Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection

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Numerous microbes establish persistent infections, accompanied by antigen-specific CD8 T cell activation. Pathogen-specific T cells in chronically infected hosts are often phenotypically and functionally variable, as well as distinct from T cells responding to nonpersistent infections; this phenotypic heterogeneity has been attributed to an ongoing reencounter with antigen. Paradoxically, maintenance of memory CD8 T cells to acutely resolved infections is antigen independent, whereas there is a dependence on antigen for T cell survival in chronically infected hosts. Using two chronic viral infections, we demonstrate that new naive antigen-specific CD8 T cells are primed after the acute phase of infection. These newly recruited T cells are phenotypically distinct from those primed earlier. Long-lived antiviral CD8 T cells are defective in self-renewal, and lack of thymic output results in the decline of virus-specific CD8 T cells, indicating that newly generated T cells preserve antiviral CD8 T cell populations during chronic infection. These findings reveal a novel role for antigen in maintaining virus-specific CD8 T cells during persistent infection and provide insight toward understanding T cell differentiation in chronic infection.
CD8 T cells, indicate a novel role for antigen in maintaining CD8 T cells in persistent infection.

RESULTS AND DISCUSSION
Polyoma virus (PyV) establishes a systemic persistent infection in mice (Fig. 1 A) (7). CD8 T cell responses to PyV infection are detected via binding of Db tetramers complexed to an immunodominant epitope encoded by aa 359–368 of the viral large T protein (LT359) (Fig. 1, B and C). After the acute phase of infection, LT359-specific CD8 T cells are detected as late as 560 d after infection (Fig. 1 B). At 246 d after infection, this population of antigen-specific cells is heterogeneous for expression of CD27, CD62L, CD127, and bcl-2 (Fig. 1 C). In addition, viral DNA is detectable in the spleen and other organs for at least that length of time (Fig. 1 A and not depicted) (7). We asked whether this phenotypic heterogeneity represented differentiation of cells primed during the acute phase of infection or resulted from an amalgam with cells newly generated during persistent infection.

We assessed the capacity of antiviral CD8 T cells to survive during persistent infection. T cells from mice infected by PyV 150 d earlier were transferred into infection-matched congenic hosts (Fig. 2 A). LT359-specific CD8 T cells of both host and donor origin were monitored over time. Whereas host CD8 T cells were maintained at a constant level, donor cells decreased over time (Fig. 2 B); the most rapid decline occurred over the first 3 wk, followed by a slower rate of attrition. This finding was unexpected because of the slower kinetics of virus-specific CD8 T cell loss during persistent infection in unmanipulated mice (Fig. 1 B). The transferred cells did not divide over 34 d (Fig. 2 C), although PyV-infected mice support homeostatic division of conventional memory CD8 T cells (Fig. 2 D), indicating that this cell loss is a cell-intrinsic phenomenon. Similarly, CD8 T cells from mice persistently infected by lymphocytic choriomeningitis virus (LCMV) fail to undergo homeostatic proliferation when transferred to naive mice (9). Because the PyV-specific host and donor CD8 T cells were elicited and maintained under identical conditions in this transfer experiment and coexist in the same environment, this data supports the hypothesis that CD8 T cells from PyV-infected mice are selectively lost during persistent infection.

Figure 1. Visualizing PyV infection. (A) Splenic PyV DNA levels ± SEM over time. (B) Number of splenic Dp-LT359 tetramer+ CD8 T cells ± SD over time. (C) Splenic CD8 T cell responses 246 d after infection. Plots are gated on CD8 T cells, and values reflect the percentage of tetramer+ cells in each quadrant (n = 3–9 mice).

Figure 2. Loss of PyV-specific CD8 T cells in an antigen-bearing host. (A) Experimental setup. (B) Host mice were bled at day 4 after transfer to establish a base level of Dp-LT359 tetramer+ cells of donor and host origin. PBLs were monitored over time, and values indicate the percentage of tetramer+ cells ± SEM of either host or donor origin normalized for input at day 4 after transfer (n = 4 mice). Two independent experiments were performed. The donor tetramer+ cell frequencies varied from 0.3–0.6% of CD8 T cells at day 4, and host tetramer+ frequencies varied from 1.3–3.8% of CD8 T cells. (C) Transferred PyV-specific cells were monitored over time for CFSE fluorescence. Plots are gated on donor tetramer+ cells. Values indicate the percentage of cells within the marked regions. (D) CFSE-labeled memory P14 cells were transferred to naive congenic mice (n = 3) or congenic mice infected by PyV 200 d earlier (n = 4). Spleens of secondary hosts were analyzed 36 d later. Plots are gated on P14 cells.
is inconsistent with the concept that antigen maintains the survival of antiviral CD8 T cells during chronic infection. To reconcile this discrepancy, we reasoned that host cells, but not donor cells, can be resupplied through thymic output, and perhaps new, naive PyV-specific CD8 T cells were being generated and subsequently primed during persistent infection.

