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Bone Marrow Is a Preferred Site for Homeostatic Proliferation of Memory CD8 T Cells

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Proliferative renewal of memory CD8 T cells is essential for maintaining long-term immunity. In this study, we examined the contributions that various tissue microenvironments make toward the homeostatic proliferation of Ag-specific memory CD8 T cells. We found that dividing memory T cells were present in both lymphoid and nonlymphoid tissues. However, the bone marrow was the preferred site for proliferation and contained a major pool of the most actively dividing memory CD8 T cells. Adoptive transfer studies indicated that memory cells migrated through the bone marrow and divided there preferentially. These results show that the bone marrow is not only the source of stem cells for generating naive T cells but also provides the necessary signals for the self-renewal of memory T cells. *The Journal of Immunology,* **2005, 174: 1269–1273.**

Following clearance of a viral infection, a stable pool of Ag-specific memory CD8 T cells is established that persists for long periods of time, often for the life of the animal (1, 2). Long-lived memory CD8 T cells are a critical component of protective antiviral immunity because they allow the animal to mount an anamnestic response that is greater in speed and magnitude than a primary response. Increased responsiveness in a recall response is due to both quantitative and qualitative changes. First, the precursor frequency of memory T cells is much greater than for naive cells (2–4). Second, memory cells are more responsive to Ag, and gain effector function more rapidly upon restimulation with Ag (5–8). These two properties ensure that a recall response is more potent than a primary response.

Memory CD8 T cells are maintained in the absence of Ag through a process of homeostatic proliferation (9, 10). Recent work using IL-15$^{-/-}$ and IL-7$^{-/-}$ mice has demonstrated that these two cytokines are critical for memory CD8 T cell maintenance (11–13). IL-7 appears to play a greater role in cell survival, whereas IL-15 is primarily responsible for driving memory cells into cell cycle. It is now well documented that memory T cells are present in both lymphoid and nonlymphoid tissues (14–16). However, it is not known whether homeostatic proliferation occurs in all tissues or whether there is a preferential site of memory T cell proliferation.

We have addressed this question using the murine model of infection with lymphocytic choriomeningitis virus (LCMV)[3] and assessed the homeostatic proliferation of virus-specific memory CD8 T cells in various lymphoid (spleen, lymph nodes, bone marrow, and blood) and nonlymphoid (liver and lung) tissues. Memory T cell turnover was analyzed by determining the DNA content of the memory cells, by in vivo BrdU labeling experiments, and by adoptive transfer of CFSE-labeled memory T cells. Staining for DNA content allowed us to determine the percentage of memory cells in cell cycle at a given time point in the various tissues, whereas BrdU incorporation provided evidence of recent DNA synthesis. This was important to verify whether cells that had recently synthesized DNA were incorporated into the peripheral memory cell pool. Adoptive transfer experiments using CFSE-labeled memory cells allowed us to study the migration of these cells to various tissues and also to monitor their state of division. All adoptive transfer experiments were done using nonirradiated mice containing a full lymphoid compartment so that homeostatic proliferation of memory T cells could be studied under normal physiological conditions. We found that memory cells underwent homeostatic proliferation in both lymphoid and nonlymphoid tissues. However, memory cells divided to a greater extent in lymphoid tissues, and most prominently in the bone marrow. We propose that the bone marrow is a uniquely important site for signaling and/or supporting the homeostatic proliferation of memory CD8 T cells.

Materials and Methods

Virus

Stocks of the Armstrong strain of LCMV were prepared as previously described (17). Mice were infected by i.p. injection of 2×10^5 PFU of the Armstrong strain of LCMV.

Mice

C57BL/6 mice were purchased from the National Cancer Institute. The generation and description of IL-15$^{-/-}$ mice has been described previously (18). IL-15$^{-/-}$ mice were obtained from M. Caligiuri (Ohio State University, Columbus, OH). IL-15$^{-/-}$ were backcrossed to B6 mice for >10 generations and maintained in our colony. P14 transgenic mice with T cells expressing the TCR specific for the LCMV epitope GP33–41 (19) were obtained from The Jackson Laboratory and backcrossed to B6 mice in our colony. Immune P14 cells were made by transferring 1×10^6 splenocytes from a P14 transgenic mouse into a naive B6 recipient by i.v. injection, and then infecting with LCMV 24 h later.

