Antiviral CD4$^+$ memory T cells are IL-15 dependent

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Survival and intermittent proliferation of memory CD4$^+$ and CD8$^+$ T cells appear to be controlled by different homeostatic mechanisms. In particular, contact with interleukin (IL)-15 has a decisive influence on memory CD8$^+$ cells, but not memory CD4$^+$ cells. Past studies of memory CD4$^+$ cells have relied heavily on the use of naturally occurring memory phenotype (MP) cells as a surrogate for antigen (Ag)-specific memory cells. However, we show here that MP CD4$^+$ cells contain a prominent subset of rapidly proliferating major histocompatibility complex (MHC) II–dependent cells. In contrast, Ag-specific memory CD4 cells have a slow turnover rate and are MHC II independent. In irradiated hosts, these latter cells ignore IL-15 and expand in response to the elevated levels of IL-7 in the lymphopenic hosts. In contrast, in normal nonlymphopenic hosts where IL-7 levels are low, memory CD4 cells are heavily dependent on IL-15. Significantly, memory CD4$^+$ responsiveness to endogenous IL-15 reflects marked competition from other cells, especially CD8$^+$ and natural killer cells, and increases considerably after removal of these cells. Therefore, under normal physiological conditions, homeostasis of CD8$^+$ and CD4$^+$ memory cells is quite similar and involves IL-15 and IL-7.

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Abbreviations used: Ag, antigen; B6, C57BL/6; B6.PL, B6.PL *Thy1a*/Cy Thy-1.1$^+$; GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; MHC-II$^{\Delta/\Delta}$, MHC II locus-deficient; MP, memory phenotype.

The prolonged lifespan of memory T cells combined with their ability to rapidly acquire effector function provides long-term protective immunity against repeated exposure to pathogens (1). The mechanisms governing the homeostasis of memory T cells have been an area of intense investigation. Unlike naive T cells, which survive largely in interphase, memory T cells persist under normal conditions with a slow but constant turnover, defined as basal homeostatic proliferation, presumably reflecting an elevated state of cell activation (1, 2). As with naive T cells, memory T cells are also capable of undergoing more rapid cell division under lymphopenic conditions, known as acute homeostatic proliferation (2). There is now a general consensus that two members of the common γ chain cytokine family, namely IL-7 and IL-15, control the homeostasis of CD8$^+$ memory cells (1, 3–5). However, the factors that govern the homeostasis of CD4$^+$ memory cells have yet to be fully defined.

To date, two types of memory T cells have been used interchangeably to study memory cell homeostasis: memory phenotype (MP) T cells that arise spontaneously in normal mice and antigen (Ag)-specific memory T cells that are generated by deliberate Ag administration (1, 3–6). For CD8$^+$ cells, both types of memory cells require IL-7 and IL-15 for their homeostasis, although they display minor differences in their relative dependence on the two cytokines (1, 3). MP CD8$^+$ cells are exquisitely dependent on IL-15 for their generation, survival, and basal homeostatic proliferation; hence, MP CD8$^+$ cells are drastically depleted in mice deficient in either IL-15 or IL-15Rα, the latter being required for presentation of IL-15 (2, 7–11). Ag-specific memory CD8$^+$ cells, on the other hand, are less dependent on IL-15, relying more on IL-7, but still require IL-15 for basal homeostatic proliferation and long-term maintenance (2, 8, 12–15). Thus, Ag-specific memory CD8$^+$ cells were efficiently generated in IL-15$^-$ or IL-15Rα$^-$–deficient mice, but these cells remained in interphase and gradually disappeared over several months (12–14).

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The discrepancy in the homeostatic requirements for Ag-specific memory versus MP cells appears even greater for CD4⁺ than CD8⁺ cells. For instance, although the two types of memory CD8⁺ cells undergo similar rates of homeostatic proliferation, MP CD4⁺ cells as a population undergo a considerably faster rate of homeostatic proliferation than Ag-specific memory CD4⁺ cells (2, 9, 15–17). Moreover, the homeostasis of MP CD4⁺ cells seems to be governed more by TCR signaling than by cytokines. Thus, the basal homeostatic proliferation rate of MP CD4⁺ cells declined dramatically after the forced down-regulation of TCR expression, and acute homeostatic proliferation of MP CD4⁺ cells in lymphopenic hosts occurred in the absence of IL-7 and/or IL-15 (9, 18, 19). In contrast, the homeostasis of Ag-specific CD4⁺ memory cells appears to be solely controlled by cytokines as these cells can survive and undergo acute homeostatic expansion in lymphopenic hosts in the absence of MHC II molecules (17, 20). Consistent with this notion, recent studies have shown that IL-7 is essential for the survival and basal homeostatic turnover of Ag-specific CD4⁺ memory cells (15, 21).

In addition to IL-7, IL-15 treatment is known to promote the proliferation of human memory CD4⁺ T cells in vitro and mouse Ag-specific CD4⁺ memory T cells in vivo (15, 22). Nonetheless, IL-15 is generally considered to be irrelevant for the homeostasis memory CD4⁺ cells (1, 3–6), especially because normal numbers of MP CD4⁺ cells are present in IL-15–deficient mice (8). Likewise, IL-15 is reported to have only a minimal role in the homeostasis of Ag-specific CD4⁺ memory cells (15). Here, however, the Ag-specific CD4⁺ memory cells were generated de novo in IL-15–deficient mice, a situation where T cells can become permanently conditioned to cope with IL-15 deficiency (23). In light of this caveat, we have reexamined the role of IL-15 in the homeostasis of CD4⁺ Ag-specific memory cells using lymphocytic choriomeningitis virus (LCMV)-specific TCR transgenic memory CD4⁺ T cells, as well as polyclonal LCMV-specific CD4⁺ memory cells, generated in normal C57BL/6 (B6) mice. We report for the first time that Ag-specific CD4⁺ memory cells are dependent on IL-15 for their basal homeostatic proliferation and long-term survival.

RESULTS

Sources of memory CD4⁺ T cells

Two types of memory CD4⁺ cells were used for this study: spontaneously arising MP cells and Ag-specific memory cells. MP cells were obtained by FACS purifying CD44^{hi} CD25^{-} NK1.1^{-} CD4⁺ cells from 6–10-mo-old normal B6.PL*Thy1a*/Cy Thy-1.1⁺ (B6.PL) spleens. Ag-specific memory cells were generated using a line of CD4⁺ TCR transgenic mice on a B6.PL background, designated Smarta, specific for LCMV glycoprotein (GP) 61-81–H2-A^{b} complexes (24). As described previously, small numbers of naive Thy-1.1⁺ Smarta cells were transferred into Thy-1.2⁺ B6 mice and immunized 1 d later with LCMV. Memory cells were purified from spleens after 5–7 wk (25).