To test this hypothesis, we induced partial hematopoietic chimerism in persistently infected mice using congenic bone marrow (Fig. 3 A). Such animals now have stem cells that give rise to a population of naive CD8 T cells distinguished either by host or donor allelic differences (Fig. 3 B). This protocol permits stem cell engraftment without irradiation, thereby limiting the perturbation of viral load or established T cell populations, as preexisting PyV-specific T cells are not affected by busulfan treatment alone (unpublished data) (7). Moreover, the emergence of donor naive T cells beginning at \( \sim 1 \) mo after busulfan conditioning is thymus dependent (16). Additionally, this protocol does not replace all bone marrow stem cells, and the population of newly developing T cells will be a mix of donor and host cells. 50 d after infusion of congenic bone marrow in busulfan-conditioned mice infected by PyV 115 d previously, animals were analyzed for PyV-specific CD8 T cells. As shown in Fig. 3 C, host-derived tetramer+ cells were detected in chimeric animals. Interestingly, a population of PyV-specific CD8 T cells originating from the donor population was also detected. This was not caused by contaminating naive or memory CD8 T cells in the donor cell inoculum, because transfer of the same number of bone marrow cells or splenocytes without busulfan did not generate a detectable donor cell population (Fig. 3 B). It has been estimated that thymic education occurs over the course of 3–4 wk (17). This is consistent with the inability to detect donor antigen-specific T cells earlier than 30 d after busulfan conditioning (unpublished data). Recruitment of recent thymic emigrants during the persistent stage of infection was not limited to PyV infection. We performed the same chimeric induction protocol in mice infected 120 d earlier by LCMV clone 13; at this time point, LCMV is only detected in the kidneys and brain (3). It is important to mention that this LCMV–mouse model is different from other models of persistent LCMV infection in which virus is detectable in the thymus (e.g., congenitally infected carrier mice, CD4-depleted LCMV clone 13–infected adult mice, and LCMV clone 13–infected adult mice approximately day

**Figure 3. Priming of new, naive antiviral CD8 T cells during persistent infection.** (A) Partial bone marrow chimerism protocol. (B) Mice infected by PyV 84 d earlier were either injected with splenocytes or bone marrow cells or treated with busulfan and injected with bone marrow cells the next day (\( n = 3–8 \) mice). Spleens were analyzed 42 d later and assessed for chimerism. Each plot contains 10^5 events, and values represent the percentage of lymphocytes within the indicated regions. (C) Mice infected by PyV 115 d earlier were treated with busulfan and injected with bone marrow cells as in A. Organs were analyzed for D-LT359 tetramer+ CD8 T cells 51 d after cell transfer. Plots are gated on donor or host CD8 T cells, and values indicate the percentage of tetramer+ cells. 10 and 8% of tetramer+ cells are of donor origin in the spleen and lung, respectively. (D) Mice infected by LCMV clone 13 120 d earlier were treated with busulfan and received bone marrow cells as in A. Organs were analyzed for D-gp276 tetramer+ CD8 T cells 48 d after cell transfer (\( n = 6 \)). Plots are gated on host or donor CD8 T cells, and values represent the percentage of CD8 T cells that are tetramer+. 7.5 and 9.4% of tetramer+ cells are of donor origin in the liver and lung, respectively. (E) Naive mice were thymectomized (thymx; \( n = 8 \)) or sham operated (sham; \( n = 6 \)). 45 d after PyV infection, spleens were analyzed for tetramer+ cells. The experiment was repeated twice with a combined p-value of 0.04.
30 after infection) (18, 19), but it more closely approximates
the low-level viral replication seen in persistent PyV infec-
tion and many persistent viral infections in humans. Fig. 3 D
depicts the presence of donor and host LCMV-specific CD8
T cells in chimeric animals.

To assess the contribution of newly primed T cells to the
long-term pool of antiviral CD8 T cells, we thymectomized
naive mice and determined the number of PyV-specific CD8
T cells after infection. Thymectomy significantly reduced the
number of tetramer+ CD8 T cells (Fig. 3 E). This is consist-
ent with our interpretation that continuous priming of new
thymic emigrants is required for the maintenance of the pop-
ulation of virus-specific CD8 T cells. Our data also support
the conclusions of a recent study exploring the contribution
of thymic emigrants to the CD4 T cell response, where pro-
longed antigen presentation occurs, despite the lack of infec-
tious virus (20).

