus-specific CD4^+ T cells was evaluated in mice that had received a large number of SMARTA cells; mice with ~10^5 SMARTA cells were maintained for 10–12 days before analysis, in an attempt to ensure that we were detecting cells that had survived the adoptive transfer. All tissues (except blood) were obtained from mice that had been perfused with saline. As shown in Fig. 3, the naive virus-specific SMARTA cells (CD4^+Ly5a^+) were, as expected, most abundant in the spleen and lymph nodes, and most of these cells were CD44^low/CD62L^high, as expected. The mice from which these SMARTA cells were derived
were not RAG-deficient, and it is known that, even in the absence of LCMV infection, some of the SMARTA cells—and, therefore, some of the transferred LCMV-naive cells—are CD44^{high}, providing an explanation for the small number of CD44^{high} cells in the spleen or brain, because the extraction procedure involves proteolytic digestion, which can denude cells of CD62L (24). Although we cannot distinguish between local cell division, and the ingress of cells that had divided elsewhere, the increased numbers of SMARTA cells observed between days 4–8 in peripheral tissues (Fig. 3) are concurrent with the burst of cell division observed in the spleen (Fig. 2).

The effector functions of CD4^{+} T cells mature over the course of infection.

CD8^{+} T cell effector functions mature in several ways during acute infection and in the early memory phase (25–27). To determine whether similar events occur for CD4^{+} T cells, the cytokine profile, peptide sensitivity, and IFN-\gamma production of SMARTA cells were measured at various times following virus infection. As previously reported for the CD8 response, there were qualitative and quantitative changes in the cytokine production by CD4^{+} T cells from days 4 to 12. First, the cells changed in the pattern of cytokines expressed following Ag stimulation (Fig. 4A). At early time points postinfection (days 4 and 6), IFN-\gamma/TNF-\alpha cells (single positive; top left quadrants) outnumbered IFN-\gamma/TNF-\alpha cells (double positive; top right quadrants) by \sim 2:5:1, but by day 8 the ratio had reversed, to \sim 1:2.5 and, by 12 days postinfection, almost 90% of the cells were double positive. This change is very similar
FIGURE 4. Effector functions of CD4+ T cells mature over the course of infection. A, C57BL/6 mice with 3 x 10^5 SMARTA cells were infected with LCMV, and, at the indicated times postinfection, the ability of the SMARTA cells to make IFN-γ and TNF in response to in vitro peptide stimulation was determined by ICCS. The plots are gated on Ly5a+ CD4+ T cells (i.e., only SMARTA cells are shown). Cells were incubated without (left column) or with (right column) gp61-80 peptide. The numbers indicate the proportion of cells in that quadrant, as a percentage of total SMARTA cells. B, The geometric mean fluorescence intensity (gMFI) of IFN-γ made by SMARTA cells at the indicated times after infection are shown. C, The relative amounts of IFN-γ produced by SMARTA cells (gMFI) at days 4 and 8 in response to a 10^6-fold range of gp61-80 concentrations (x-axis) were measured. For each of the two time points, the maximum response was assigned the value 100%, and the responses at lower peptide concentrations were plotted as a fraction of the maximum (y-axis). The dotted lines indicate the peptide concentrations required to drive a half-maximal response. D, P14 cells (CD8+ T cells carrying a transgenic receptor for the LCMV MHC class I epitope gp33) and SMARTA cells were adoptively transferred into different C57BL/6 mice, and, 8 days after LCMV infection, the proportion of each population that made IFN-γ in response to cognate peptide stimulation was determined by ICCS. The p value was calculated using the Student t test.

to that observed in CD8+ T cells during LCMV (27) or influenza virus (28) infections. In addition, the level of IFN-γ produced by the responding cells changed markedly over this time period, increasing in a near-linear manner, and more than doubling between days 4 and 12 (Fig. 4B). The cells also increased their ability to respond to low doses of peptide—that is, in their functional avidity. When the ability of the cells to express maximal levels of IFN-γ was determined over a 10^6-fold range of peptide concentration, day 8 cells required ~6-fold less peptide than did day 4 cells (Fig. 4C); an almost identical increase in functional avidity occurs between days 4 and 8 postinfection in P14 transgenic CD8+ T cells (26). Together, the data indicate that CD4+ T cells, like CD8+ T cells, mature functionally from days 4 to 8. Finally, we determined the proportion of SMARTA cells that were capable of making cytokine in response to Ag stimulation; this calculation was facilitated by our ability to readily identify all of the transferred Ag-specific T cells by surface staining. For comparative purposes, we conducted the same experiment using P14 mice, which bear the receptor for the LCMV gp41, epitope (10). SMARTA and/or P14 cells were adoptively transferred into wild-type mice, which were infected with LCMV and, 8 days later, the abundances of the two T cell populations were measured by ICCS (Fig. 4D). Based on 24 independent measurements of P14 responses, and 19 of SMARTA responses, the proportion of CD4+ T cells that respond to Ag contact by producing IFN-γ (~61%) is significantly lower than the proportion of CD8+ T cells that respond (~90%; p < 0.001). This result also suggests that, if IFN-γ expression was the sole criterion for enumerating virus-specific CD4+ T cells, prior measurements of their numbers may have underestimated their frequency.