For the initial characterization of Smarta memory cells, two different doses of naive cells were transferred into B6 hosts: a high dose (10^5 cells/mouse) and a lower physiologically relevant dose (10^3 cells/mouse; reference 26). Smarta cells at either precursor frequency (10^4 or 10^2, assuming 10% engraftment of injected cells) displayed kinetics of virus-induced expansion, contraction, and maintenance phases comparable to those described for polyclonal CD4⁺ T cells (Fig. 1 A; reference 27). However, the high precursor frequency of naive Smarta cells led to the generation of 10-fold more

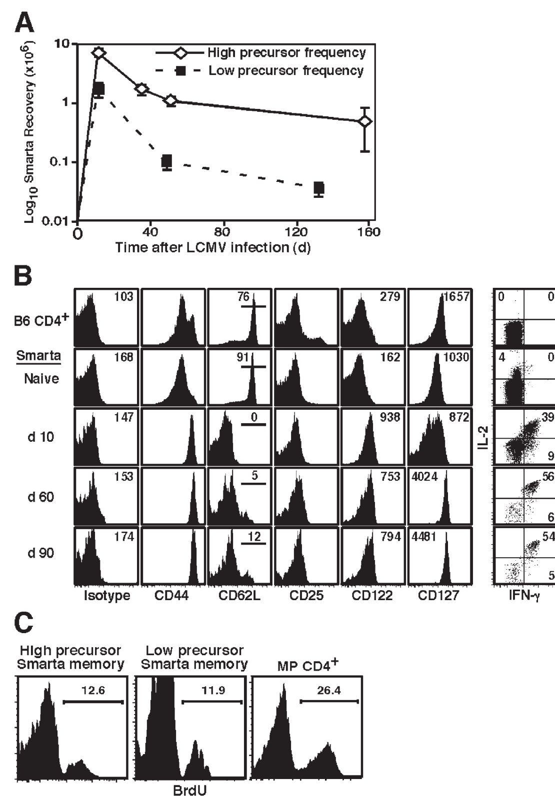

Figure 1. Generation and characterization of Smarta memory cells. (A) The responses of naive Smarta CD4⁺ cells transferred into B6 hosts at a high or low precursor frequency. B6 mice were injected with 10^5 or 10^3 naive Thy-1.1⁺ Smarta cells and infected with LCMV Armstrong 1 d later. The recovery of Smarta cells in host spleen was analyzed at the indicated time points by staining for Thy-1.1 and CD4. (B) Characterization of Smarta naive, effector, and memory cells generated from 10^5 injected precursors. Histograms indicate expression of activation markers and cytokine receptors on Smarta cells before and after LCMV infection, shown in comparison with total polyclonal B6 CD4⁺ cells. Numbers inside histograms indicate mean fluorescence intensity. Dot plots show the expression of intracellular IL-2 and IFN-γ by Smarta cells after a 5-h in vitro stimulation with agonist GP61-80 peptide. Data are representative of four separate experiments, with at least three mice per time point. (C) Basal homeostatic proliferation rate of Smarta memory and CD4⁺ MP cells. B6 mice harboring a high or low precursor frequency of Smarta memory cells (at 72 d after LCMV infection) were given BrdU in the drinking water for 5 d, and the incorporation of BrdU on memory CD4⁺ cells was detected as described in Materials and methods. Similar results were obtained in two other experiments.

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memory cells with a slightly longer half-life (72 vs. 64 d) than the low precursor frequency (Fig. 1 A). Smarta memory cells generated from either precursor frequency were virtually identical in terms of their ability to synthesize cytokines and their expression of activation markers and cytokine receptors (Fig. 1 B and Fig. S1, which is available at http://www.jem.org/cgi/content/full/jem.20061805/DC1). Smarta cells at memory time points (>40 d after virus infection) displayed a CD25⁻ CD44^{hi} CD62L^{lo} phenotype and increased expression of CD122, a shared component of the IL-15 (and IL-2) receptor (28), as well as CD127 (IL-7Rα) relative to naive Smarta cells (Fig. 1 B). A small population of CD62L^{hi} Smarta memory cells arose at later time points, but the implication of this is not clear. Additionally, ~50% of Smarta memory cells synthesized IFN-γ and/or IL-2 upon in vitro stimulation with cognate peptide (Fig. 1 B).

To visualize the basal homeostatic proliferation of Smarta memory cells, mice containing long-term primed (72 d) Smarta memory cells were given drinking water containing the nucleotide analogue BrdU, which is incorporated into the DNA of proliferating cells. After 5 d, similar proportions of high and low precursor frequency Smarta memory cells incorporated BrdU, indicating that they underwent comparable rates of basal homeostatic proliferation (Fig. 1 C). Notably, the basal homeostatic proliferation rate of Smarta CD4$^+$ memory cells was less than half the rate displayed by MP CD4$^+$ cells, consistent with previous results obtained with polyclonal Ag-specific memory CD4$^+$ cells (15, 16). Overall, Smarta memory cells generated from low or high precursor frequencies displayed comparable phenotype, functionality, and basal rates of turnover to those previously observed for polyclonal Ag-specific CD4$^+$ memory cells (15, 27, 29). Consequently, Smarta memory cells derived from high precursor frequencies were used to study the homeostatic requirements of Ag-specific CD4$^+$ memory cells.

MP CD4$^+$ cells are a heterogeneous population of cells

MP CD4$^+$ cells have been used as surrogates for Ag-specific memory CD4$^+$ cells in studies of T cell homeostasis with the assumption that the two types of cells are interchangeable (9, 19). To test the validity of this supposition, we directly compared the acute homeostatic turnover of both types of cells in irradiated B6 hosts for 1 wk (Fig. 2 A). Lymphopenia-induced homeostatic proliferation in irradiated hosts provides a convenient and sensitive model to compare the homeostatic requirements of various T cell subsets. Smarta memory cells underwent a homogeneous, slow rate of acute homeostatic proliferation. In contrast, MP CD4$^+$ cells comprised two distinct populations of cells that underwent either an extremely rapid or a slow rate of cell division (Fig. 2 A). Because the rapidly dividing MP CD4$^+$ cells resembled cells undergoing proliferation driven by foreign Ags, we tested whether MP CD4$^+$ cells would undergo an even greater expansion in RAG-1⁻ hosts. This rationale is based on our recent finding that the density of foreign Ags recognized by T cells is much higher in congenitally T-deficient hosts