Because the T cell priming environment may vary be-
tween acute and persistent phases of infection and differen-
tially affect T cell programming, we also analyzed PyV-specific
CD8 T cells for the expression of markers of differentiation.
As shown in Fig. 4 A, there are variations in expression of
CD27, CD62L, CD127, and bcl-2 between the cell populations
primed at different times. When the expression of either
CD27 or CD62L is monitored over time on the bulk popul-
ation of PyV-specific T cells, an interesting pattern emerges

![Figure 4](image-url)

**Figure 4. Dynamic phenotype of PyV-specific CD8 T cells.** Mice
infected by PyV 35 (A) or 95 (C) d earlier received busulfan and bone mar-
row cells as in Fig. 3 A. Organs were analyzed for tetramer+ CD8 T cells
45 (A) or 140 (C) d after cell transfer. Plots are gated on tetramer+ cells.
Host cells (black), donor cells (red), and host CD44+ CD8 T cells (blue) are
shown. In A, 6–7% of tetramer+ cells are of donor origin; in C, 5–6% of
tetramer+ cells are of donor origin (n = 3–8 mice). SPL, spleen; LG, lung.
(B) CD27 and CD62L expression on splenic tetramer+ cells over time.
Plots are gated on CD8 T cells, and values in plots indicate the percentage
of tetramer+ cells that express high levels of either CD27 or CD62L. The
plot for CD62L at day 246 is the same as in Fig. 1 C (n = 3–12 mice per
time point).
of newly emerging CD8 T cells to persisting antigens has primed to viral antigens long after initial infection. Priming late in persistent infection may be derived from more recent events, such as bcl-2 levels, by 140 d after induction of chimerism, consistent with the concept that newly primed T cells, being CD27hi and CD62Lhi (Fig. 4 A), are productively contributing to the pool of antigen-specific CD8 T cells and modify the phenotype of the entire virus-specific CD8 T cell population. Current evidence suggests that CD27hi T cells do not regain expression of CD27 and preferentially die (21). The data in Fig. 4 (A and B) are in line with the interpretation that the CD27hi PyV-specific CD8 T cell population is undergoing progressive attrition. Because PyV antigen is present up to 300 d after infection (Fig. 1 A), it is likely that antigen modifies the population of tetramer+ cells in at least two ways: antigen is required to prime recent thymic emigrants, which have a CD27hi CD62Lhi phenotype, but antigen can also induce down-regulation of CD27 and CD62L. Antigen-independent events may modulate the expression of these molecules as well.

Newly recruited antiviral CD8 T cells may differ qualitatively from their older counterparts. We have found that the number of newly primed cells making IFN-γ closely corresponds to the number of Db-LT359 tetramer+ cells (unpublished data). A comprehensive comparison of the functional attributes of new and old antiviral CD8 T cells should prove particularly informative in elucidating the contribution of each cell population to viral control.

Analysis of chimeric animals at late time points (Fig. 4 C) reveals a narrowing in the phenotype of virus-specific CD8 T cells primed over the course of persistent infection (compare with Fig. 4 A). Although some differences are still apparent, such as bcl-2 levels, by 140 d after induction of chimerism, splenic CD27 levels by host and donor PyV-specific T cells are similar. Because chimerism induction does not displace the original host stem cells (Fig. 3 B), new naive T cells can originate from either donor or recipient precursors. The priming environment is also dynamic over time in terms of antigen load and virus-associated inflammation, with these changes affecting the strength and frequency of T cell activation and, therefore, longevity. Jelley-Gibbs et al. recently demonstrated that influenza virus–specific CD4 T cells primed late in the response survive better than those recruited earlier, suggesting that early and protracted antigen encounter is detrimental to T cell survival (20). Collectively, these findings imply that many of the virus-specific CD8 T cells late in persistent infection may be derived from more recently primed cells.

Recent studies indicate a dependence on antigen for maintenance of antiviral CD8 T cells in chronic infections, although there are some exceptions (8–10, 22). Our data demonstrate that new, naive virus-specific CD8 T cells are primed to viral antigens long after initial infection. Priming of newly emerging CD8 T cells to persisting antigens has been suggested before, and a recent report shows de novo CD4 T cell responses to chronic infections in baboons after autologous bone marrow transplant (23–25). In a chronic LCMV infection model, continuous thymic output does not appear to be required to maintain CD8 T cell responses, although a T cell deficit was observed at day 30 after infection (26). In contrast, thymectomy is associated with loss of CTL activity in cats persistently infected by a feline lentivirus (27). Our data are consistent with reports describing antigen dependence for chronic pathogen-specific T cell maintenance, especially as newly primed cells may be important for preserving the population of virus-specific T cells (Fig. 3 E) (8–12). Moreover, the new, naive antigen-specific CD8 T cells are able to survive thymic education, despite persisting antigen, and exit to the periphery.