Cell surface staining

Single-cell suspensions were prepared from the spleen and from the brachial, axillary, inguinal, lumbar, and mesenteric lymph nodes. Bone marrow was obtained by flushing two femurs with cold RPMI 1640. Total number of cells in bone marrow was calculated as follows: no. in two femurs × 7.9 (20, 21). Lymphocytes from the blood, lungs, and liver were

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; P, postnatal day; PI, propidium iodide; DC, dendritic cell.

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obtained as described (11). All Abs were purchased from BD Pharmingen. Preparation of MHC tetramers and staining for various cell surface markers were done as described (2).

CFSE labeling and adoptive transfer

Splenocytes were prepared from LCMV-immune mice. Cells were labeled with CFSE (Molecular Probes) by incubating at 5 mM in PBS, quenching with FCS, and washing as described previously (22). Cells were resuspended in PBS, and 4×10^7 total splenocytes were transferred by i.v. injection into naive B6 or IL-$15^{-/-}$ mice.

Cell cycle analysis

A total of 1×10^6 cells was stained for surface markers, and then permeabilized in PBS containing 0.3% saponin. Propidium iodide (PI; 10 μg/ml) and RNase (200 μg/ml) were added, and cells were incubated for 20 min at room temperature. Cells were acquired on a FACSCalibur in their staining solution. Doublet discrimination was done by gating out events with high signal width as described (23). Cell cycle analysis of DNA histograms was done with FlowJo software (Tree Star).

BrdU incorporation

Immune mice containing P14 cells were given 2 mg of BrdU in PBS by i.v. injection. Twenty-four hours later, mice were sacrificed, and lymphocytes were prepared from all tissues. Cells were stained for surface markers, fixed in ethanol, and stained for BrdU incorporation as described (9).

Poly(I:C) treatment

B6 or IL-$15^{-/-}$ mice treated with 150 mg of poly(I:C) dissolved in 0.5 ml of PBS or PBS alone by i.p. injection (24). Mice were analyzed 2 days later for cycling of memory CD8 T cells by surface staining and PI staining as described above. In addition, some mice were given CFSE-labeled splenocytes from immune animals, rested for 1 day, then treated with poly(I:C) or PBS. Three days later, mice were sacrificed and CFSE-labeled memory CD8 T cells were analyzed in all tissues.

Results

The bone marrow contains a large population of memory CD8 T cells

B6 mice were infected with the Armstrong strain of LCMV, which induces a vigorous primary CD8 T cell response that clears the virus within 1 wk. These immune mice then generate memory CD8 T cells that persist for the life of the animal. Lymphocytes were isolated from several lymphoid (spleen, lymph nodes, bone marrow, PBMCs) and nonlymphoid (liver, lung) tissues of these LCMV immune mice (40–60 days after infection) and examined for the presence of virus-specific CD8 T cells. Memory cells specific for the LCMV epitope DbGP33–41 could be found in all tissues analyzed (Fig. 1*A*). The absolute number of Ag-specific cells was then determined for each tissue (Fig. 1*B*). The bone marrow contained one of largest pools of Ag-specific cells, second only to the spleen. In addition to studying polyclonal responses to LCMV, we also analyzed memory responses using the P14 transgenic T cells specific for the DbGP33–41 LCMV epitope. P14 transgenic cells expressing the congenic marker Thy1.1 were transferred into naive B6 mice 1 day before LCMV infection, and then memory cells were analyzed at ~60 days postinfection. P14 memory cells were present in all tissues and had a similar tissue distribution to polyclonal DbGP33–41 CD8 T cells generated in B6 mice (Fig. 1*B*).

