Programmed death-1 (PD-1) defines a transient and dysfunctional oligoclonal T cell population in acute homeostatic proliferation

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The host responds to lymphopenic environments by acute homeostatic proliferation, which is a cytokine- and endogenous peptide-driven expansion of lymphocytes that restores the numbers and diversity of T cells. It is unknown how these homeostatically proliferating (HP) cells are ultimately controlled. Using a system where lymphocytic choriomeningitis virus–immune C57BL/6 splenocytes were transferred into lymphopenic T cell–deficient hosts and allowed to reconstitute the environment, we defined the following three populations of T cells: slowly dividing Ly6C+ cells, which contained bona fide virus-specific memory cells, and more rapidly dividing Ly6C− cells segregating into programmed death (PD)-1+ and PD-1− fractions. The PD-1+ HP cell population, which peaked in frequency at day 21, was dysfunctional in that it failed to produce interferon γ or tumor necrosis factor α on T cell receptor (TCR) stimulation, had down-regulated expression of interleukin (IL)-7Rα, IL-15Rβ, and Bcl-2, and reacted with Annexin V, which is indicative of a preapoptotic state. The PD-1+ HP cells, in contrast to other HP cell fractions, displayed highly skewed TCR repertoires, which is indicative of oligoclonal expansion; these skewed repertoires and the PD-1+ population disappeared by day 70 from the host, presumably because of apoptosis. These results suggest that PD-1 may play a negative regulatory role to control rapidly proliferating and potentially pathogenic autoreactive CD8+ T cells during homeostatic reconstitution of lymphopenic environments.

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Abbreviations used: AAD, amino-actinomycin D; HP, homeostatically proliferating; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescence intensity; PD, programmed death; PD-L, PD-1 ligand.

Naive CD8+ T cells proliferate after being adoptively transferred into mice rendered lymphopenic by genetics (T cell knockout [KO] or SCID), irradiation, or cytokine induction by Toll receptor agonists or viral infections (1). This acute homeostatic proliferation is a compensatory mechanism to replenish the peripheral T cell pool. It has been estimated that only ∼30% of the naive CD8+ T cell population rapidly proliferate under lymphopenic conditions (2), perhaps because acute homeostatic proliferation of CD8+ T cells occurs by interaction between the TCR and MHC-expressing self- or environmental peptides (3, 4). These lymphopenia-driven CD8+ T cells have "memory-like" features, including the expression of the activation/memory marker CD44 and the ability to produce IFNγ on TCR stimulation (5–7).

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Previous studies have demonstrated that those naive CD8+ T cells whose TCR have high affinity to endogenous or self-antigens have an advantage for undergoing acute homeostatic proliferation (8). This suggests a potential to skew the TCR repertoire toward oligoclonal expansion, though this is sometimes difficult to see because so many T cells proliferate. However, autoreactive T cells have an enhanced ability to initiate autoimmunity during the reconstitution of lymphopenic environments, such as in the nonobese diabetic mouse model (9). In humans, T cell lymphopenia has been associated with autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and insulin-dependent diabetes mellitus (10–13). These findings suggest that lymphopenic conditions are potentially hazardous for immune regulation, and imply that some mechanism must regulate these processes to prevent higher incidences of autoimmunity. Thus far, it is unclear

which peripheral tolerance mechanisms are involved in the regulation of T cells responding to self- or environmental antigens during homeostatic reconstitution.

Acute homeostatic proliferation involves the division of T cells with different histories of antigenic exposure. The fate of bona fide antigen-specific memory cells, i.e., those cells that have undergone the full differentiation program after encountering a foreign antigen, differs from other memory-like cells undergoing acute homeostatic proliferation and expressing memory cell antigens. Our laboratory has shown that lymphocytic choriomeningitis virus (LCMV)– and Pichinde virus–specific CD8+ memory T cells of no fewer than seven specificities are relatively poor at undergoing acute homeostatic proliferation and become substantially reduced in frequency in comparison to other cells responding to a lymphopenic environment (1). We show that a molecule that distinguishes between the slowly dividing Ly6C+ bona fide memory cells and the rapidly dividing Ly6C− memory-like cells is programmed death-1 (PD-1).

PD-1 is a costimulatory molecule originally isolated by subtractive hybridization in a cell death–induced T cell line (14). PD-1 is a member of the CD28 gene family and is expressed on activated T, B, and myeloid cells. PD-1 has two known ligands, PD-1 ligand-1 (PD-L1) and PD-L2, which both belong to the B7 superfamily. PD-L1 is expressed on many cell types, such as T, B, dendritic, endothelial, and tumor cells. In contrast, PD-L2 is narrowly expressed on APCs (15). The cytoplasmic domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif, which can recruit the phosphatase SHP-2 after ligand engagement and inhibit TCR signaling (15). This inhibitory function of PD-1 can occur during autoimmunity, allergy, allograft rejection, antitumor immunity, and chronic virus infection, leading to dysfunctional T cells (16–18). C57BL/6 PD-1−/− mice develop a lupus-like syndrome, whereas BALB/c PD-1−/− mice have dilated cardiomyopathy, which is caused by autoantibodies against cardiac troponin I (19–21). Moreover, a single nucleotide polymorphism of PD-1 is associated with higher incidences of systemic lupus erythematosus, type 1 diabetes, and rheumatoid arthritis in humans (22–24). These findings suggest that PD-1 plays an important role to negatively regulate immune responses.

We show that PD-1 is expressed on a large subset of acute homeostatically proliferating (HP) cells isolated from lymphopenic environments. These PD-1+ HP cells undergo oligoclonal expansion, are dysfunctional, react with the apoptosis indicator Annexin V, and then disappear, suggesting PD-1 involvement in the elimination of autoreactive CD8+ T cells and the prevention of autoimmunity.

RESULTS

PD-1 is transiently expressed during reconstitution of lymphopenic environments

Our earlier study demonstrated that when CFSE-labeled LCMV- or Pichinde virus–immune spleen leukocytes were transferred into T cell–deficient hosts, the bona fide virus-specific memory cells underwent relatively limited cell division and were substantially diluted in frequency by other more extensively proliferating cells originating from that donor cell population (1). This pattern of division is seen in Fig. 1 A, where, 12 d after cell transfer, the viral peptide–specific CD8 T cells, as detected by intracellular IFNγ assay, were mostly in the CFSE-positive cells that had undergone limited numbers of divisions. We questioned how the slowly dividing population, which contained bona fide memory cells, differed from the rapidly dividing cells, which contained memory-like cells. As a preliminary screen, we performed a comparative genome-wide microarray analysis of genes expressed on sorted, rapidly proliferating (CFSE-low) and slowly proliferating (CFSE-high) CD8 cell populations, as described in Materials and methods. One of the most dramatic differences was in PD-1 mRNA levels, which were high in the CFSE-low population, but below reliable detection in the CFSE-high population. In addition, mRNA levels for IL-7Rα, IL-15Rβ, and Bcl-2 were 3–13-fold lower in the CFSE-low population than in the CFSE-high population. Based on the information obtained from the gene array experiment, we stained HP cells with PD-1–specific antibody 13 d after transfer and confirmed that this molecule was highly expressed on a large proportion of the CFSE-low population (28.6 ± 7.2%, $n = 4$), but on only a few cells in the CFSE-high population (1.0 ± 0.3%, $n = 3$; Fig. 1 B). Very little (5.8 ± 3.3%, $n = 3$) expression of PD-1 was seen in donor CD8 cells before adoptive transfer or on donor cells that had been transferred into normal C57BL/6 mice, where acute homeostatic proliferation does not occur because of the replete immune system (Fig. 1 B). We next measured the kinetics of PD-1 expression on CD8+ donor cells during reconstitution of the lymphopenic environment. Barely detectable before transfer, PD-1 expression slightly increased at day 7, peaked at day 21, and returned to pretransfer levels by day 70 (Fig. 1 C). As the PD-1+ cell frequency declined from day 21 to 70, the total donor cell number only moderately increased (Fig. 1 D), indicating a substantial loss in PD-1+ cell number. Expression of PD-1 on CFSE-low donor cells similar to those in Fig. 1 B was also seen in LCMV-immune donor cell populations transferred into B6 mice rendered lymphopenic by 600 Gy irradiation. Fig. 1 E is a representative plot from an experiment where 16 ± 9% ($n = 5$) of cells were PD-1+ and CFSE-low, whereas 1.4 ± 0.2% ($n = 5$) of cells were PD-1+ CFSE-high, at day 32. Because PD-1 is reported to regulate the expansion of self-reactive autoimmune T cells, we also tested the fate of transgenic HY male antigen-specific donor cells in a T cell KO environment (Fig. 1 F). Spleen CD8 T cells from female HY-transgenic mice contain ∼30% of cells expressing both the α and β TCR transgenes, whereas the rest represent a diverse T cell population expressing different TCR α proteins. In this and a second experiment, transferred transgenic HY CD8+ T cells dramatically expanded by day 6 in male T cell KO mice ($3.8 \pm 2 \times 10^5$, $n = 4$) in comparison to those in female KO mice ($1.9 \pm 1.4 \times 10^4$, $n = 3$) or lymphocyte-replete B6 male mice ($2.3 \pm 0.89 \times 10^4$, $n = 4$)