The precursor frequency of naive CD4 T cells specific to a single viral epitope is ~10^-3.

The frequency of naive splenic CD8+ T cells specific for a single viral MHC class I epitope has been identified by the adoptive transfer of various numbers of TCR transgenic CD8+ T cells specific for a known viral epitope, and is estimated at ~10^-5 (5). However, the precursor frequency for CD4+ T cells has not been previously determined. To address this issue, differing numbers of SMARTA cells were transferred into recipient mice, and the relative proportions of SMARTA cells to gp61-80-specific host cells were determined by ICCS at 8 days postinfection. SMARTA cells could be enumerated using Thy1.1 and CD4 costaining, but to identify gp61-80-specific host cells, it was necessary to use ICCS. As shown in Fig. 5A, using IFN-γ-producing cells as the criterion, mice with ~10^4 SMARTA cells at the time of infection mounted gp61-80-specific responses that were almost entirely composed of donor cells, while the responses in mice with only a few (~1) SMARTA cells were reliant on host CD4+ T cells. The host and SMARTA responses were almost equivalent in mice with ~100 SMARTA cells, providing a first approximation of the precursor frequency of gp61-80 T cells in a naive C57BL/6 mouse. More detailed data are presented in Fig. 5B, in which a 10^5-fold range of SMARTA cells was used; these experiments suggest that a naive mouse contains ~60 gp61-80-specific CD4+ T cells. One factor complicating interpretation of these data is that the take of the donor cells could vary over the very wide range of cell numbers transferred, and any variation in the take could skew the estimates of precursor frequency. For example, if recipient mice can accommodate only a limited number of transferred donor cells, then the relationship between the number of cells transferred, and the number of cells retained by the recipient, may be nonlinear. To overcome this problem, a second approach was taken, in which different numbers of SMARTA cells were mixed with an excess (2 x 10^6) of C57BL/6 “filler” spleen cells; thus, recipient mice all received almost identical numbers of adoptively transferred cells, but with differing content of SMARTA cells. The data derived from one such experiment (two mice per group) are shown in Fig. 5C, and indicate a naive gp61-80-specific cell number of ~150 per mouse spleen. Taken together, the data suggest that the number of
LCMV-naive gp101–80-specific T cells is ~100, similar in magnitude to that reported for naive gp33–41-specific CD8 T cells (5). Thus, the ~10- to 50-fold difference in abundance of CD4+ and CD8+ T cells that is present during the anti-LCMV response (29, 30) is unlikely to be attributable to a difference in frequency of LCMV-naive precursors.

Discussion

Of the many factors that may influence the overall size of the adaptive T cell response, perhaps the most obvious is the frequency of naive precursor cells capable of responding to any given MHC/peptide complex. In theory, as many as 10^15 different αβ TCRs could be generated through somatic recombination (31). This number far exceeds the total number of naive T cells in a mouse spleen (~2 × 10^5) and it is, at first blush, surprising that so much potential diversity may be absent from individual animals. However, our conclusion has some caveats. First, it is possible that we have underestimated the number of naive gp101–80-reactive cells, because some wild-type TCRs may have weaker affinity than the SMARTA TCR for the I-Ab + gp101–80 complex, and such cells may not expand as vigorously as SMARTA cells. Second, a recent report indicated that LCMV-specific CD8+ T cells can kill target cells in vivo (37), raising the possibility that the transferred SMARTA cells might have directly reduced the viral load. However, CD4+ T cell-mediated in vivo killing appears to be relatively inefficient, because the half-life of peptide-coated target cells was ~20 h (37); this compares unfavorably with the in vivo cytolytic effects of CD8+ T cells, for which the half-life of epitope-expressing target cells is 1–4 h (12, 38, 39). Consistent with this, we found no detectable CD4-dependent in vivo cytotoxicity of gp101–80-coated target cells using a 1 h in vivo assay, during which 90–100% of target cells coated with LCMV CD8 epitope peptides (gp13 or nucleoprotein (NP)396) were lysed (data not shown). Moreover, CD4+ T cells alone are incapable of resolving the infection: CD8-deficient mice given SMARTA cells were unable to control LCMV infection (data not shown). Finally, SMARTA cell transfer did not negatively affect the virus-specific CD8+ T cell response, which is central to LCMV control and eradication (Fig. 1E).