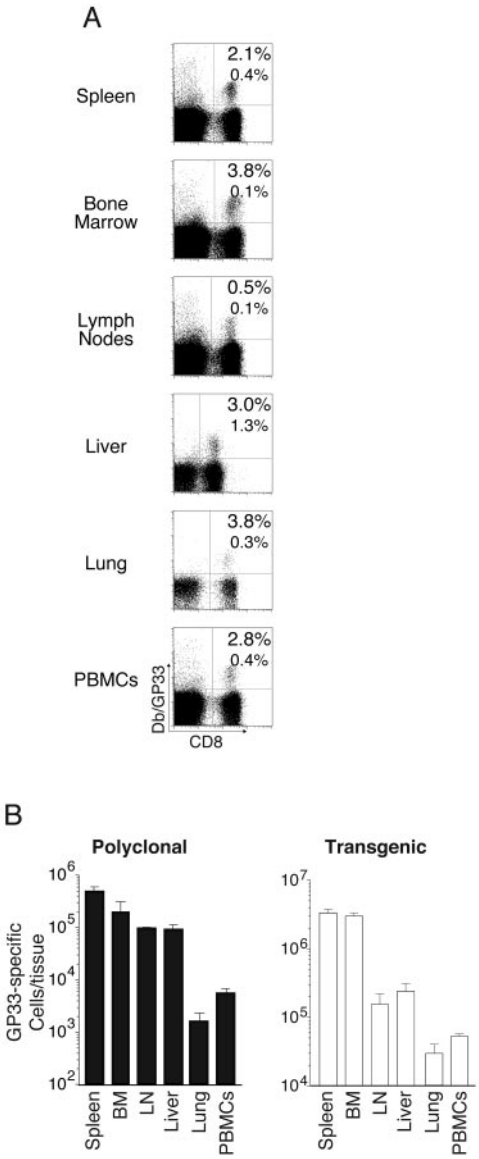


FIGURE 1. The bone marrow contains a large population of virus-specific memory CD8 T cells. *A*, B6 mice were infected with LCMV, and 60 days postimmunization, lymphocytes isolated from the indicated tissues were stained using MHC tetramers specific for the GP33–41 epitope. *Upper number* indicates the percentage of GP33-specific CD8 T cells per total CD8 T cells in the respective tissue, whereas the *lower number* indicates the percentage of Ag-specific CD8 T cells per total number of lymphocytes present in the tissue. *B*, Ag-specific cells were quantified from each tissue of LCMV-immune B6 mice and B6 mice that had been transferred with P14 transgenic T cells before infection.

The bone marrow contains the most actively dividing pool of memory CD8 T cells

To examine the homeostatic proliferation of memory cells, three experimental approaches were used. First, memory cells in each tissue were stained for DNA content using PI. DNA content provided a snapshot of cells actively undergoing division in each tissue at a given time. Second, immune mice were injected with BrdU, and incorporation was measured over 24 h to provide evidence of recent DNA synthesis. This was used to assess whether memory cells that had recently synthesized DNA were maintained in the peripheral pool. Finally, splenocytes from LCMV-immune mice were labeled with CFSE and adoptively transferred into uninfected animals. Dilution of CFSE provided a division history of the cells recovered from each tissue, allowing us to examine the long-term migration of cells undergoing homeostatic proliferation. Together, these techniques allowed visualization of both the cells that had recently completed division and those that were actively dividing.

We first examined memory cells from each tissue for evidence of active cell division by DNA staining using PI (Fig. 2*A*). Cells actively synthesizing DNA (i.e., in cell cycle) could be identified