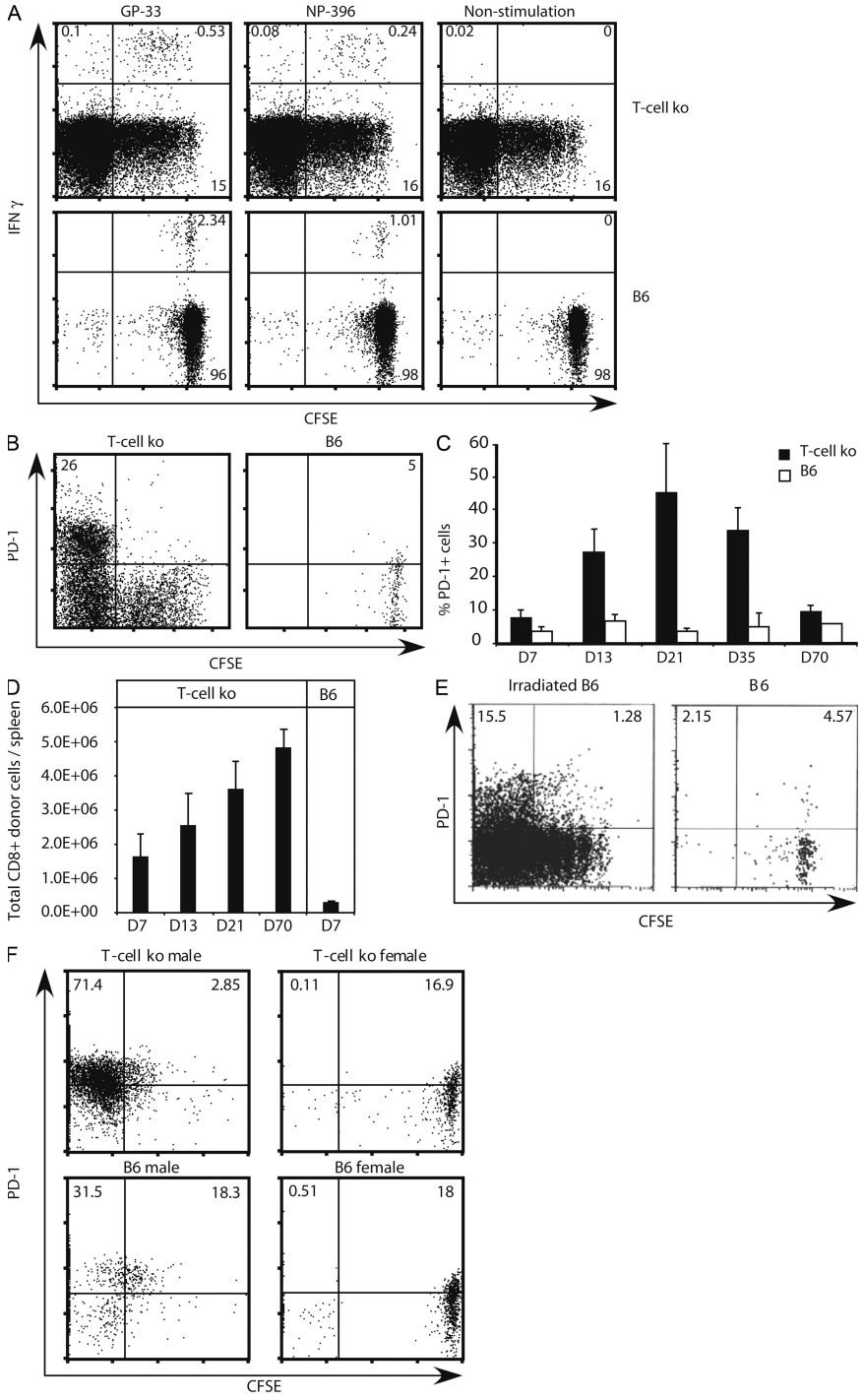

Figure 1. Dynamics of memory cell division and PD-1 expression on HP cells. Spleen leukocytes were isolated from Ly5.1-LCMV–immune mice, labeled with CFSE, and adoptively transferred into male T cell KO or B6 mice. (A) After day 12, leukocytes were stimulated with peptides and stained for surface makers and intracellular IFNγ. The epitope-specific IFNγ-producing CD8+ T cells were analyzed for intensity of CFSE. (B) On day 13, leukocytes were harvested and stained with anti-CD8, -Ly5.1, and –PD-1 antibodies. Gated CD8+ Ly5.1+ donor cells were analyzed for CFSE versus PD-1 intensity. This is representative of three similar experiments with two to five mice per group. (C) The time kinetics of expression of PD-1 on CD8+ Ly5.1+ donor cells in T cell KO or B6 mice. This is representative of two similar experiments with two to five mice per group. (D) The time kinetics of expansion of CD8+ Ly5.1+ donor cells in T cell KO mice or B6 mice. (E) Naive B6 mice were irradiated with 600 Gy. The day after irradiation, spleen leukocytes were isolated from Ly5.1-LCMV–immune mice, labeled with CFSE, and adoptively transferred into irradiated or unirradiated B6 mice. On day 32, leukocytes were harvested from irradiated B6 or unirradiated B6 mice and stained with anti-CD8, -Ly5.1, and –PD-1 antibodies. Gated CD8+ Ly5.1+ donor cells were analyzed for CFSE versus PD-1 intensity. (F) Spleen leukocytes were isolated from HY female mice, labeled with CFSE, and adoptively transferred into T cell KO or normal male or female B6 mice. On day 6, leukocytes were harvested are stained with anti-CD8, -HY TCR, and –PD-1 antibodies. Gated CD8+HY+ donor cells were analyzed for CFSE versus PD-1 intensity. This is representative of two similar experiments.

or female mice ($2.2 \pm 1.8 \times 10^4$, $n = 3$). The expression of PD-1 on the CSFE-low HY cells in the T cell KO male mice was elevated on >70% of the cells (Fig. 1 F, gated on the transgenic cells). In female mice, the transferred HY transgenic CD8+ T cells did not lose CSFE, and few expressed PD-1. In contrast, only $6.8 \pm 7.8\%$ ($n = 4$) of non-transgenic donor cells from HY mice expressed PD-1 on the CFSE-low cell population 6 d after transfer into T cell KO