Next, we evaluated the early events in CD4+ T cell expansion. Ag-driven proliferation and differentiation is responsible for most of the increase in T cell numbers, and the roles of Ag quantity and longevity in dictating the magnitude and quality of the primary and memory responses are areas of active investigation in several laboratories. For CD8+ T cells, even an abbreviated period of Ag stimulation leads to a primary response that is relatively normal in size, suggesting that CD8+ T cell expansion may be preprogrammed (40–42). Some evidence indicates that the same is true for CD4 T cells, because shortening the infectious period has no deleterious effect on the magnitude of the response (43, 44); however, other data indicate that CD4+ T cell responses are reduced in situations of low/brief Ag stimulation (42, 45). In Fig. 2, we show that the kinetics of the antiviral CD4+ T cell response is nonlinear, and has two key features: an initial lengthy lag phase, followed by an explosive 2-day proliferation. The lag phase, which extends for 4 days following LCMV infection (Fig. 2C), cannot be ascribed to the absence of LCMV-naive SMARTA cells from the spleen or lymph nodes (Fig. 3). Using two-photon microscopy (46), others have shown that naive T cells form transient, then stable, interactions with Ag-presenting dendritic cells during the first ~24 h following Ag delivery, and only then do they begin to proliferate (a simulation of the early events in T cell responses is proposed in Ref. 8). Thus, it is possible that, during the first 2 days of infection, Ag presentation is a limiting factor, preventing extensive CD4+ T cell expansion; however, very high titters of LCMV (~10^5 PFU/g) are present by 2 days following infection (47). Indeed, LCMV...
titers in the spleen peak at 3 days postinfection, and descend rapidly from days 3 to 6 (47), during which time CD4+ T cell expansion is maximal. Thus, it is possible that there may be active, Ag-independent regulation of CD4+ T cell expansion. For example, the delayed initiation of proliferation could reflect a dependence of CD4+ T cell responses on accessory molecules (29), which either stabilize the immunological synapse (48) or directly signal into the cells. Alternatively, the constraint on expansion may result from active inhibition, perhaps mediated by local inflammation; when this subsides, proliferation can begin. Once proliferation commences, it proceeds quickly, and appears to be uninterrupted, because all cells become CFSElow during a 2-day period. Previous estimates of CD4+ T cell proliferation assumed that it was continuous from the time of infection, and concluded that the doubling time of CD4+ T cells must be ~11 h, substantially slower than that of CD8+ T cells (49); however, our demonstration that the cells undergo an ~150-fold expansion in 2 days indicates that CD4+ T cells must divide every 6–7 h during this period. An elegant comparison of CD4+ T cell responses with CD8+ T cell responses led to the conclusion that CD4+ T cells are programmed to undergo limited proliferation, and that many are arrested after very few divisions (21). We could not identify any such proliferative CD4+ T cell arrest in this study. There are several possible reasons for this discrepancy; for example, different models of infection were used. It also may be significant that, in the published study, large numbers (2 × 10^6) of TCR-transgenic cells were transferred; others have shown that, when a high number of clonotypic cells is present, their ability to proliferate is limited (22), and we demonstrate herein a plateau effect when >10^4 cells are present at the time of infection (Fig. 2B). This in vivo restriction on CD4+ T cell proliferation may reflect a physiological control mechanism which sets the upper limit of clonotypic T cell expansion, a phenomenon that also affects CD8+ T cells (50, 51).