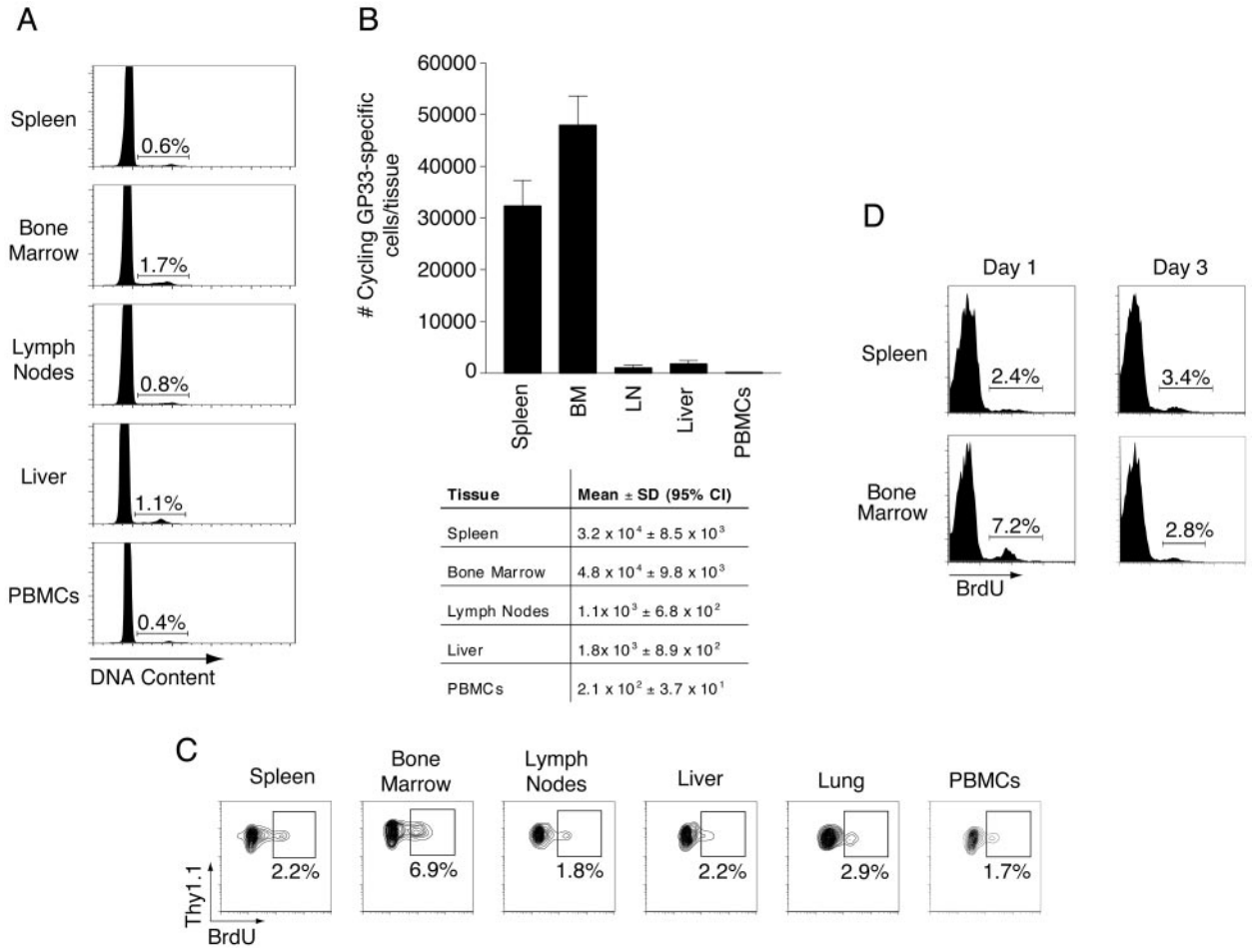


FIGURE 2. The bone marrow contains the most actively dividing pool of memory CD8 T cells. *A*, Memory P14 transgenic T cells from immune mice were stained for DNA content using PI. After gating on Thy1.1$^+$ P14 cells, histograms of DNA content were analyzed. Numbers indicate the percentage of memory P14 cells in cycle (S+G_2/M). One representative mouse is shown of six mice analyzed. *B*, The total number of P14 memory cells in cell cycle was calculated for each tissue. Results indicate the mean of four mice in one representative experiment of three. Mean and SD are given for the experiment shown. *C*, Immune mice containing P14 memory cells were injected with 2 mg of BrdU in PBS i.v. Twenty-four hours later, T cells were isolated from several tissues and analyzed for BrdU incorporation. Numbers indicate the percentage of P14 memory cells from each tissue that had incorporated BrdU. One representative mouse is shown. *D*, LCMV-immune B6 mice were injected with BrdU for pulse-chase studies of BrdU incorporation. CD44^{high} memory CD8 T cells were stained for BrdU incorporation in the spleen and bone marrow 1 and 3 days following injection. One representative mouse of four is shown for each time point.