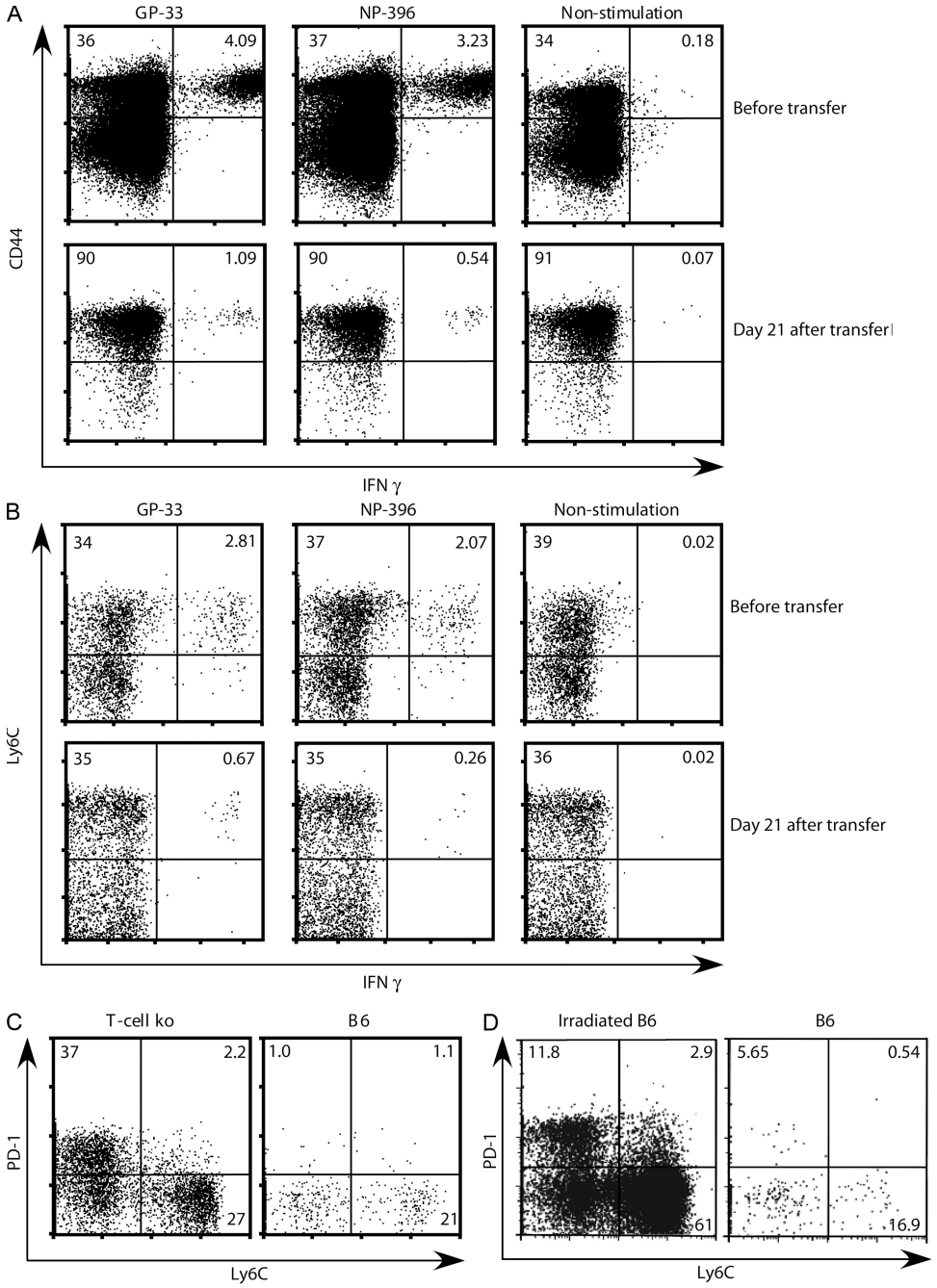

Figure 2. Ly6C distinguishes bona fide memory HP cells from PD-1+ HP cells. Spleen leukocytes were isolated from Ly5.1-LCMV–immune mice, and before or after adoptive transfer into T cell KO, leukocytes were stimulated with the indicated peptides and stained for surface antigens and intracellular IFNγ. The LCMV-specific memory T cells were identified by CD44 (A) or Ly6C (B) and IFNγ costaining before or after adoptive transfer into T cell KO mice. (C) At day 21, splenocytes from T cell KO or B6 mice were harvested and stained with anti-CD8, -Ly5.1, -Ly6C, and –PD-1 antibodies, revealing three distinct cell populations. This is representative of three similar experiments with three to five mice per group. (D) Spleen leukocytes were isolated from Ly5.1-LCMV–immune mice, and adoptively transferred into irradiated or unirradiated B6 mice. At day 32, splenocytes were harvested and stained with anti-CD8, -Ly5.1, -Ly6C, and –PD-1 antibodies, revealing three distinct cell populations. This plot is of one representative mouse out of five mice.

mice (unpublished data). These results suggest that self-reactive cells rapidly divide in a lymphopenic environment and express PD-1.

PD-1+ HP cells belong to the Ly6C− population

Bona fide memory CD8+ T cells express high levels of CD44 and Ly6C on their cell surface (25, 26). Fig. 2 A shows that most HP cells expressed CD44+ during homeostatic proliferation, so that molecule is not useful for differentiating bona fide memory cells from memory-like HP cells (Fig. 2 A). This experiment also confirms that virus-specific memory cells are diluted after homeostatic proliferation (1). In contrast, the overall percentage of Ly6C+ CD8 T cells was similar before and after homeostatic proliferation, and virtually all of the LCMV-specific bona fide memory cells were in this subset (Fig. 2 B). This result suggested that Ly6C might be a good marker to distinguish bona fide memory cells from at least some of the memory-like HP cells. We next addressed whether Ly6C was expressed on PD-1+ CD8 cells 21 d after transfer, which is when PD-1 expression was at its peak. As shown in Fig. 2 C, PD-1 + cells were nearly exclusively located within the Ly6C− population. Thus, by using PD-1 and Ly6C costaining, we could define three major populations: 45 ± 16% PD-1+Ly6C− (n = 5), 24 ± 8% PD-1−Ly6C− (n = 5), and 30 ± 8% PD-1−Ly6C+ (n = 5), with the first two subsets contained mostly within the rapidly dividing CSFE-low population, and the last subset containing virtually all of the slowly dividing bona fide memory cells. Donor cells transferred into WT C57BL/6 mice remained PD-1− and contained Ly6C+ and Ly6C− subsets (Fig. 2 C). Similar results were seen when irradiated mice were used as hosts. Fig. 2 D compares PD-1 to Ly6C expression and shows that 12 ± 8% (n = 5) of cells are PD-1+Ly6C− HP cells, 25 ± 5% (n = 5) are PD-1−Ly6C− HP cells, and 60 ± 8% (n = 5) are PD-1−Ly6C+ HP cells at day 32 in reconstituted irradiated C57BL/6 mice. We questioned whether these three subpopulations had other unique properties that distinguished them from each other.

Expression of activation molecules and growth factor receptors on the three HP subtypes

To characterize the three major populations distinguished by PD-1 and Ly6C costaining, we examined changes in expression of the activation markers CD43 and CD62L. CD43 is normally expressed on all CD8+ T cells, but a unique glycosylated form, detected by mAb 1B11, is expressed on activated T cells. 21 d after transfer, 79 ± 2% (n = 4) of the PD-1+Ly6C− HP cells and 34 ± 12% (n = 4) of the

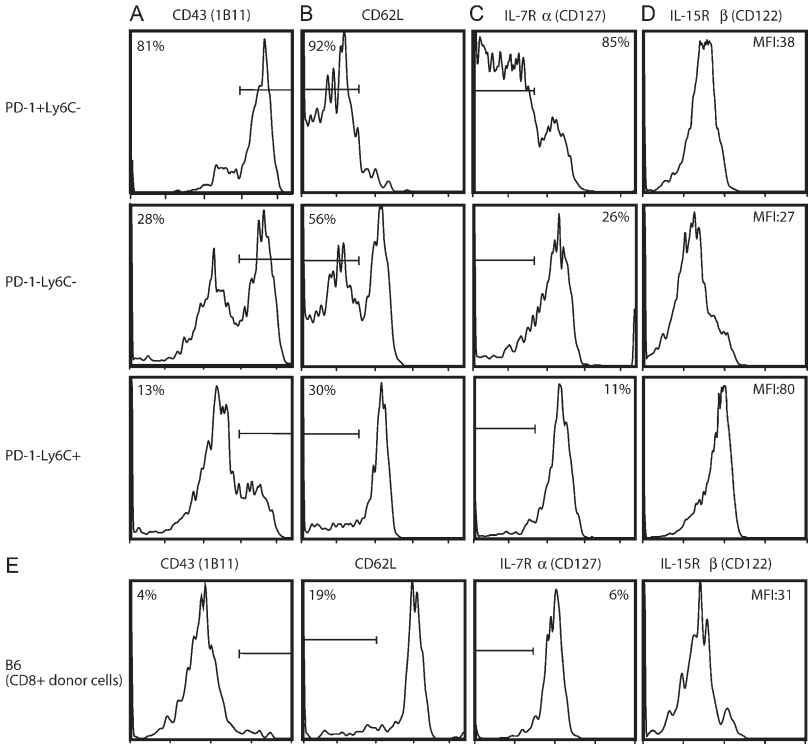

Figure 3. Expression of glycosylated CD43, CD62L, IL-7Rα, and IL-15Rβ on HP cells. Spleen leukocytes were isolated from Ly5.1-LCMV–immune mice, labeled with CFSE, and adoptively transferred into T cell KO or B6 mice. After day 21, splenocytes from T cell KO or B6 mice were harvested and stained with anti-CD8, -Ly5.1, -Ly6C, -PD-1, -CD43, -CD62L, -CD127, or -CD122 antibodies. The expression of glycosylated CD43 (A), CD62L (B), CD127 (C), and CD122 (D) in the individual populations was analyzed after gating on PD-1 and Ly6C costaining. This is representative of three similar experiments with three to five mice per group. (E) The expression of CD43, CD62L, CD127, and CD122 on CD8+ Ly5.1+ donor cells in B6 mice. Numbers on graphs, MFI.