It has been suggested that memory–like CD8 T cells in chronic infections go through an abortive differentiation pathway, as dissected through phenotypic analysis of CD8 T cells in infected individuals (4, 15, 28). The lineage relationship between these stages is comprehensible only if the end cell stems from the beginning cell or its progeny. Our data indicate that new naive cells are continuously being recruited during the chronic phase of infection and that the time point of priming influences the phenotype of CD8 T cells. These observations affect our understanding of T cell differentiation pathways during chronic infections, as the T cell population analyzed will potentially contain cells primed at any time after infection.

In summary, we propose that ongoing selection and priming of naive CD8 T cells during persistent infection contributes to the maintenance of chronic memory–like cells. Phenotypic heterogeneity indicates programming differences in cells primed more recently, and their continual entry into the virus-specific T cell reservoir complicates interpretation of current models of lineage differentiation. In other situations in which antigen persists, such as cancer, autoimmunity, and organ transplantation, it will be important to address whether naive T cells continue to be recruited. Our findings may offer new avenues for therapeutic vaccination, as newly recruited cells may be more amenable to boosting strategies.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) female mice were purchased from the National Cancer Institute. B6/CD45.1 female mice were purchased from Taconic or bred in our facility. P14 transgenic mice bearing the TCR specific for the gp33-41 epitope of LCMV were bred on a B6/CD90.1 background in our facility. All mice were used between 4 and 10 wk of age. Thoracic thymi were removed from anesthetized mice, which were rested for at least 2 wk before infection. All protocols for animal studies were approved by the Institutional Animal Care and Use Committee of Emory University.

Viral DNA detection. DNA was extracted from frozen tissue using a DNA mini kit (QIAamp; Qiagen). PyV genomic copy numbers were determined by quantitative PCR (TagMan; Glen Research Corporation), as described previously (7). PyV DNA quantity is expressed in genome copies per milligram of tissue and is calculated based on a standard curve of known PyV genome copy numbers versus threshold cycle of detection. The detection limit with this assay is 10 copies of genomic viral DNA.
Viruses and cell transfers. Mice were infected with 2 × 10^6 PFU of PyV (strain A2) s.c.; 2 × 10^6 PFU LCMV clone 13 was administered i.v. Spleens from PyV-infected mice were harvested at the indicated time points and depleted of IgG^+ cells by panning on plates coated with anti-IgG antibodies (The Jackson Laboratory). Cells were labeled with 5 μM CFSE (Invitrogen) for 10 min at 37°C. 50 × 10^6 CFSE-labeled cells were transferred to infection-matched congenic hosts. Naïve B6/CD90.2 mice received 5 × 10^5 naive P14 cells i.v. 1 d before i.p. infection by 2 × 10^6 PFU of LCMV-Armstrong. 120 d after infection, memory P14 cells were isolated from spleens and labeled with CFSE, and 20 × 10^6 cells were transferred to naïve B6/CD90.2 mice or B6/CD90.2 mice infected by PyV 200 d earlier.

Cell isolation and flow cytometry. Femurs and tibias were crushed to isolate bone marrow cells. Single cell suspensions were made of RBC-lysed spleens. Animals were perfused before lung and liver isolation. Lung pieces were treated with EDTA and digested with collagenase with subsequent density centrifugation to isolate lymphocytes, as previously described (29). Single-cell suspensions were made from liver by passing tissue through nylon mesh, followed by density centrifugation to isolate lymphocytes (29). Antibodies to KLRG1 were purchased from Southern Biotechnologies, Inc. Antibodies to CD27, CD45.1, CD45.2, CD62L, and CD127 were purchased from eBioscience. Antibodies to KLRL1 were purchased from Southern Biotechnologies, Inc. Tetramers were constructed as previously described (7). All samples were collected on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.).

Bone marrow chimerism induction. Mice persistently infected either by PyV or LCMV clone 13 at least 35 d earlier received 600 μg bsulfafan i.p. (Busulfe; Orphan Medical). The next day, 25 × 10^6 cells isolated from the bone marrow of congenic mice were injected i.v.

Statistical analysis. Statistical significance for individual experiments was determined using the unpaired Student’s t test. The Mack-Skillings test was used to determine statistical significance of multiple experiments. A p-value ≤0.05 was considered statistically significant.

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