Several studies have followed CD4+ T cell trafficking to peripheral sites after infection (20, 23, 52–54). Influenza, Sendai, and respiratory syncytial virus infections recruit virus-specific CD4+ T cells into the lung and, after influenza infection, virus-specific CD4+ T cells in peripheral and lymphoid tissues express a variety of cytokines (20). In this study, the ability of LCMV-specific CD4+ T cells to traffic to several nonlymphoid tissues was examined (Fig. 3). Ten days after transfer, surviving LCMV-naive CD4+ T cells were present in the liver and lung. This contrasts with transferred LCMV-naive CD8+ cells, which showed very little trafficking into the lung or liver (55); however, in those studies, the transferred cells were analyzed 4 h after injection, suggesting the possibility that LCMV-naive T cells may, over a period of days, enter peripheral nonlymphoid tissues. The marker phenotype of LCMV-naive CD4+ T cells was evaluated, and naive cells in the spleen and lymph nodes were CD44low, as expected; surprisingly, the naive SMARTA cells in the liver of the same animals were uniformly CD44high, despite never having been exposed to LCMV Ag. Because the transferred SMARTA cells were obtained from an otherwise immunocompetent donor, it is possible that some of the cells carried an alternate TCR, allowing them to be activated by an undetermined Ag, and causing them to become CD44high; thus, it is possible that the accumulation in the liver may be specific for some Ag other than LCMV. Furthermore, others have recently shown that, during a virus infection, previously primed CD4+ T cells (but not naive cells) can be recruited to a peripheral tissue (lung) in a non-Ag-specific manner (56). However, we propose that the SMARTA cells in the liver may be physiologically distinct from those in other tissues, leading to their being selectively retained in that organ, as has been reported for activated T cells (57). It may be relevant that, in naive “Yeti” mice, which express yellow fluorescent protein under the control of the IFN-γ promoter, 30–90% of T cells in the liver are fluorescent, while T cells in lymphoid tissues are not (58). The liver has been proposed as a “graveyard” for T cells (59); perhaps the presence of these particular CD44high LCMV-naive SMARTA (and Yeti) cells in the liver reflects a physiological status that condemns them to imminent execution.

After infection, there was a striking increase in the percentage of SMARTA cells in the peripheral tissues. The percentages of SMARTA cells in the lung and IEL escalated concurrently with the onset of rapid proliferation in the spleen and, at 8 days postinfection, SMARTA cells were highly abundant in the liver, which has also been reported for CD8+ T cells after infection (24, 60–62). Nearly all of the SMARTA cells found in these organs after infection were activated, as indicated by elevated CD44 and reduced CD62L expression, consistent with what has been seen for CD4+ T cells after Sendai virus infection, and CD8+ T cells after Sendai, vesicular stomatitis virus, Rotavirus, and Listeria infections (23, 63, 64). Interestingly, activated SMARTA cells were particularly abundant in the intestinal epithelium after infection; the virus-specific population represents a major fraction of the IEL, implying directed (perhaps, Ag-dependent) accumulation at this site. An earlier analysis identified LCMV-specific CD8+ T cells among IEL, but failed to detect viral Ag (65); however, Ag was sought by immunohistochemistry, which may not have been a sufficiently sensitive approach. CD4+ T cell migration to the lung has been reported after influenza infection (20), and activated CD4+ T cells can be found in lung, liver, and intestinal lamina propria in addition to lymphoid tissues following Listeria infection (66). Furthermore, autoreactive CD4+ T cells travel to the CNS in models of experimental allergic encephalomyelitis (67). These data, from a number of different animal models, indicate that CD4+ T cells carry out widespread immunosurveillance, like their CD8+ counterparts. We have not yet determined whether the LCMV-specific CD4+ T cells in the peripheral tissues divide in situ, or if cell division occurs elsewhere. It also will be interesting to determine whether CD4+ memory T cells persist at these sites.

Finally, we show that CD4+ T cells improve functionally early in the antiviral response (Fig. 4). Like CD8 T cells, they acquire a double-positive phenotype for IFN-γ and TNF, and their sensitivity to peptide is increased; recent evidence indicates that the CD4 molecule increases the association of Lck with the immunological synapse, thus increasing sensitivity to Ag (68). However, despite this increased sensitivity, the concentration of peptide required to trigger IFN-γ production is greater for CD4+ T cells than for CD8+ T cells, consistent with a previous analysis (69); at day 8, the half-maximal response for gp140-specific CD4+ T cells is ~20 nM, ~10- to 100-fold greater than that reported for LCMV-specific CD8+ T cells (26). These changes improve the ability of these cells to respond to trace amounts of Ag. Interestingly, these events occur concurrent with the period of greatest expansion, suggesting a link between cell division and differentiation.

A major conclusion from these studies is that virus-specific CD4+ T cells and CD8+ T cells share similar precursor frequencies of ~100 cells that are able to respond to a given epitope, and this number may be common to mice and humans. The effector functions of both cell types mature over the days following virus infection, and both populations rapidly migrate to nonlymphoid tissues, where they can exert their physiological responses to infection. However, they differ both in the kinetics of expansion, and in their ultimate abundance. Our data show that the CD4+ T cell expansion phase itself has three phases: an early delay in the presence of Ag, followed by a fast expansion that leads up to a plateau that is markedly lower than the “set point” for CD8+ T cells. The
identification of these separate stages, and the factors that deter-
mine the set points for these two T cell populations, beg further
characterization at the molecular level. Ongoing studies are fo-
cused on dissecting the interactions, both positive and negative,
between these two populations, and on better understanding the
factors that regulate their expansion, contraction, and passage into
the memory phase.

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