PD-1−Ly6C− HP cells had elevated glycosylated CD43 expression (Fig. 3 A). However, only 14 ± 8% (n = 4) of the Ly6C+ HP cells had high levels of glycosylated CD43, suggesting that they were in a lower state of activation (Fig. 3 A). Naive and most memory spleen CD8+ T cells generally express high levels of CD62L, but CD62L is down-regulated on activated T cells (25, 26). On day 21, expression of CD62L was down-regulated on 90 ± 2% (n = 4) of the PD-1+ Ly6C− HP cells and 62 ± 5% (n = 4) of the PD-1− Ly6C− HP cells, but only on 36 ± 6% (n = 4) of the Ly6C+ HP cells (Fig. 3 B). These data suggest that the PD-1+ population was highly activated; that the Ly6C+ population was mostly resting, despite undergoing some limited division; and that the PD-1−Ly6C− population may be in some intermediate state. This is consistent with the concept that the PD-1+ population may be undergoing the greatest degree of homeostatic proliferation.

Previous studies have demonstrated that IL-7 or -7/-15 plays an important role in homeostatic expansion of naive or memory CD8+ T cells (27, 28), so we analyzed whether the expression levels of IL-7Rα (CD127) and -15Rβ (CD122) correlated with the proliferation status of the three major populations. As shown in Fig. 3 C, 76 ± 11% (n = 4) of the PD-1+ HP cells had dramatically decreased expression of IL-7Rα, but IL-7Rα was expressed at normal high levels on the other two populations (PD-1−Ly6C−, 72 ± 4%, n = 4; PD-1−Ly6C+, 86 ± 3%, n = 4; Fig. 3 C). Other studies have demonstrated that memory CD8 T cells express IL-15Rβ, which is important for IL-15–mediated induction of proliferation and survival signals. As shown in Fig. 3 D, Ly6C+ HP cells, which contain the bona fide memory cells, were IL-15Rβ–high (mean fluorescence intensity [MFI] = 72 ± 7, n = 3), PD-1+ HP cells were IL-15Rβ–intermediate (MFI = 33 ± 4, n = 3), and PD-1−Ly6C− HP cells were IL-15Rβ–low (MFI = 25 ± 2, n = 3). Fig. 3 E portrays the expression of CD43, CD62L, IL-7Rα, and IL-15Rβ on the total transferred CD8+ T cells in WT C57BL/6 mice 21 d after transfer.

Most PD-1+ cells react with Annexin V+

In other systems of T cell activation, the down-regulation of IL-7Rα expression correlates with reduced viability and impending apoptosis (29, 30). Many of the PD-1+ cells were low in IL-7Rα expression and did not express the high levels of IL-15Rβ that might be needed to compensate for that loss. We thus questioned whether the PD-1+ HP cells, despite their high levels of activation and proliferation, might be a population with a propensity for cell death. To address whether cell death was involved in the progression of homeostatic proliferation, CD8+ donor cells were stained with Annexin V 21 d after transfer. The CFSE-low populations were 36 ± 10% Annexin V+ cells (n = 7), but the CSFE high population had only 2.4 ± 1% Annexin V+ cells (n = 7; not depicted), suggesting that high levels of apoptosis might accompany homeostatic proliferation. When the three subpopulations were analyzed for reactivity with Annexin V, the PD-1+ HP population contained most of the Annexin V+ cells (MFI = 3,553 ± 320, n = 5), and the other two subsets had few Annexin V+ cells (PD-1−Ly6C− HP cells, MFI = 1,246 ± 86, n = 5; PD-1−Ly6C+ HP cells, MFI = 1,054 ± 126, n = 5; Fig. 4 A). Many of the PD-1+ HP cells expressed low levels of IL-7Rα (Fig. 3), and -7 is an important survival factor of T cells and acts to maintain the expression level of survival

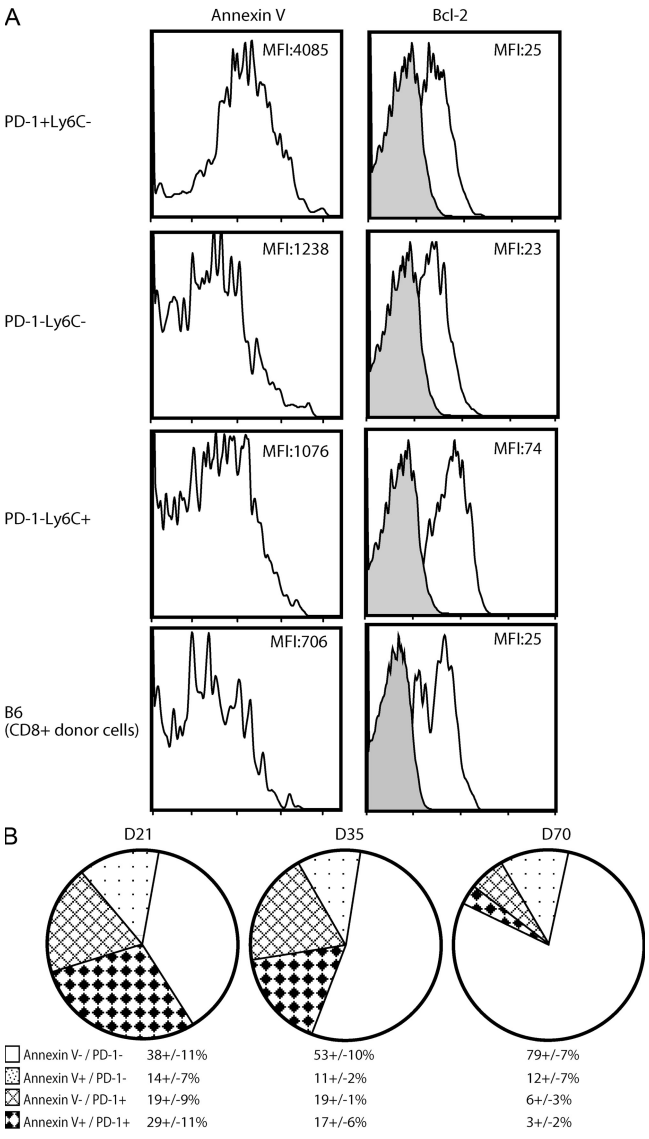

Figure 4. Expression of Annexin V–binding reactivity and Bcl-2 on HP cells. Spleen leukocytes were isolated from Ly5.1-LCMV–immune mice, labeled with CFSE, and adoptively transferred into T cell KO or B6 mice. After day 21, leukocytes from T cell KO or B6 mice were harvested and stained with anti-CD8, -Ly5.1, –PD-1, -Ly6C, –Annexin V, or 7AAD antibodies. (A) Annexin V binding or Bcl-2 expression (filled region, isotype control; open region, Bcl-2) in the individual populations was analyzed after gating on PD-1 versus Ly6C populations. (B) The time kinetics of expression of PD-1 versus Annexin V binding at days 21, 35, and 70. This is representative of two to three similar experiments with three to five mice per group.

molecules such as Bcl-2 (31). Fig. 4 A shows that PD-1+Ly6C− and PD-1−Ly6C− HP cell populations both expressed low levels of Bcl-2 (PD-1+Ly6C− HP cells, MFI = 17.2 ± 6.9, $n = 3$; PD-1−Ly6C− HP cells, MFI = 17.2 ± 5.2, $n = 3$). In contrast, the Ly6C+ HP cells, which contained the bona fide memory cells, expressed much higher levels of Bcl-2 (MFI = 51.1 ± 20.6, $n = 3$). The bottom row of Fig. 4 compares this to the binding of Annexin V (MFI = 746 ± 56, $n = 2$) and the expression of Bcl-2 (MFI = 24.5 ± 0.4, $n = 2$) on total transferred CD8+ cells in WT C57BL/6 21 d after transfer. Fig. 4 B shows that 61% of gated PD-1+ HP cells stained with Annexin V+ at day 21 (comparing PD-1+/Annexin V+ and PD-1+/Annexin V− populations), suggesting that most of the PD-1+ HP cells were in a preapoptotic state 21 d after homeostatic proliferation. Fig. 4 B shows that this apoptotic cell population declines over time; as 29 ± 11% ($n = 12$) of the HP cells were PD-1+Annexin V+ at day 21, decreasing to 17 ± 6% ($n = 6$) at day 35, and then to 3 ± 2% ($n = 8$) at day 70, when there were very few remaining PD-1+ cells. These results demonstrated that PD-1 expression correlated with the Annexin V+ preapoptotic state and the presumed elimination of a cell population during homeostatic proliferation.