by their increased DNA content, allowing us to determine in which tissue(s) active cell division was occurring. Memory cells were prepared from LCMV-immune mice containing P14 memory cells and stained for DNA content. A larger percentage of memory cells was actively synthesizing DNA in the bone marrow than in any other tissue (Fig. 2*A*). Although the difference was slight, it was reproducible over four separate experiments. The total number of actively cycling memory CD8 T cells was then calculated in each tissue based on the percentage of cells in cycle by PI staining (Fig. 2*B*). The largest number of cycling Ag-specific memory CD8 T cells in LCMV-immune mice was consistently found in the bone marrow. The number of memory cells actively undergoing DNA synthesis at any one time was small, and the difference between the bone marrow and spleen did not reach statistical significance (Fig. 2*B*).

Homeostatic proliferation was next assessed by analyzing the incorporation of BrdU. To accurately examine the differences in memory cell proliferation in different tissues, it was necessary to give a short pulse of BrdU. Longer treatment with BrdU obscured the differences among the various tissues, probably due to the migration of dividing cells among the tissues. Immune mice containing P14 memory cells were injected i.v. with 2 mg of BrdU. Mice were sacrificed 24 h later, and BrdU incorporation was measured in P14 transgenic memory T cells (identified by Thy1.1 expression) obtained from several tissues (Fig. 2*C*). A higher percentage of memory cells had incorporated BrdU in the bone marrow than in any other tissue analyzed. Although the extent of BrdU incorporation varied over three experiments, the percentage of BrdU$^+$ memory cells in the bone marrow (4.5–9.7%) was consistently two to three times that in the next closest tissue, the spleen (2.2–4.2%). The same pattern was observed when the entire population of CD44^{high} memory phenotype CD8 T cells was analyzed (data not shown).

In an additional series of experiments, mice were injected with BrdU, and memory CD8 T cells from the spleen and bone marrow were examined 24 h and 3 days later (pulse-chase). One day after injection, the bone marrow again contained the largest proportion of BrdU$^+$ memory cells (Fig. 2*D*). By day 3, however, the percentage of BrdU$^+$ cells in the bone marrow had declined, whereas the percentage in the spleen had remained stable. This suggests that memory cells divide preferentially in the bone marrow, and then rapidly migrate out into the periphery. Alternatively, dividing memory cells may be triggered to migrate into the bone marrow. We cannot exclude the possibility that BrdU$^+$ memory cells in the