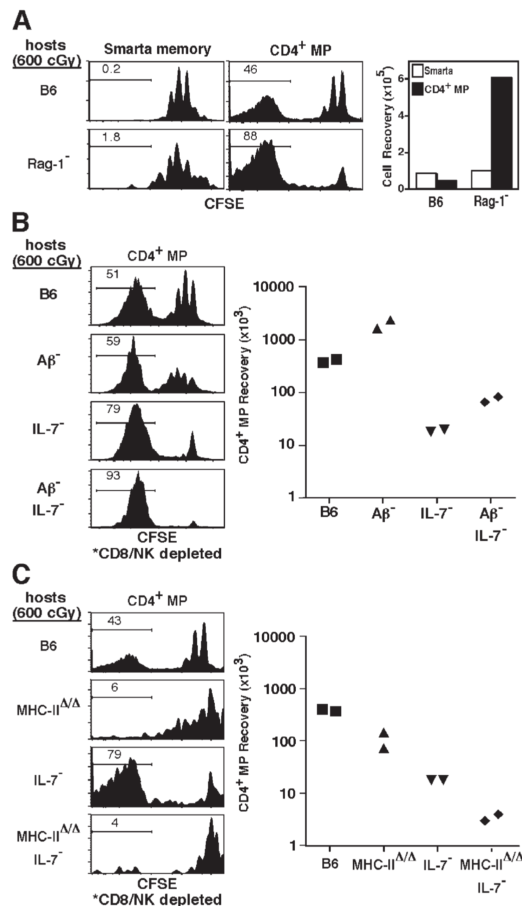

Figure 2. MP CD4$^+$ cells are comprised of heterogeneous populations of cells in terms of their homeostatic characteristics. (A) Comparison of acute homeostatic proliferation between Smarta memory and MP CD4$^+$ cells. A mixture of CFSE-labeled Thy-1.1$^+$ Smarta memory cells (4×10^5) and CD45.1$^+$ MP CD4$^+$ cells (2×10^5) was injected into irradiated B6 and Rag-1⁻ mice, and the donor cells in the host spleen and LNs were analyzed 1 wk later by staining for Thy-1.1, CD45.1, and CD4. Histograms show CFSE profiles of the two donor cell types in the host spleen, and the bar graphs show mean donor cell recovery from pooled host spleen and LNs. Data are representative of two separate experiments, with two mice per host type analyzed individually. (B) MP CD4$^+$ cells recognize a hybrid MHC II molecule in Aβ⁻ mice. Small numbers (3×10^5) of CFSE-labeled purified Thy-1.1$^+$ CD4$^+$ MP cells were injected into irradiated and CD8/NK-depleted B6, Aβ⁻, IL-7⁻, and Aβ⁻IL-7⁻ mice and were analyzed 1 wk later by staining splenocytes for CD4 and Thy-1.1. Aβ⁻ B6 mice lack the H2-A β chain. CFSE dilution and recovery from one of two experiments, with two mice per treatment are shown. (C) The role of MHC II and IL-7 in expansion of MP CD4$^+$ cells in lymphopenic hosts. A small dose (3×10^5) of purified MP CD4$^+$ cells was injected into irradiated and CD8/NK cell–depleted B6, MHC-II$^{Δ/Δ}$, IL-7⁻, and MHC-II$^{Δ/Δ}$ IL-7⁻ mice. Donor cells from the host spleen were analyzed 1 wk later by staining for Thy-1.1 and CD4. MHC-II$^{Δ/Δ}$ B6 mice lack all H2-A and E chains. Histograms and a log scale scatter-plot show proliferation and recovery of donor cells in the spleen. Data are representative of two separate experiments, with two mice per host type analyzed individually.

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than in irradiated normal hosts (30). Indeed, the magnitude of the fast-dividing MP $CD4^+$ cells was greatly increased in RAG-1⁻ hosts, leading to an ~10-fold increase in donor cell recovery when compared with irradiated B6 hosts (Fig. 2 A). For Smarta memory cells, in contrast, the homeostatic proliferation rate and the recovery in RAG-1⁻ hosts were virtually identical to those in irradiated B6 hosts (Fig. 2 A). These results demonstrate that MP $CD4^+$ cells are a heterogeneous population of cells. Although the majority of the cells display homeostatic characteristics similar to Ag-specific cells and undergo slow homeostatic proliferation, a fraction of cells divide very rapidly, possibly driven by nonhomeostatic mechanisms, e.g., through contact with foreign Ags. In partial support of this idea, the rapidly dividing donor MP $CD4^+$ cells recovered from irradiated B6 hosts displayed some properties of effector cells (31). Thus, these cells were $CD43^{hi}CD62L^{lo}$, a fraction down-regulated CD127, but remained $CD122^{lo}$ $CD69^-$ $CD25^-$ PD-1⁻, and they did not readily produce IFN-γ or IL-2 after anti-CD3 stimulation (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20061805/DC1, and not depicted).

One possibility is that MP $CD4^+$ cells in lymphopenic hosts are driven by TCR signaling rather than by the increased levels of homeostatic cytokines. According to this idea, the population of rapid-dividing cells would not be apparent if CFSE-labeled MP $CD4^+$ cells were transferred into irradiated syngeneic hosts deficient in the expression of MHC II molecules. However, when irradiated H2-Aβ⁻ mice were used as hosts, the fast-dividing MP $CD4^+$ cells emerged after 1 wk just as readily as in control irradiated B6 hosts (Fig. 2 B). Unexpectedly, for these studies we had to use H2-Aβ⁻ mice that were depleted of $CD8^+$ and NK cells, as unmanipulated H2-Aβ⁻ hosts promptly rejected donor MP $CD4^+$ cells despite the irradiation. The mechanism involved in the rejection of wild-type B6 MP $CD4^+$ cells by H2-Aβ⁻ hosts is not known, but one possible cause is a genetic difference at the nonclassical MHC region between B6 and H2-Aβ⁻ mice, which are derived from 129 stem cells (17). The rejection was not observed when H2-Aβ⁻ hosts were depleted of $CD8^+$ and NK cells, and hence, adoptive transfer into MHC II–deficient hosts was performed after such a lymphocyte depletion.

Because H2-Aβ⁻ mice are reported to express low levels of chimeric H2-AαEβ molecules that can be recognized by mature T cells (32), the above experiment was repeated using MHC II locus-deficient (MHC-$II^{\Delta/\Delta}$) mice that lack all chains of the H2-A and E molecules (33). Again, MHC-$II^{\Delta/\Delta}$ mice were depleted of $CD8^+$ and NK cells to prevent the rejection of donor MP $CD4^+$ memory cells. Strikingly, in MHC-$II^{\Delta/\Delta}$ hosts, the fast-dividing population of donor MP $CD4^+$ cells was virtually absent, even though the slow-dividing cells were largely unaffected; in control B6 hosts, both fast- and slow-dividing populations were observed (Fig. 2 C).

To determine whether IL-7 plays a role in the homeostasis of fast-dividing MP $CD4^+$ cells, IL-7⁻ and MHC-$II^{\Delta/\Delta}$ IL-7⁻ mice were also included as hosts in the above experiment. Significantly, a population of fast-dividing cells was prominent in IL-7⁻ hosts but was almost undetectable in MHC-$II^{\Delta/\Delta}$ IL-7⁻ hosts, indicating that IL-7 is not required for the fraction of MP $CD4^+$ cells that undergoes rapid proliferation (Fig. 2 C). Nonetheless, the recovery of donor cells declined severely in IL-7⁻ hosts (Fig. 2 C), suggesting that IL-7 can augment the survival of fast-dividing cells. It should also be noted that the majority of the slow-dividing cells did not proliferate or survive in the absence of IL-7, thus severely reducing the recovery of donor cells in MHC-$II^{\Delta/\Delta}$ IL-7⁻ hosts as compared with MHC-$II^{\Delta/\Delta}$ hosts and causing the proportion of fast-dividing cells to increase in IL-7⁻ hosts (Fig. 2 C). Collectively, these findings indicate that although both fast- and slow-dividing MP $CD4^+$ cells are dependent on IL-7 for their survival, the former cells do not require IL-7 for fast proliferation, whereas the latter cells are dependent on IL-7 for their slow homeostatic proliferation. Moreover, slow-dividing cells do not require contact with MHC II for their homeostatic proliferation, but the fast-dividing cells do require contact with MHC II, possibly loaded with foreign peptides, and are largely responsible for the high basal turnover rate of MP $CD4^+$ cells as a population. Attempts to remove the fast-dividing population among MP $CD4^+$ cells on the basis of cell surface phenotype have thus far failed (not depicted), so it is not currently possible to specifically remove these cells. All the above findings indicate that MP $CD4^+$ cells are comprised of distinct subsets of MHC II–dependent and –independent cells. Hence, MP $CD4^+$ cells cannot be used as a surrogate for characterizing the homeostatic requirements of Ag-specific memory $CD4^+$ cells.