Deficient cytokine production by the PD-1+ HP cells

Previous studies have shown that cytokine production can be inhibited in virus-specific PD-1+ CD8+ T cells during chronic viral infection, and that interference with PD-1 signaling can restore the cytokine production in exhausted CD8+ T cells (32–34). We found that in the CSFE-low populations 21 d

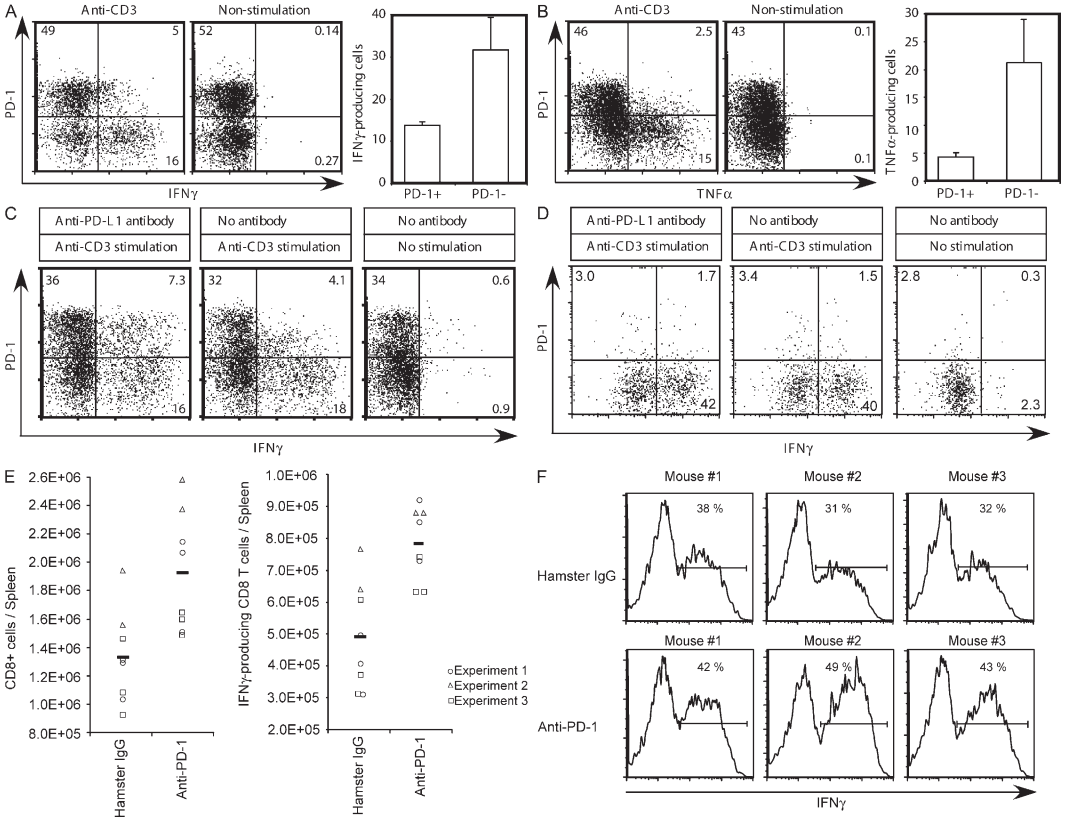

Figure 5. Poor IFNγ- and TNFα-production by PD-1+ HP cells. Spleen leukocytes were isolated from Ly5.1-LCMV–immune mice, labeled with CFSE, and adoptively transferred into T cell KO mice. After day 20 (A) or 21 (B), splenocytes from T cell KO mice were harvested and stained with anti-CD8, -Ly5.1, –PD-1, -IFNγ, or -TNFα antibodies. (A or B) The expression of IFNγ or TNFα versus PD-1 was analyzed on gated CD8+ Ly5.1+ donor cells. This is representative of three similar experiments with three to five mice per group. (C) Splenocytes from T cell KO mice after 14 d of reconstitution were harvested, and 10^6 cells were seeded into 96-well plates and treated with 25 μg/ml anti–PD-L1 antibody for 3 d. Cells were stained with anti-CD8, -Ly5.1, –PD-1, and -IFNγ antibodies. The expression of IFNγ versus PD-1 was analyzed on gated CD8+Ly5.1+ donor cells. This is a representative result of nine mice tested in three experiments. (D) Splenocytes from T cell KO mice ($n = 4$) after 14 d of reconstitution were harvested, and CD8+PD-1+ cells were removed by cell sorting. 10^6 cells of the CD8+PD-1+–depleted population were seeded into 96-well plates and treated with 25 μg/ml anti–PD-L1 antibody for 3 d. The expression of IFNγ versus PD-1 was analyzed on gated CD8+Ly5.1+ donor cells after stimulation for 4 h with anti-CD3. This is a representative result of two experiments (four mice per group). (E) This displays the total number of CD8+Ly5.1+ donor cells or IFNγ-producing CD8+Ly5.1+ donor cells harvested from 20–23-d reconstituted T cell KO mice, which had been treated with hamster IgG ($n = 8$) or anti-PD-1 antibody ($n = 8$) for 6 d. (F) Increase in CD8+ IFNγ-producing cells after 6 d treatment of anti–PD-1 antibody in vivo. This displays the FACS profiles from the experiment in E that were designated by circles.

after transfer, 20 ± 6% (n = 10) of the cells produced IFNγ and 12 ± 6% (n = 7) produced TNFα after anti-CD3 stimulation, suggesting that some memory-like cells have the capacity to make cytokines while undergoing acute homeostatic proliferation (not depicted). Experiments designed to examine the functional capacity of PD-1+ cells showed, on average, that only 13 ± 4% (n = 4) of the PD-1+ cells produced IFNγ after anti-CD3 stimulation, in contrast to 32 ± 3% (n = 4) of the PD-1− HP cells (Fig. 5 A). Similarly, only 4 ± 1% (n = 3) of the PD-1+ HP cells produced TNFα, whereas 21 ± 8% (n = 3) of PD-1− HP cells produced TNFα (Fig. 5 B). Those PD-1+ cells that did produce cytokines had lower levels per cell than did the PD-1− cells (Fig. 5, A and B).

Because the bona fide memory cells in the Ly6C+ PD-1− population may have biased the results by being the major contributors to cytokine production, we also compared the production of IFNγ and TNFα in the Ly6C− population, separated into PD-1+ or PD-1− cells. Only 10 ± 1.1% (n = 4) of PD-1+Ly6C− cells produced IFNγ after anti-CD3 stimulation, in contrast to 37 ± 1.8% (n = 4) of the PD-1−Ly6C− cells (not depicted). Similarly, only 4.1 ± 2.5% (n = 3) of the PD-1+Ly6C− cells produced TNFα, in contrast to 40 ± 7.2% (n = 3) of the PD-1−Ly6C− cells. The abilities of these three fractions to synthesize IFNγ or TNFα after anti-CD3 stimulation are summarized in Table I.

We next tested whether the expression of PD-1 may be a cause of the T cell dysfunction by incorporating 25 μg/ml anti–PD-L1 mAb into cultures of day 14 HP cells for 3 d. The frequencies of PD-1+ cells producing IFNγ were significantly enhanced in the presence of anti–PD-L1 mAb (No antibody treatment, 6.6 ± 2.5%, n = 9; anti–PD-L1 mAb treatment, 9.3 ± 2.2%, n = 9; P = 0.03; Fig. 5 C), whereas the percentage of PD-1− IFNγ-producing cells was not significantly changed (No antibody treatment, 16 ± 5.5%, n = 9; anti–PD-L1 mAb treatment, 15 ± 5.4%, n = 9; P = 0.6; Fig. 5 C), suggesting that the anti–PD-L1 blocking mAb only affected PD-1−expressing cells. Of note is that there was hardly any IFNγ production by the highest expressing PD-1+ cells, but anti–PD-L1 mAb enabled some of these high-expressing cells to make IFNγ (Fig. 5 C). Because it was possible that the high-expressing PD-1+ cells that made IFNγ had been derived from a PD-1− population within the culture, we sorted out CD8+PD-1+ cells from the ex vivo cell populations before putting them into culture. Fig. 5 D shows that PD-1+ cells did not appear in these PD-1–depleted 3-d cultures, and that the proportion of cells in the culture making IFNγ was higher than that in the cultures retaining the PD-1+ cells. In those PD-1–depleted cultures, there was no effect of the anti–PD-L1 antibody. Thus, the anti–PD-L1 treatment seems to restore cytokine production by PD-1+ cells. Similar results were seen in the HY transgenic T cell system, where 7.5 ± 1.2% (n = 4) of the PD-1+ HY cells derived from 6-d transferred T cell KO mice produced IFNγ after HY male peptide stimulation in vitro, in contrast to 16 ± 4.9% (n = 4) of the PD-1− HY cells (not depicted). These results with HP cells are consistent with published work in other systems on the inhibitory properties of these molecules (32, 33, 35).