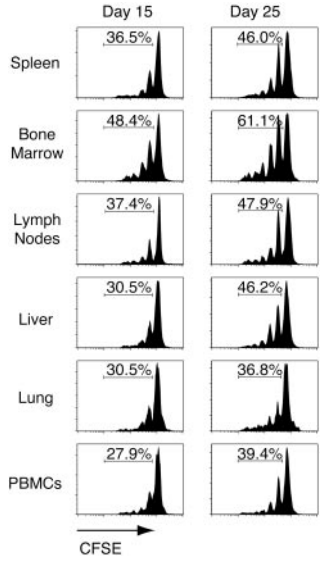


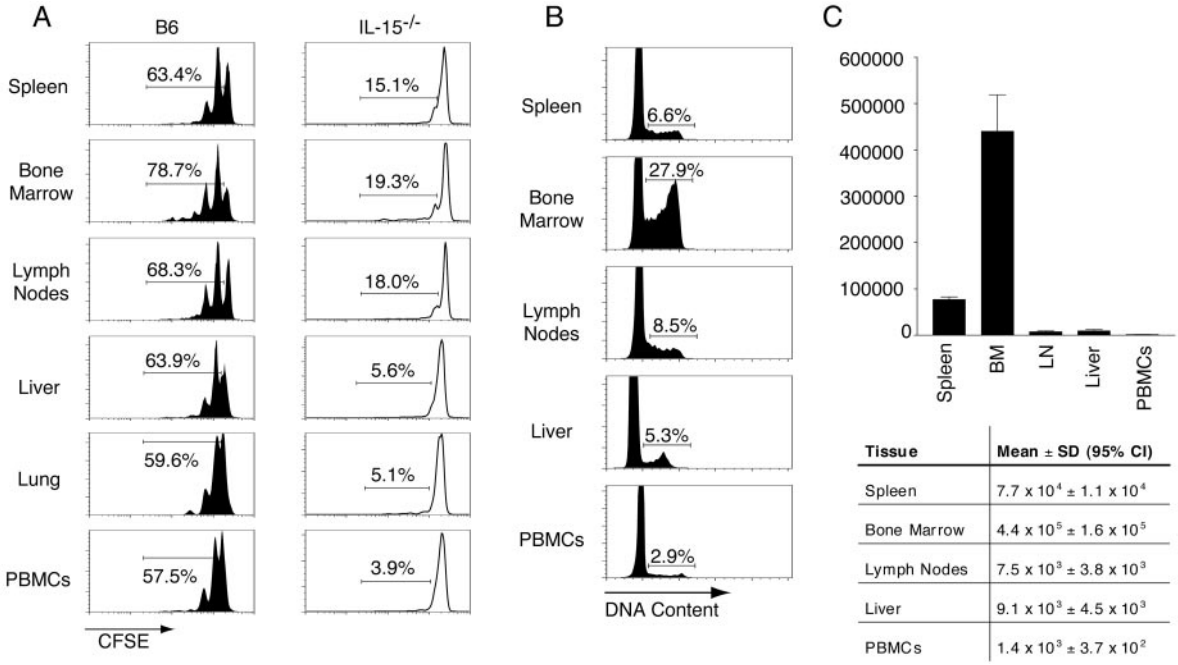
FIGURE 3. Adoptively transferred memory CD8 T cells undergo homeostatic proliferation preferentially in the bone marrow. Splenocytes were prepared from LCMV-immune mice, labeled with CFSE, and adoptively transferred into naive B6 recipients. The division of labeled $CD44^{high}$ CD8 T cells memory cells was assessed in cohorts of mice at 15 and 25 days posttransfer. Numbers indicate the percentage of memory cells that had undergone one or more divisions at the indicated time points. Results shown are a representative mouse from each time point from one representative experiment of three. Similar results were obtained for P14 transgenic memory cells (data not shown).

bone marrow will go on to die preferentially. However, the subsequent accumulation of $BrdU^+$ cells in the spleen on day 3 makes this possibility less likely.

Memory cell division was next assessed in each tissue by CFSE dilution. Memory cells from the spleens of LCMV-immune mice were labeled with CFSE and adoptively transferred into naive recipients. At 15 and 25 days after transfer, the division of labeled T cells was examined in each tissue (Fig. 3). Although the differences were subtle, memory CD8 T cells recovered from the bone marrow had consistently divided more extensively than those from other lymphoid and nonlymphoid tissues. Results are shown for $CD8^+$, $CD44^{high}$ cells, and identical results were obtained for Ag-specific cells (data not shown).

IL-15-dependent proliferation of memory CD8 T cells occurs preferentially in the bone marrow

Although memory cell division was consistently higher in the bone marrow than in other tissues, the slow rate of homeostatic proliferation made the differences slight. To induce a higher rate of proliferation and more readily identify the site(s) of memory cell division, mice were treated with poly(I:C). This treatment selectively induces proliferation of memory CD8 T cells in an IL-15-dependent manner, mimicking the signals for homeostatic proliferation (25). First, B6 or IL-$15^{-/-}$ mice received CFSE-labeled P14 memory cells 1 day before they were injected with poly(I:C). Division of the CFSE-labeled memory cells was then analyzed 3 days later (Fig. 4*A*). The rate of memory T cell proliferation was more extensive in all tissues in the mice injected with poly(I:C). Lymphoid tissues contained more divided cells than nonlymphoid tissues, and the bone marrow once again contained the most extensively divided cells. There was little division in IL-$15^{-/-}$ mice, illustrating the IL-15 dependence of poly(I:C)-driven stimulation as shown before (25). In a separate experiment, immune mice containing P14 memory cells were injected with poly(I:C). Two days later, memory cells were prepared from the indicated tissues and stained for DNA content. In poly(I:C)-treated mice, the bone marrow stood out even more