The homeostasis of Ag-specific memory $CD4^+$ cells is cytokine dependent

To determine whether Ag-specific memory $CD4^+$ cells require contact with MHC II for their homeostasis, CFSE-labeled Smarta memory cells were transferred to irradiated $CD8^+$/NK-depleted B6 and MHC-$II^{\Delta/\Delta}$ hosts and analyzed 1 wk later. The proliferation and recovery of Smarta memory cells in either host were nearly identical, indicating that Ag-specific memory $CD4^+$ cells do not require MHC II contact to undergo acute homeostatic proliferation or survival (Fig. 3 A). Furthermore, MHC II contact did not affect the turnover of Smarta memory cells in partially lymphopenic $CD8^+$/NK-depleted hosts or increase the survival of these cells in the absence of IL-7 and IL-15 cytokine signaling (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20061805/DC1). Smarta memory cells thus resemble the slow-proliferating MP $CD4^+$ cells (Fig. 2 C) and probably require only cytokines for their homeostasis.

To define the cytokines that support the acute homeostatic turnover of Ag-specific $CD4^+$ memory cells, CFSE-labeled Smarta memory cells were transferred into irradiated B6, IL-7⁻, IL-15⁻, and IL-7⁻15⁻ mice, and their proliferation and recovery were determined 2 wk later. Similar numbers of Smarta memory cells were found to initially engraft in the various hosts (not depicted). Slow proliferation of Smarta

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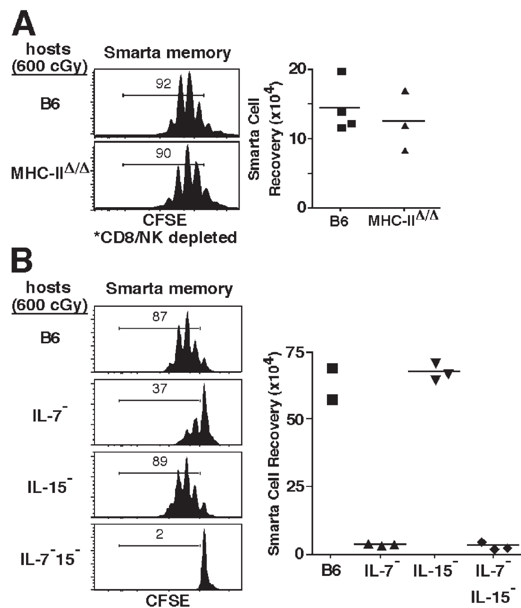

Figure 3. IL-7, but not MHC II, is essential for acute homeostatic proliferation of Smarta memory cells. (A) Contact with MHC II is not required for acute homeostatic proliferation of Smarta memory cells. A small dose (3×10^5) of CFSE-labeled Thy-1.1$^+$ Smarta memory cells was injected into irradiated, thymectomized, and CD8/NK cell–depleted B6 and MHC-II$^{\Delta/\Delta}$ mice, and the donor cells in the host spleen and LNs were analyzed 1 wk later by staining for Thy-1.1 and CD4. The CFSE profiles and recoveries of donor Smarta cells are shown. Data are representative of two separate experiments, with three to four mice per host type analyzed individually. (B) Acute homeostatic proliferation of Smarta memory cells is chiefly driven by IL-7. A small dose (5×10^5) of CFSE-labeled Thy-1.1$^+$ Smarta memory cells was injected into irradiated B6, IL-7$^-$, IL-15$^-$, and IL-7$^-$15$^-$ mice, and host spleens were analyzed 2 wk later by staining for Thy-1.1 and CD4. The CFSE profiles and recoveries of donor Smarta cells are shown. Data are representative of three experiments, with two to three mice per host type analyzed individually.

memory cells in irradiated B6 hosts seems to be driven largely by IL-7 because proliferation was much reduced in IL-7$^-$ hosts but maintained in IL-15$^-$ hosts (Fig. 3 B). Nevertheless, a fraction of Smarta memory cells did undergo one to two cell divisions in IL-7$^-$ hosts, and this proliferation was completely abolished in IL-7$^-$15$^-$ hosts, indicating that IL-15 does have a significant though minor role in driving the acute homeostatic proliferation of Smarta memory cells (Fig. 3 B). The recovery of donor Smarta memory cells in B6 and IL-15$^-$ hosts was comparable but ~20-fold lower in IL-7$^-$ and IL-7$^-$15$^-$ hosts. Even when normalized to account for differences in proliferation, the recovery of donor cells was consistently 5–10-fold lower in IL-7$^-$ and IL-7$^-$15$^-$ hosts. These findings are consistent with the idea that the homeostasis of memory CD4$^+$ cells is primarily controlled by IL-7 (15, 19, 21, 34).

IL-15 drives the basal homeostatic proliferation of Ag-specific memory CD4$^+$ cells in nonlymphopenic hosts

The low rate of proliferation of Smarta memory cells in irradiated IL-7$^-$ hosts (Fig. 3 B) resembled the basal homeostatic proliferation of Smarta memory cells in intact nonirradiated hosts (Fig. 1 C). This finding suggests that IL-15 may play a significant role in supporting basal turnover of memory CD4$^+$ cells under normal T cell–sufficient conditions. In this regard, previous studies have reported that exogenous IL-15 is not mitogenic for MP CD4$^+$ cells but is stimulatory for Ag-specific memory CD4$^+$ cells under in vivo conditions (14, 15, 35).

To directly compare the in vivo effects of IL-15 for memory cell subsets, whole splenocytes from LCMV-primed B6 mice containing Thy-1.1$^+$ Smarta memory cells were CFSE labeled and transferred into nonirradiated CD45.1 congenic B6 hosts. The host mice were then injected with 1.5 μg IL-15 or PBS, and the proliferation of donor Smarta memory, MP CD4$^+$, and MP CD8$^+$ cells (all three distinguishable by CD45 and Thy-1 alleles) was measured 5 d later. As expected, IL-15 injection caused a marked increase in the proliferation of donor MP CD8$^+$ cells but did not seem to influence MP CD4$^+$ cells, which displayed a prominent population of fast-dividing cells (Fig. 4 A). In contrast to MP CD4$^+$ cells, a fraction of Smarta memory cells clearly proliferated in response to IL-15 treatment, although not as strongly as MP CD8$^+$ cells (Fig. 4 A). This finding confirms that MP CD4$^+$ cells, as a population, are indifferent to IL-15, but shows that Smarta memory cells, like polyclonal Ag-specific memory CD4$^+$ cells (15), are capable of responding to IL-15 under in vivo conditions.