Having shown that interference with PD-1 signaling enhances IFNγ production in vitro, we tested whether interference with PD-1 signaling in vivo would lead to an increase in the number of CD8 T cells. Sufficient quantities of antibody to PD-1 were available to us, but we needed to test different timing regimens and doses to establish an effect. We found that giving day 20–23 T cell KO mice (reconstituted with LCMV-immune splenocytes) 2 i.p. injections of 200 μg of antibody to PD-1 (clone J43; or control hamster IgG) in 200 μl PBS 3 d apart was effective at increasing CD8 T cell numbers 3 d later, or a total of 6 d after initiation of treatment. In 3 experiments with a total of 8 mice for each treatment, anti–PD-1 treatment, in comparison to a control hamster antibody, caused a 56, 43, and 36% increase in total donor CD8 T cell number, and a 105, 26, and 56% increase in the total number of donor CD8 T cells capable of synthesizing IFNγ after anti-CD3 treatment in a 5-h in vitro assay. This amounted to averages of 1.9 ± 0.42 × 10^6 CD8 T cells (treated) versus 1.3 ± 0.33 × 10^6 cells (control; P = 0.007; Fig. 5 E) and 7.9 ± 1.2 × 10^5 IFNγ-producing CD8 cells (treated) versus 4.9 ± 1.7 × 10^5 cells (control; P = 0.001; Fig. 5, E and F). Fig. 5 E shows all of the individual values from three experiments, where each experiment is designated by a unique symbol. Fig. 5 F shows the IFNγ-secreting cell FACS profiles of individual mice from one of the experiments. These results are consistent with the concept that PD-1 signaling inhibits the outgrowth of functional T cells, at least at this time point of homeostatic proliferation.

Bona fide memory transgenic T cells are PD-1− Annexin V− and can produce IFNγ during acute homeostatic proliferation

We have interpreted our failure to find LCMV-specific memory CD8 T cells in the PD-1+ population to indicate that bona fide memory cells do not fall into this population. However, we used peptide-induced IFNγ production to monitor LCMV-specific T cells, and Fig. 5 shows that the PD-1+ population is functionally defective in regards to

Table I. The phenotypes of the three major populations during homeostatic proliferation

	CD43	CD62L	IL-7Rα	Il-15Rβ	Annexin V	Bcl-2	IFNγ	TNFα
PD-1+Ly6C−	high	low	low	intermediate	high	low	low	low
PD-1− Ly6C−	low/high	low/high	high	low	low	low	high	high
PD-1−Ly6C+	low	high	high	high	low	high	high	high

cytokine production. By these criteria we cannot rule out the presence of dysfunctional bona fide memory cells within the PD-1+ population. It is difficult to get convincing tetramer staining of these low frequencies of memory CD8 T cells under these conditions of acute homeostatic proliferation, so we did analyses using P14 transgenic memory CD8 T cells, which can be identified by anti-Vα2 and -Vβ8.1/8.2 staining. We generated P14-transgenic memory CD8 T cells in C57BL/6 mice, as described in the Materials and methods, and then adoptively transferred the memory T cell–containing splenocytes into T cell KO mice to address this question. As shown in Fig. 6 A, 94% of the P14-transgenic memory CD8 T cells remained negative for expression of PD-1 in T cell KO or C57BL/6 hosts 20 d after transfer. Furthermore, P14-transgenic memory CD8 T cells were mostly Annexin V− during homeostatic proliferation, in contrast to some of the nontransgenic Ly5.2+ CD8+ donor cells within the same population (Fig. 6 B). In addition, Fig. 6 C shows that 96% of the P14-transgenic memory CD8 T cells produced IFNγ after GP-33 peptide stimulation. These results indicate that bona fide memory CD8 cells remain functional and do not express PD-1 under conditions of acute homeostatic proliferation.

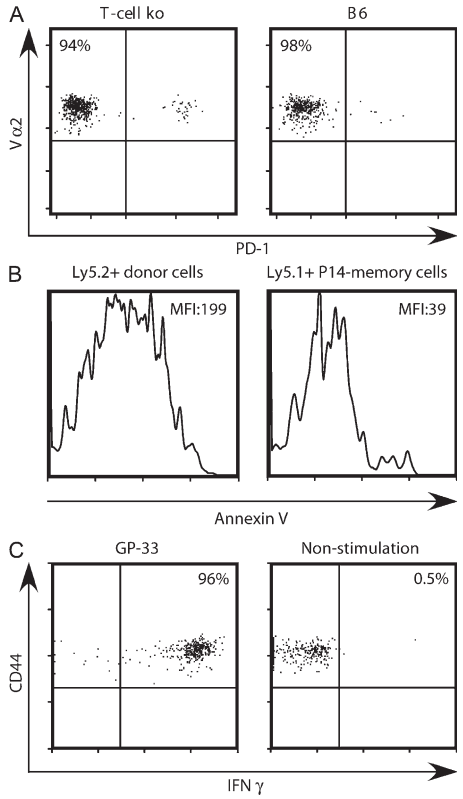

Figure 6. P14-transgenic memory CD8 T HP cells are PD-1− Annexin V− and can produce IFNγ after GP-33 stimulation. The generation of Ly5.1+ P14-transgenic memory CD8 T cells is described in the Materials and methods. Spleen leukocytes were isolated from Ly5.2-LCMV–immune mice containing Ly5.1+ P14 memory CD8 T cells. Cells were labeled with CFSE and adoptively transferred into T cell KO mice or B6 mice. Leukocytes from these reconstituted mice were then harvested and analyzed on day 20. (A) The expression of PD-1 on P14-transgenic memory CD8 T cells was analyzed on gated CD8+ Ly5.1+ Vα2+ cells. (B) Annexin V binding on Ly5.1+ P14-transgenic memory CD8 T cells was analyzed after gating on 7AAD-, CD8+, Ly5.1+, and Vα2+ cells. For Ly5.2 donor cells, Annexin V binding was analyzed after gating on CD8+7AAD− cells. (C) The production of IFNγ by P14-transgenic memory CD8 T cells was analyzed on gated CD8+, Ly5.1+, and Vα2+ cells after GP-33 peptide stimulation.

PD-1+ HP cells undergo oligoclonal expansion

Previous studies have demonstrated that PD-1 becomes expressed on T cells that are repeatedly and strongly stimulated through their TCR (36, 37). Consistent with those findings, the PD-1+ HP cells observed here had a highly activated phenotype, suggesting frequent stimulation through their TCR (Fig. 3). We questioned whether this strong stimulation would select for specific T cell clones outcompeting other clones. To address this question, we used all available Vβ monoclonal antibodies (Vβ2-14) to investigate the TCR Vβ repertoires at day 21. Vβ staining of the total HP population revealed polyclonal populations with moderate TCR skewing, so we analyzed the three subpopulations of HP cells defined by PD-1 and Ly6C. As shown in Fig. 7 A, considerable TCR Vβ skewing was seen in the PD-1+ population, indicating oligoclonal patterns of expansion, and there was great variation among mice regarding the Vβ usage of the skewed population. This high variation between mice was unusual, given that mice 1–5 and 6–8 received the same pooled donor cells, respectively. This may indicate that the progenitors for these oligoclonal expansions were of very low frequency in the donor populations. The skewed oligoclonal expansions were almost exclusively within the PD-1+ subset of the Ly6C− cells, as Fig. 7 A shows little skewing of TCR in either the PD-1−Ly6C− or in the PD-1−Ly6C+ populations, which remained polyclonal. The polyclonal Vβ profiles of those populations seemed very similar to the Vβ profiles of CD8 T cells from LCMV-immune mice used as donors (Fig. 7 B). Thus, the oligoclonal expansions, which are indicative of highly proliferating T cell populations, were exclusively within the PD-1+ cell populations. These results, along with those in Fig. 4, suggest that discrete clones of donor nonmemory T cells might interact with endogenous antigens with sufficiently high affinity such that they dramatically expand, are induced to express PD-1, and then become apoptotic and disappear under these conditions of acute homeostatic proliferation.