Tissue	Mean ± SD (95% CI)
Spleen	$7.7 \times 10^4 \pm 1.1 \times 10^4$
Bone Marrow	$4.4 \times 10^5 \pm 1.6 \times 10^5$
Lymph Nodes	$7.5 \times 10^3 \pm 3.8 \times 10^3$
Liver	$9.1 \times 10^3 \pm 4.5 \times 10^3$
PBMCs	$1.4 \times 10^3 \pm 3.7 \times 10^2$

FIGURE 4. IL-15-dependent proliferation of memory CD8 T cells occurs preferentially in the bone marrow. *A*, P14 memory cells were prepared from LCMV-immune mice, labeled with CFSE, and transferred into B6 or IL-$15^{-/-}$ recipients. Mice were injected with poly(I:C) the following day and were analyzed 3 days later. Numbers indicate the percentage of P14 memory cells that had undergone one or more divisions. *B*, LCMV-immune B6 mice containing memory P14 cells were injected with poly(I:C). Mice were sacrificed 2 days later, and lymphocytes were isolated from the indicated tissues and analyzed for DNA content by PI staining. Numbers indicate the percentage of P14 memory cells in cell cycle ($S+G_2/M$). One representative mouse of five is shown. *C*, The total number of memory P14 cells in cell cycle was calculated for the five mice in each group. Mean and SD are given in the table below.

strikingly as the site of greatest memory cell proliferation, with ~30% of P14 memory cells in cycle in the bone marrow just 2 days after treatment (Fig. 4*B*). In addition, the total number of memory cells in cycle was greater in the bone marrow than any other tissue analyzed (Fig. 4*C*). This further supports a key role for the bone marrow in promoting memory CD8 T cell proliferation.

Discussion

In conclusion, using three different measures of cell division, PI staining, BrdU, and CFSE, we found memory cells dividing in multiple tissues and identified the bone marrow as a preferred site for memory CD8 T cell homeostatic proliferation. Although it is difficult to ascertain the exact site of cell division, because it is only possible to examine one static time point, pulse-chase experiments of BrdU incorporation strongly suggest that memory cells migrate to the bone marrow, synthesize DNA as they divide, and then migrate back out into the periphery.

A recent report has demonstrated very efficient interactions between T cells and dendritic cells (DCs) in the bone marrow microenvironment (26). In that study, efficient T cell-DC interactions promoted rapid priming of a primary T cell response against a peptide Ag in the bone marrow. Similarly, in our study of IL-15-dependent homeostatic proliferation, the bone marrow stood out as a preferred site of T cell division. It may be that the same environment that promotes T cell priming also triggers homeostatic proliferation of established memory cells. Perhaps, as has been suggested for plasma cells, a unique combination of the cytokine milieu and contact-dependent interactions in the bone marrow supports memory CD8 T cell homeostasis (27).

It remains an important problem to identify the soluble and/or cellular interactions specific to the bone marrow that may regulate memory cell homeostasis. In this context, it is worth noting that IL-15 is "*trans*-presented" to memory CD8 T cells by non-T cells expressing the high affinity IL-15R, IL-15Rα (28). It is possible that the bone marrow is enriched for these cells capable of efficiently presenting IL-15 to memory CD8 T cells and providing the signals to undergo proliferation. This might explain the finding that low levels of memory cell division were observed in all tissues. DCs or other APCs capable of *trans*-presenting IL-15, although present in many tissues, are enriched in the bone marrow.

Finally, it is well established that the bone marrow is the major reservoir of long-lived plasma cells that are critical for maintaining long-term humoral immunity (29, 30). Our studies now show that a large number of memory CD8 T cells are also present at this site. It is interesting to note that several homing molecules known to be important for migration to the bone marrow, including LFA-1, integrin α_4, and CD44 are preferentially up-regulated on memory cells over naive cells (31, 32). It is intriguing that both long-lived plasma cells and memory T cells home preferentially to the bone marrow microenvironment. Taken together, these results highlight the importance of the bone marrow in immunological memory and protective immunity.

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