To determine the role of IL-15 in supporting basal homeostatic proliferation of Ag-specific CD4$^+$ memory cells under normal physiological conditions, CFSE-labeled Smarta memory cells were transferred into nonirradiated IL-15$^-$ mice and analyzed 51 d later. B6, IL-7$^-$, and IL-7$^-$15$^-$ mice were included as controls (Fig. 4 B). Consistent with their low basal homeostatic proliferation rate (Fig. 1 C), 70–80% of Smarta memory cells underwent one to three cell divisions in B6 hosts during the 51-d period. Strikingly, in IL-15$^-$ hosts, proliferation of Smarta memory cells was severely curtailed as only a small fraction of cells underwent one cell division. The residual cell division in IL-15$^-$ hosts was driven by IL-7 as complete abrogation of proliferation was observed in IL-7$^-$15$^-$ hosts. A severe reduction in the proliferation of Smarta memory cells was also observed in IL-7$^-$ hosts. These findings indicate that IL-7 and IL-15 play codominant roles in supporting efficient basal homeostatic proliferation of Smarta memory cells. However, in terms of cell survival, IL-7 plays a more prominent role than IL-15 (Fig. 4 B). Thus, the recovery of Smarta memory cells in IL-7$^-$ and IL-7$^-$15$^-$ hosts was approximately fivefold less than that obtained from B6 hosts, whereas the recovery of Smarta memory cells in IL-15$^-$ hosts was two- to threefold lower than that found in B6 hosts. Notably, Smarta memory cells continued to decline slowly as a population after transfer to B6 hosts at the same rate observed in LCMV-infected B6 hosts (Fig. 1 A); thus, only 60% of the original engrafted population was recovered on day 51 (unpublished data).

To determine whether a requirement for IL-15 during basal homeostatic turnover and survival is generally applicable

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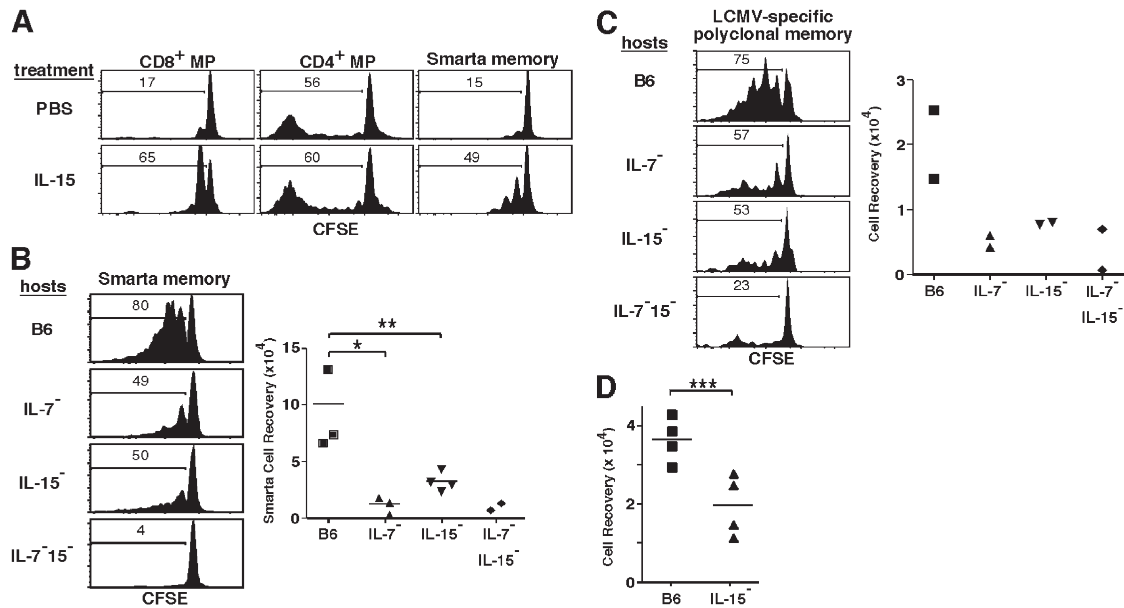

Figure 4. Ag-specific memory CD4$^+$ cells require IL-15 to undergo homeostatic proliferation and to survive under normal non-irradiated conditions. (A) Exogenous IL-15 can induce slow proliferation of Smarta memory cells. A dose of 5×10^6 splenocytes from LCMV-primed B6 mice containing Thy-1.1$^+$ Smarta memory cells was CFSE labeled and transferred to nonirradiated CD45.1$^+$ B6 mice. Recipients were then injected with 1.5 μg of recombinant murine IL-15 or PBS on days 0 and 2, and the donor cells in the host spleen were analyzed on day 5 by staining for Thy-1.1, CD45.2, CD44, CD4, and CD8. The CFSE profiles of donor MP CD8$^+$, MP CD4$^+$, and Smarta memory cells are shown. Data are representative of two experiments using two recipients per treatment analyzed individually. (B) IL-15 is essential for basal homeostatic proliferation and survival of Smarta memory cells. A small dose (8×10^5) of CFSE-labeled Thy-1.1$^+$ Smarta memory cells was injected into nonirradiated B6, IL-7$^-$, IL-15$^-$, and IL-7$^-$IL-15$^-$ mice. Approximately 20% of the donor cells could be recovered from the spleen on day 1 in all hosts. Spleens were analyzed 51 d later by staining for Thy-1.1 and CD4, and the CFSE profiles and recovery of donor cells are shown. Data are representative of three experiments using two to four recipients per host type analyzed individually. *, $P < 0.01$; **, $P < 0.05$ by one-way ANOVA, Bonferroni's multiple comparison test. (C) Polyclonal-derived Ag-specific CD4$^+$ secondary memory cells require IL-15 for their basal turnover. A dose of 2×10^7 purified CFSE-labeled CD4$^+$ cells from Thy-1.1$^+$ B6 mice previously immunized and boosted with LCMV (comprising $\sim 1.5 \times 10^5$ LCMV-specific polyclonal secondary memory CD4$^+$ cells as detected by IFN-γ production) were injected into nonirradiated B6, IL-7$^-$, IL-15$^-$, and IL-7$^-$IL-15$^-$ mice. The LCMV-specific polyclonal donor CD4$^+$ memory cells were detected 51 d later by staining for Thy-1.1, CD4, and IFN-γ after a 5-h in vitro stimulation with agonist GP61-80 peptide. Data are representative of three experiments using two to four recipients per host type analyzed individually. (D) IL-15 sustains polyclonal-derived Ag-specific CD4$^+$ memory cells. Experiments were performed as in C, except twofold higher numbers (3×10^5) of primary LCMV-specific polyclonal memory CD4$^+$ cells were transferred into only B6 and IL-15$^-$ mice, and the recovery of donor cells was analyzed 65 d later. ***, $P = 0.0127$ by two-tailed unpaired *t* test.

to all Ag-specific memory CD4$^+$ cells, the above experiment was repeated with polyclonal Ag-specific memory CD4$^+$ cells. Thus, splenic CD4$^+$ cells were purified from B6.PL mice that were previously immunized with LCMV, CFSE labeled, and then transferred into nonirradiated B6, IL-7$^-$, IL-15$^-$, and IL-7$^-$15$^-$ mice. These hosts displayed comparable engraftment of donor cells. The fate of LCMV-specific polyclonal donor cells was analyzed 51 d later by staining for donor Thy-1.1$^+$ cells that synthesized IFN-γ upon in vitro stimulation with LCMV GP61-81 peptide. An advantage of this system is that the LCMV-specific CD4$^+$ memory cells are generated from physiological numbers of precursors, as recent results have indicated that precursor numbers can impact the homeostasis of the resultant memory cells (36, 37). Significantly, polyclonal Ag-specific memory CD4$^+$ cells behaved similarly to Smarta memory cells, both in their basal rate of turnover and their dependence on IL-15 (Fig. 4 C). Thus, although polyclonal memory CD4$^+$ memory cells underwent considerable basal homeostatic proliferation in B6 hosts, their proliferation in IL-15$^-$ and IL-7$^-$ hosts was equally severely reduced and virtually abolished in IL-7$^-$15$^-$ hosts. Moreover, the recovery of polyclonal CD4$^+$ memory cells in IL-15$^-$ hosts was significantly lower as compared with control B6 hosts and was even further reduced in IL-7$^-$ and IL-7$^-$15$^-$ hosts (Fig. 4, C and D). Collectively, these findings indicate that Ag-specific memory CD4$^+$ cells depend on both IL-7 and IL-15 for their basal homeostatic proliferation and prolonged survival.