An alternative hypothesis, however, is that the oligoclonally expanded cells lost their PD-1 expression but remained in the host. To test for this, we examined the TCRβ usage in the Ly6C− and Ly6C+ cells 70 d after transfer, after the PD-1+ cells had virtually disappeared (Fig. 7 C). At this time point, no TCR skewing in either HP fraction was seen, and the two fractions looked nearly identical. Further, of the 8 tested T cell populations from day 21 displayed in Fig. 7 A, expanded Vβ populations could be easily seen in nonsubdivided donor CD8 T cell populations from all but mouse 6, which had a relatively low (27%) frequency of PD-1+ cells

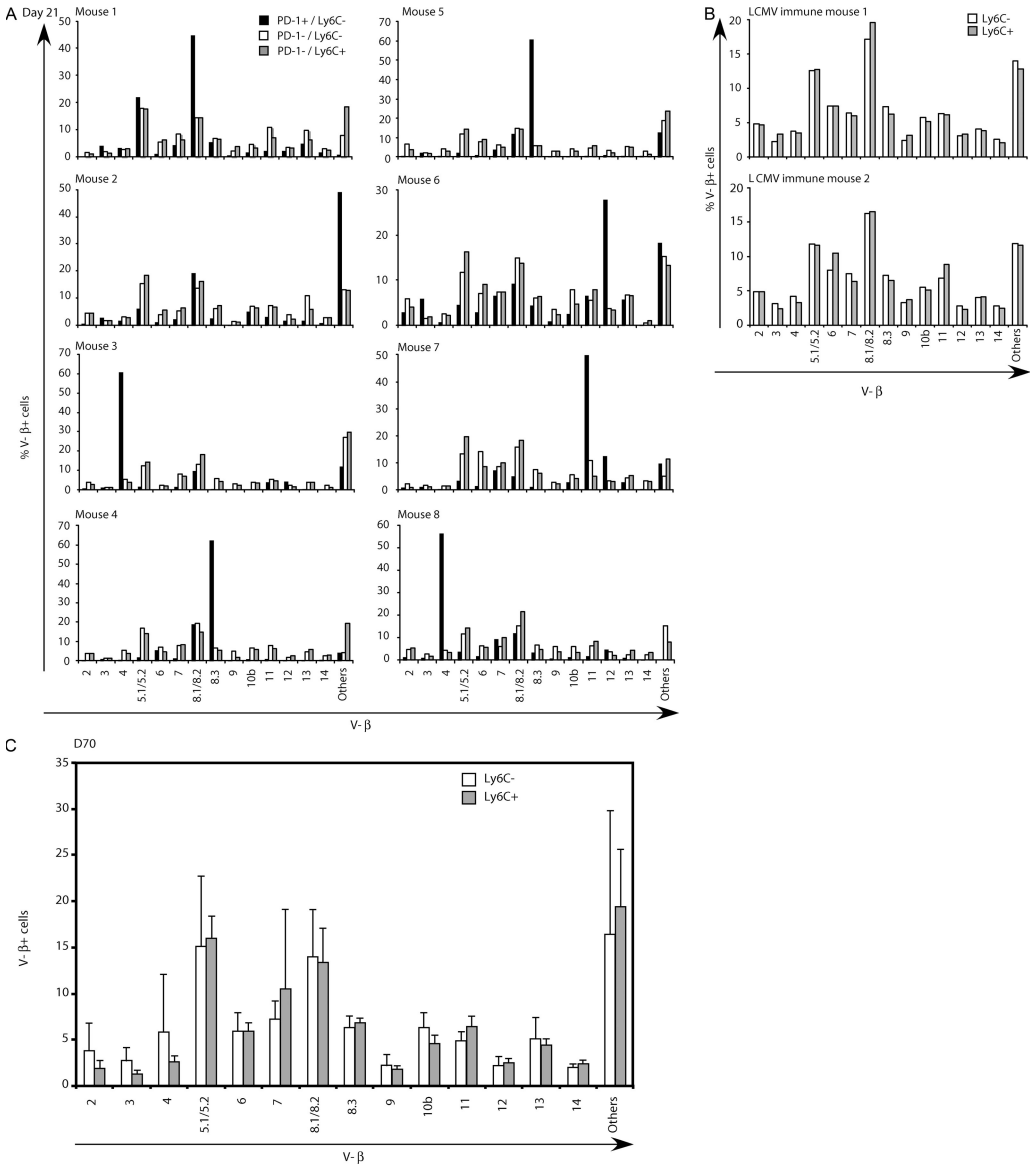

Figure 7. PD-1+ HP cells undergo oligoclonal expansion. Spleen leukocytes were isolated from Ly5.1-LCMV–immune mice and adoptively transferred into T cell KO mice. After day 21 or 70, leukocytes were harvested and stained with anti-CD8, -Ly5.1, -PD-1, -Ly6C, and -Vβ antibodies. (A) The percentage of Vβ usage in the individual populations was calculated. The percentage of Vβ usage on PD-1+Ly6C− cells (black bar), on PD-1−Ly6C− donor cells (white bar), and PD-1−Ly6C+ (gray bar) in 8 individual mice 21 d after transfer. Groups 1–5 and 6–8 received pooled donor cells from Ly5.1-LCMV–immune mice. (B) The percentage of Vβ usage on Ly6C− (white bar) and Ly6C+ (gray bar) CD8+ cells in LCMV immune mice. (C) The percentage of Vβ usage on Ly6C− (white bar) and Ly6C+ (gray bar) HP cells at day 70 after transfer. These are mean expressions in six mice ± the SD.

(not depicted). Expansions of a single defined Vβ population representing 30–42% of the total CD8 T cells were observed in mice 3–5, 7, and 8. In contrast, expanded Vβ populations of >23% were not found in any of the 6 tested nonsubdivided day 70 populations (individual samples not depicted). Given that Fig. 1 D shows that between day 21 and 70 there is only a modest increase in total CD8 T cell number that would not substantially dilute out the oligoclonal populations, we interpret these data to indicate that the expanded oligoclonal populations have been drastically reduced in frequency by day 70. We also found that the number of transferred HY transgenic CD8+ T cells, which were mostly expressing PD-1, had dramatically decreased by 54 d after transfer, in comparison to the HY cell numbers 6 d after transfer (day 6, $5.5 \pm 0.65 \times 10^5$, $n = 2$; day 54, $5.8 \pm 0.47 \times 10^4$, $n = 2$). This strongly argues that those cells have been eliminated from the host, which is consistent with the Annexin V+ phenotype of the PD-1+ cells when they were present (Fig. 4).

DISCUSSION

The expression of PD-1 is induced after TCR engagement, and PD-1 is known to down-regulate immune responses to

self-antigens or pathogens (16). We found that HP cell populations present during acute homeostatic proliferation could be divided into the following three subsets defined by expression of PD-1 and Ly6C: PD-1+Ly6C−, PD-1−Ly6C−, and PD-1−Ly6C+. The properties of these HP subsets are summarized in Table I. The PD-1+ population was mostly devoid of bona fide memory cells, consisted of dramatically expanded distinct oligoclonal Vβ populations, and expressed a highly activated antigenic phenotype. The PD-1+ cells had reduced growth factor receptor expression, low Bcl-2, and presented with a preapoptotic phenotype, as shown by their reactivity with Annexin V. These cells were in a dysfunctional state, as they produced cytokines poorly on anti-CD3 stimulation. T cells that react with Annexin V can remain viable for some time and, in contrast to those found here, continue to secrete cytokines. For example, most Annexin V+ CD8 T cells present during acute LCMV infection maintain their ability to produce cytokines (29, 38). Even though Annexin V reactivity can be a property of actively dividing and still functioning T cells, studies have shown that those Annexin V+ T cells are far more susceptible to death than Annexin V− cells, when placed into in vitro culture or transferred in vivo (32, 38). PD-1+ CD8 cells during persistent LCMV infection, where they receive repeated signaling through their TCR, have a similar phenotype to these PD-1+ HP cells in that they fail to produce cytokines on TCR stimulation; treatment of mice with anti–PD-L1 can restore their functional phenotype (32–34). We show in our model of acute homeostatic proliferation that anti–PD-L1 can enhance IFNγ production by the PD-1+ cells in vitro, and that anti–PD-1 can increase the number of total and IFNγ-producing CD8 T cells in vivo, arguing for an important functional role for PD-1 in this system.

The ultimate fate of these highly active oligoclonally expanded PD-1+ HP cells during acute homeostatic proliferation appears to be elimination from the host, as most of them disappear by 70 d after transfer. We interpret this to mean that those cells that are most self-reactive or reactive with other endogenous ligands initially undergo dramatic oligoclonal proliferation, express PD-1, become functionally inactive, and are eliminated from the host. This is consistent with previous observations that PD-1 KO mice spontaneously develop autoimmune diseases and suggests an important role for PD-1 under conditions of lymphopenia.

The properties of the PD-1+ T cell subset differed dramatically from the other subsets (Table I). Naive T cells are induced to express CD44 during acute homeostatic proliferation (5, 6), but we have reported that bona fide CD44+ antigen-specific memory cells, which have undergone the full differentiation series as a consequence of the immune response, only divide at a modest level and are diluted during acute homeostatic proliferation (1). Because of similarities in CD44 expression, it has been difficult to distinguish memory cells from memory-like cells during this process. We show that Ly6C may be useful for this process. It is expressed on virtually all bona fide memory cells before transfer into the lymphopenic environment and stays expressed as they divide within that environment. Fig. 2 shows that virtually all of the bona fide memory cells were Ly6C+, but it is not clear whether all Ly6C+ cells originated as bona fide memory cells. Nevertheless, the phenotype is remarkably different from that of the other subsets. Despite the fact that these cells are dividing, albeit slowly, they do not have an activation antigen phenotype. In addition, they function normally in regard to cytokine production on TCR stimulation, which is best illustrated with the transgenic P14-transgenic memory CD8 T cells, where 96% produced IFNγ after stimulation (Fig. 6). The Ly6C+ HP cells express high levels of growth factor receptors, high levels of Bcl-2, and are viable, as indicated by their lack of reactivity with Annexin V. This population shows no indication of oligoclonal expansion.

The PD-1+ oligoclonally expanding cell population appears to be eventually lost (Fig. 7), and the bona fide memory cell population, staining with Ly6C and dividing slowly, is gradually diluted. Although virtually all of the bona fide memory cells expressed Ly6C, this does not mean that all Ly6C+ cells are bona fide memory cells and that Ly6C+ can be a marker to reliably distinguish bona fide memory cells from other HP cells, and it is possible that some Ly6C− HP cells might eventually convert to expressing the Ly6C marker. The day 21 PD-1−Ly6C− subset, however, has its own unique phenotype in that, while actively dividing, it has no evidence of oligoclonal expansion, suggesting that it consists of cells not reacting with high affinity to self- or endogenous antigens. Although expressing low levels of Bcl-2, these cells are functional and viable, in that they make cytokines on anti-CD3 stimulation and fail to react with Annexin V. Bcl-2 is sometimes down-regulated in activated dividing cells without being associated with apoptosis (31). Down-regulation of IL-7Rα is frequently correlated with a preapoptotic state, but no such down-regulation was seen in this population.

Thus, it would seem that acute homeostatic proliferation selects against those cells that are reacting strongly to either self-antigens or other endogenous antigens, such as those expressed by microbial flora. These cells initially expand oligoclonally, and but then undergo apoptosis, as the immune system is gradually repopulated with a less vigorously expanding polyclonal population.

MATERIALS AND METHODS

Mice. C57BL/6 male mice (Ly5.2+) were purchased from The Jackson Laboratory and used at 8–12 wk of age. B6.SJL (Ly5.1+) mice, which are congenic to C57BL/6 mice, except for the Ly5.1 marker, were purchased from Taconic Farms at 4–5 wk of age. P14 TCR (Ly5.1+) transgenic mice (expressing transgenic TCR specific for the D^b-restricted LCMV epitope GP33–41) (39), B6.129P2 $Tcrbtml^{Mom}Tcrdtml^{Mom}$ (αβγδ KO) mice (T cell-KO) (40), and C57BL/6 HY-transgenic mice, whose transgenic TCR is specific for the H-$2D^b$–restricted male antigen HY (41, 42), were bred within the Department of Animal Medicine at the University of Massachusetts Medical School. All of these mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and age- and sex-matched before use.

Immunofluorescent staining of antigen-specific T cells. Spleen leukocytes were incubated with 5 μM of synthetic peptide or 0.2 μg/ml anti-CD3 antibody, 10 U/ml of human recombinant IL-2, and 0.2 μl GolgiPlug (BD Biosciences) for 5 h at 37°C. After FcR blockade (anti-FcRII), the cells were stained with fluorescently labeled mAbs against CD8, CD44, CD122, CD43, Vα2, Vβ8, IL15Rβ, CD62L, Ly6G/6C, Ly6C, Ly5.1, PD-1, H-Y TCR, or IL-7Rα for 30 min at 4°C. The mAb to Ly6G/C was clone RB6-8C5, which cross-reacts with a common epitope of Ly6G and Ly6C, but only Ly6C is expressed on memory CD8+ T cells (43). After staining, cells were permeabilized with Cytofix/Cytoperm for 20 min at 4°C and stained with mAb to IFNγ or TNFα in perm/wash buffer (BD Biosciences) for 25 min at 4°C. Finally, cells were washed and resuspended in FACS buffer (PBS with 2% FCS and 0.1% sodium azide) and analyzed by flow cytometry. All mAbs were purchased from BD Biosciences, except for antibodies specific for Ly5.1, PD-1, H-Y TCR, and IL-7Rα, which were purchased from eBioscience.

Adoptive transfer of LCMV-immune splenocytes into lymphopenic environments. LCMV-immune T cell populations were prepared by inoculating Ly5.1+ mice i.p. with 5×10^4 PFU of the Armstrong strain of LCMV and waiting at least 6 wk before use. For the creation of LCMV-specific transgenic memory CD8 cells, splenocytes were harvested from naive P14-transgenic mice (Ly5.1+), which contained ∼90% of Vα2+ and Vβ8.1/8.2+ CD8 T cells. 2×10^6 splenocytes were resuspended in 200 μl HBSS (Invitrogen) and adoptively transferred via the tail vein into Ly5.2+ B6 mice. 1 d later, B6 recipients were infected i.p. with 5×10^4 PFU of the Armstrong strain of LCMV. After 6 wk, ∼25% of the spleen CD8+ T cells were memory P14-transgenic CD8+ T cells. Splenocytes from normal or LCMV-immune Ly5.1, from Ly5.2+ containing memory P14-transgenic T cells, and from HY-transgenic Ly5.2 mice were harvested and isolated, suspended in HBSS at 2.0×10^7 cells/ml, and incubated in 5 μM CFSE (Invitrogen) solution for 12 min at 37°C. After incubation, these donor cells were washed twice with HBSS, resuspended in 200 μl HBBS, and 1.5×10^7 cells were adoptively transferred via the tail vein into Ly5.2+ T cell KO, irradiated, or normal B6 mice.

Annexin V staining. Cells that had been incubated with anti–surface marker antibodies for 30 min at 4°C were washed and incubated in Annexin V binding buffer (BD Biosciences) with allophycocyanin/Annexin V at a 1:20 dilution for 15 min at room temperature. The cells were washed and resuspended in Annexin V binding buffer with 7-amino-actinomycin D (7AAD) at a 1:250 dilution. Cells were analyzed by flow cytometry immediately thereafter, and 7AAD+ cells were excluded from the analysis.

Gene microarray analysis. CFSE-labeled splenocytes from T cell KO hosts that had received splenocytes from LCMV-immune Ly5.1 donors were stained with anti-CD8 antibody, anti-Ly5.1 antibody, and 7AAD. 7AAD− cells were gated, and CFSE-low (greater than eight divisions) and CFSE-high (zero to six divisions) cells were sorted by flow cytometry using a FACStar^{PLUS} sorter (BD Biosciences). Total RNA was extracted from CFSE-low or -high cells using an RNeasy Mini kit (QIAGEN). T7-$(dT)_{24}$ primer (GENSET Corp.) and SuperScript DS cDNA synthesis kits were used to direct cDNA synthesis. The biotin-labeled cDNA was transcribed to cRNA and labeled using an RNA transcript labeling kit (Enzo Diagnostics, Inc.). Fragmented cRNA was hybridized to MG-U74Av2 microarray chips (Genomic Core Facility at University of Massachusetts Medical School), and expression levels were analyzed with Microarray Suite 5.0 (Affymetrix).

In vitro and in vivo antibody blockade. For stimulations in vitro, splenocytes were harvested from T cell KO mice 14 d after reconstitution, and 10^6 splenocytes or CD8+PD-1+–depleted splenocytes were seeded into 96-well plates in RPMI supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 50 mM 2-mercaptoethanol, 2 mM L-glutamine, 1% (vol/vol) nonessential amino acid solution, and 10 mM Hepes, and treated with 25 μg/ml anti–PD-L1 blocking antibody (clone MIH5; eBioscience) for 3 d. For anti–PD-1 antibody treatments in vivo, day 20–23 T cell KO mice (reconstituted with LCMV-immune splenocytes) were given 2 i.p. injections of 200 μg of antibody to PD-1 (clone J43; BioExpress) or control antibody (hamster IgG; BioExpress) in 200 μl PBS at day 0 and 3. Splenocytes were harvested at day 6. IFNγ was induced in these splenocytes by incubating them with 0.2 μg/ml anti-CD3 antibody, 10 U/ml of human recombinant IL-2, and 0.2 μl GolgiPlug for 4–5 h at 37°C. After FcR blockade, the cells were stained with fluorescently labeled mAbs against CD8, Ly5.1, or PD-1 for 30 min at 4°C. After staining, cells were permeabilized with Cytofix/Cytoperm for 20 min at 4°C and stained with mAb to IFNγ in perm/wash buffer for 25 min at 4°C. Finally, cells were washed and resuspended in FACS buffer and analyzed by flow cytometry.

TCR Vβ analysis. 21 or 70 d after reconstitution of T cell KO mice with splenocytes from LCMV-immune Ly5.1+ mice, spleen cells were harvested and stained with mAb to CD8, Ly5.1, PD-1, Ly6C, and FITC-labeled Vβ-specific mAbs (Vβ2-14; BD Biosciences kit). Data represent the percentage of CD8 cells staining with the Vβ mAb. The percentage for "other Vβ" was calculated by subtracting the sum of individual Vβ from 100%.

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