Ag-specific CD4$^+$ memory cells compete with CD8$^+$ MP and NK cells for IL-15

In contrast to memory CD8$^+$ cells, whose basal homeostatic proliferation is primarily driven by IL-15 (2, 12, 13), the above findings indicate that the basal turnover of Ag-specific memory CD4$^+$ cells is equally dependent on both IL-15 and IL-7. One likely explanation for this difference in cytokine requirements

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is that memory CD4$^+$ and CD8$^+$ cells express different levels of the receptors for IL-15 and IL-7. Indeed, MP CD4$^+$ and Smarta memory cells express much lower levels of CD122 (approximately fourfold less) than MP CD8$^+$ and NK cells (Fig. 5 A). CD127 expression levels on memory CD4$^+$ and CD8$^+$ cells are much more comparable, but it is notable that MP CD4$^+$ cells express a much wider range of CD127 than Smarta memory cells (Fig. 5 A).

One possible consequence of low CD122 expression on Smarta memory cells is that these cells may encounter strong competition for IL-15 from CD122^{hi} MP CD8$^+$ and NK cells. Accordingly, Smarta memory cells might display increased responsiveness to IL-15 in the absence of CD8$^+$ and NK cells. To test this idea, CFSE-labeled Smarta memory cells were transferred into nonirradiated B6 mice, and the effect of selectively depleting host CD8$^+$ or/and NK cells was measured during a 3-wk period. As controls, IL-7$^-$ and IL-15$^-$ hosts were also included. Notably, the basal homeostatic proliferation rate of Smarta memory cells in normal B6 hosts was not affected by the removal of NK cells, slightly increased by the removal of CD8$^+$ cells, and significantly elevated by the depletion of both CD8$^+$ and NK cells (Fig. 5 B). Significantly, these effects were even more striking in IL-7$^-$ hosts (Fig. 5 B). Thus, the severely depressed rate of Smarta memory cell proliferation apparent in untreated IL-7$^-$ hosts was partially restored with removal of either CD8$^+$ or NK cells, and completely restored with the joint removal of both CD8$^+$ and NK cells. The reason why the removal of NK cells alone had a greater effect in IL-7$^-$ hosts than in B6 hosts could reflect the fact that IL-7$^-$ mice possess very few CD8$^+$ cells to compete for IL-15, but have normal numbers of NK cells (38, 39). It is also possible that the available levels of IL-15 are higher in IL-7$^-$ mice due to the near absence of naive CD8$^+$ cells, which are partially IL-15 dependent (7, 8). In control IL-15$^-$ hosts, the removal of CD8$^+$ and NK cells had little effect in restoring the reduced proliferation of Smarta memory cells (Fig. 5 B), confirming that the depleted cells competed with Smarta memory cells by sequestering IL-15.

As a control, the basal turnover rate of Smarta memory cells was compared with the proliferation of CD8$^+$ P14 memory cells transferred into nonirradiated B6, IL-7$^-$, and IL-15$^-$ hosts for a similar duration of time (Fig. 5 B). In B6 hosts, the proliferation rate of P14 memory cells was similar to that of Smarta memory cells. However, the proliferation of P14 memory cells was not reduced in IL-7$^-$ hosts, presumably because CD8$^+$ memory cells are less dependent than CD4$^+$ memory cells on IL-7 for undergoing basal homeostatic proliferation. The slight increase in proliferation of P14 memory cells in IL-7$^-$ hosts probably reflects the increased availability of IL-15 in these mice. As expected, both subsets of memory cells did not proliferate in the absence of IL-15. The key finding in the above experiment is that the restricted capacity of memory CD4$^+$ cells to use IL-15 for normal homeostasis presumably reflects competition for IL-15 by CD8$^+$ cells and NK cells. Only in the absence of these cells can memory CD4$^+$ cells use IL-15 about as efficiently as memory CD8$^+$ cells.

DISCUSSION

In this study we have resolved the controversy on the factors controlling the homeostasis of memory CD4$^+$ cells. By

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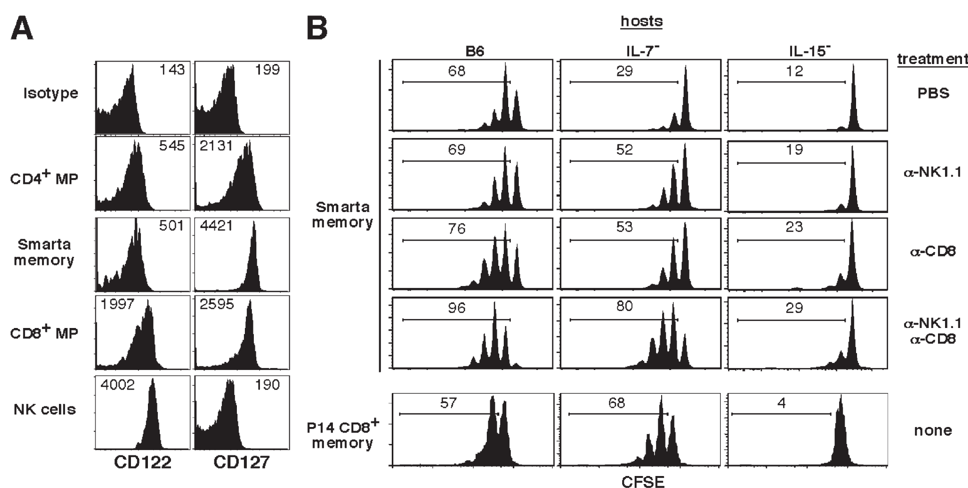

Figure 5. Smarta memory cells compete with CD8$^+$ MP and NK cells for IL-15. (A) Comparative expression levels of CD122 and CD127 on various cell types. Representative histograms of CD122 and CD127 analyzed on CD4$^+$ MP, Smarta memory, CD8$^+$ MP, and NK cells from 3–4-mo-old B6 mice after staining for CD4, CD8, NK1.1, CD44, CD122, or CD127. Numbers indicate mean fluorescence intensity. (B) Depletion of CD8 and/or NK cells increases basal homeostatic proliferation rate of Smarta memory cells. A small dose (5×10^5) of CFSE-labeled Thy-1.1$^+$ Smarta memory cells was injected into B6, IL-7$^-$, or IL-15$^-$ mice that were treated with PBS or mAbs to deplete NK and/or CD8 cells. The CFSE profiles of donor cells analyzed 21 d later by staining for Thy-1.1 and CD4 are shown. As a comparison, CD8$^+$ P14 memory cells undergoing basal homeostatic proliferation for 21 d in indicated hosts are shown. Data are representative of two experiments using two recipients per host type analyzed individually.

studying LCMV-specific TCR transgenic Smarta memory and polyclonal Ag-specific memory CD4$^+$ cells, we find that the homeostasis of memory CD4$^+$ cells is regulated by two cytokines, namely IL-7 and IL-15, and appears to be completely independent of MHC II molecules. In confirmation of previous work (15, 21, 34), memory CD4$^+$ cells were found to be exquisitely dependent on IL-7, both for survival and homeostatic proliferation. Nevertheless, we demonstrate for the first time that IL-15 plays an essential role in supporting memory CD4$^+$ cell homeostasis. Significantly, IL-15 is not required under conditions of excess IL-7, such as in lymphopenia. However, under normal physiological conditions of limiting IL-7 availability, IL-15 is crucial for both survival and homeostatic proliferation of memory CD4$^+$ cells. These findings demonstrate that, in a normal environment, memory CD4$^+$ cells closely resemble memory CD8$^+$ cells in their dependency on both IL-7 and IL-15 for their homeostasis.

Why was the role of IL-15 in memory CD4$^+$ cell homeostasis not found previously? We believe this reflects the limitations inherent in the three previous approaches used to study homeostatic requirements of memory CD4$^+$ cells. First, as shown here, using MP CD4$^+$ cells as surrogates for Ag-specific memory CD4$^+$ cells is problematic because MP CD4$^+$ cells are a heterogeneous population and are not equivalent to Ag-specific memory CD4$^+$ cells in terms of their homeostatic requirements (9, 19). Second, the prior use of a CD4$^+$ TCR transgenic line that could not undergo homeostatic proliferation precluded revealing a connection between IL-15 and homeostatic proliferation (21). Third, studying memory CD4$^+$ cells generated in IL-15$^-$ mice appears to be inappropriate as these cells seem to have found an alternative way to sustain their homeostasis in the absence of IL-15 (15).

As with the CD8$^+$ cell subset, MP CD4$^+$ cells are widely used for homeostasis studies with the assumption that these cells are equivalent to Ag-specific memory CD4$^+$ cells in their homeostatic requirements. Hence, we and others had previously reported that MP CD4$^+$ cells are able to undergo rapid acute homeostatic proliferation in the absence of IL-7 and/or IL-15 (9, 19). However, it is now becoming increasingly clear that, as a population, MP CD4$^+$ cells display different homeostatic characteristics than Ag-specific CD4$^+$ cells. In particular, MP CD4$^+$ cells contain a subset of cells that undergoes a very fast rate of homeostatic proliferation. This rapidly dividing subset depends on contact with MHC II for proliferation but does not require IL-7 or IL-15, although IL-7 promotes survival of these cells. The fact that these cells proliferate and expand even more rapidly in RAG$^-$ hosts suggests that they are responding to foreign Ags, probably originating from an enteric source. However, the possibility that self-Ags also play a role cannot be ruled out. Thus, preliminary experiments on B6 mice raised under germ-free conditions showed that the relative turnover rate of MP CD4$^+$ cells in these mice is very similar to that observed with conventionally raised mice (unpublished data). The intriguing question of why foreign or self-peptides induce strong proliferation of a subset of MP CD4$^+$ cells is still unclear. However, until the subset of fast-dividing cells can be effectively identified and removed, which is not currently possible, normal MP CD4$^+$ cells cannot be used as surrogates for Ag-specific memory CD4$^+$ cells.

With the exception of the fast-dividing cells, the remaining MP CD4$^+$ cells are relatively quiescent as they either remain in interphase or undergo only very slow turnover. Their limited turnover rate together with dependence on IL-7 suggests that most MP CD4$^+$ cells closely resemble Ag-specific CD4$^+$ cells in their homeostatic requirements. For IL-15, defining the role of this cytokine on MP CD4$^+$ cells is complicated by the presence of the fast-dividing subset of these cells. Nonetheless, because MP CD4$^+$ cells and Smarta memory cells express similar levels of CD122, and because polyclonal Ag-specific memory CD4$^+$ cells are dependent on IL-15, it is likely that most MP CD4$^+$ cells are also reliant on IL-15 for their homeostasis. If so, as for MP CD8$^+$ cells, one would expect MP CD4$^+$ cells to be depleted in IL-15$^-$ and IL-15Rα^- mice, but this is not the case (7, 8). This finding may reflect that the MP CD4$^+$ cells in these hosts have become conditioned to survive in the absence of IL-15, possibly by being more dependent on IL-7. This idea is currently being tested.

The idea that memory CD4$^+$ cells can adapt to the absence of IL-15 by using a compensatory mechanism could explain the previous report of Lenz et al. (15) that IL-15 is largely dispensable for basal homeostatic turnover of Ag-specific memory CD4$^+$ cells. Thus, this latter study analyzed polyclonal memory CD4$^+$ cells that were produced de novo in IL-15$^-$ mice rather than in normal mice. The notion that memory T cells adjust to IL-15 deficiency is also supported by the finding that memory CD8$^+$ cells produced de novo in IL-15$^-$ mice are considerably less dependent on IL-15 for homeostatic proliferation than analogous cells produced in normal mice (13, 15). Another potential complication of studying memory T cells generated de novo in IL-15$^-$ hosts is that naive T cells may be suboptimally activated because of a dendritic cell defect in IL-15$^-$ mice (40, 41). This is a relevant concern as it is becoming increasingly clear that the strength of initial stimulation during the priming of naive T cells influences the homeostatic requirements for the resultant memory T cells (36, 37).

Although we and others have found that LCMV-specific memory CD4$^+$ cells appear to decline slowly over time, significant populations of these cells persist for years in mice, and Ag-specific memory cells survive for decades in humans, indicating prolonged persistence of CD4$^+$ T cell immunity (6, 27, 42, 43). Thus, IL-7 and IL-15 play significant roles in preserving this immunity as we find a rapid loss of memory CD4$^+$ cells in the absence of either cytokine. Furthermore, the memory CD4$^+$ cell dependence on IL-7 and IL-15 for homeostasis closely resembles the requirements for homeostasis of memory CD8$^+$ cells. A minor discrepancy is that under normal physiological conditions, memory CD4$^+$ cells require both IL-7 and IL-15 for homeostatic

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proliferation, whereas homeostatic proliferation of memory $CD8^+$ cells is exclusively driven by IL-15 (2, 12, 13, 44). This slight difference probably reflects the fact that memory $CD4^+$ cells express considerably lower levels of CD122 than memory $CD8^+$ cells. Hence, $CD122^{lo}$ memory $CD4^+$ cells are likely to encounter severe competition for IL-15 from $CD122^{hi}$ cells, such as memory $CD8^+$ cells and NK cells. For this reason, memory $CD4^+$ cells could have evolved to use both IL-7 and IL-15 for homeostatic proliferation. However, despite their lower level of CD122 expression, memory $CD4^+$ cells do not display compensatory higher levels of CD127 (IL-7-Rα) than memory $CD8^+$ cells; indeed, CD127 levels on the two subsets of memory cells are very similar. Hence, memory $CD4^+$ cells are likely to be at a disadvantage relative to memory $CD8^+$ cells for competition for IL-15 during normal homeostasis. One significant implication here is that competition from memory $CD8^+$ cells may cause the gradual decline of memory $CD4^+$ cell numbers over time (Fig. 1; reference 27), especially in old age where a marked increase in MP $CD8^+$ cells presumably increases competition for IL-15 (45, 46). In addition, memory $CD4^+$ cells may receive competition from the fast-dividing population of MP $CD4^+$ cells that presumably consume substantial amounts of both IL-7 and IL-15. For these reasons, memory $CD4^+$ cells would be expected to survive indefinitely in the absence of competition from memory $CD8^+$ and MP $CD4^+$ cells. This prediction is currently under investigation.

MATERIALS AND METHODS

Mice. B6, B6.PL, and CD45.1 congenic mice were purchased from the breeding colony at The Scripps Research Institute (TSRI). $A\beta^-$ mice were provided by T. Laufer (University of Pennsylvania, Philadelphia, PA; reference 47). $MHC\text{-}II^{\Delta/\Delta}$ mice were purchased from The Jackson Laboratory. $IL\text{-}7^-$ (38) and $IL\text{-}15^-$ (8) mice were gifts from DNAX, Immunex Corporation. The Smarta mice (Smarta2), transgenic for an MHC II–restricted TCR recognizing LCMV GP61-81–H2-A^b complexes on the B6 background (24), were provided by H. Hengartner (University Hospital, Zurich, Switzerland) and bred to a B6.Thy-1.1^+ background. $IL\text{-}7^-IL\text{-}15^-$ and $IL\text{-}7^-$ $IL\text{-}15^-$ $MHC\text{-}II^{\Delta/\Delta}$ mice were bred at TSRI. All recipient mice were backcrossed at least 10 times to the B6 background and housed under specific pathogen-free conditions. Recipient mice were either irradiated with 600 cGy or left untreated. Donor cells were transferred to hosts by i.v. injection 1 d later. Where the depletion of $CD8^+$ T cells and NK cells was necessary, recipient mice were injected i.p. with depleting antibodies specific for CD8 (YTS-169) and NK1.1 (PK136) on days −7, −3, 0, and 3 relative to $CD4^+$ memory T cell transfer, and then once a week thereafter. PBS dialyzed ascites fluid containing at least 0.1 mg of each depleting antibody was used for each depletion injection. Where stated, depleted mice were also thymectomized a week before the transfer of donor cells. All experiments were approved by the TSRI Institutional Animal Care and Use Committee.

Virus treatment and adoptive transfer of $CD4^+$ memory T cells. A treatment of 2×10^5 PFUs of the Armstrong strain of LCMV, clone 53b, was used for all experiments. Memory $CD4^+$ T cells were generated as follows: 10^5 naive Smarta cells (resulting in 10^4 precursors) were transferred i.v. into B6 mice at 8–12 wk of age that were injected 1 d later with LCMV i.p. At memory time points (>50 d after virus), mice were killed and spleen cell suspensions were prepared in DMEM supplemented with 2% FCS and Hepes. Purified cells were labeled with the intracellular fluorescent dye CFSE (Invitrogen) as described previously (48). Smarta memory cells were isolated to >95% purity at memory time points by positive selection using anti–Thy-1.1 biotin (HIS51; grown and conjugated in house), streptavidin particles, and an IMag magnet as per the manufacturer's instructions (BD Biosciences). Polyclonal memory $CD4^+$ cells were generated by treating B6.PL mice with LCMV and allowing the mice to rest for at least 50 d. Spleen suspensions were stained with anti-CD4 (RM4-5) biotin (eBioscience), followed by anti-biotin tetramers and magnetic colloid as per the manufacturer's instructions (Stem Cell Technologies Inc.). Cells were passed through MACS LS separation columns (Milteyni Biotec) to collect bound $CD4^+$ cells. The 2×10^7 transferred, CFSE-labeled $CD4^+$ cells contained $\sim 2 \times 10^5$ polyclonal memory $CD4^+$ cells. For some experiments, polyclonal secondary memory cells were isolated from mice that had been boosted with 10^6 PFUs LCMV i.v. 50 d earlier with similar results. Thy1.1^+P14 $CD8^+$ memory cells were generated from 10^5 injected naive precursors and were also isolated using the IMag protocol described above.

Flow cytometry and cytokines. Spleen cells were prepared and stained for donor cells as described previously (48). The following antibodies were used for flow cytometry: CD4 PE-CY7 (RM4-5; eBioscience), NK1.1 FITC and PE (PK136), CD25 PE (PC61), CD44 FITC (IM7), CD45.1 PE (A20), CD42.5 PE-Cy5.5 (104), CD90.1 PE (HIS51), CD127 PE (A7R34), CD122 PE (5H4), rat IgG2a isotype-PE (eBR2a; BD Biosciences), CD3 PerCP-CY5.5 (145-2C11) MHC II biotin (M5/114.15.2), rat IgG2b isotype biotin (KLH/G2b1-2), and CD90.1 CY5 (HIS51 was grown and conjugated in house). Samples were run on an LSR II (Becton Dickinson), and data were analyzed with FlowJo software (TreeStar). Cell sorting was performed using a Becton Dickinson FACSAria. Murine IL-15 was purchased from eBioscience.

BrdU treatment. Mice were injected with 0.8 mg BrdU (Acros) i.p., and then supplied with daily prepared drinking water containing 0.8 mg/ml BrdU. Detection of BrdU incorporation was performed as described previously (16).

Intracellular cytokine staining. 10^6 splenocytes were incubated in 96-well flat-bottomed plates with 1 μl/ml Golgistop (BD Biosciences) in the presence or absence of 0.1 μg/ml GP61-81. After a 5-h incubation at 37°C, cells were washed in FACS buffer (0.5% FCS, 0.2% sodium azide in PBS) and stained for CD4 and Thy1.1 to identify donor cells. Cells were washed twice in FACS buffer, and then fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Cells were then stained for intracellular cytokines using IFN-γ APC (clone XMG1.2) and IL-2 PE (clone JES6-5H4), and acquired as described above. Intracellular cytokine staining was used to identify donor Thy-1.1^+ polyclonal memory $CD4^+$ T cells transferred to different nonirradiated hosts. In this instance, 3×10^7 splenocytes were incubated in six-well plates, and then prepared and analyzed as described above.

Statistics. All statistical tests listed were performed using Prism software (GraphPad).

Online supplemental material. Fig. S1 shows the characterization of Smarta effector and memory cells generated from 10^3 injected precursors. The expression of activation markers and cytokine receptors on Smarta cells were examined before and after LCMV infection. Fig. S2 shows the expression levels of various activation markers that were examined on CFSE-labeled MP $CD4^+$ cells 10 d after their transfer to irradiated mice. Fig. S3 shows that MHC II does not enhance the homeostatic turnover or survival of Ag-specific $CD4^+$ memory cells. Figs. S1–S3 are available at http://www.jem.org/cgi/content/full/jem.20061805/DC1.

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