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Frontline

Memory CD8$^+$ T cells colocalize with IL-7$^+$ stromal cells in bone marrow and rest in terms of proliferation and transcription

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It is believed that memory CD8$^+$ T cells are maintained in secondary lymphoid tissues, peripheral tissues, and BM by homeostatic proliferation. Their survival has been shown to be dependent on IL-7, but it is unclear where they acquire it. Here we show that in murine BM, memory CD8$^+$ T cells individually colocalize with IL-7$^+$ reticular stromal cells. The T cells are resting in terms of global transcription and do not express markers of activation, for example, 4-1BB (CD137), IL-2, or IFN-γ, despite the expression of CD69 on about 30% of the cells. Ninety-five percent of the memory CD8$^+$ T cells in BM are in G_0 phase of cell cycle and do not express Ki-67. Less than 1% is in S/M/G_2 of cell cycle, according to propidium iodide staining. While previous publications have estimated the extent of proliferation of CD8$^+$ memory T cells on the basis of BrdU incorporation, we show here that BrdU itself induces proliferation of CD8$^+$ memory T cells. Taken together, the present results suggest that CD8$^+$ memory T cells are maintained as resting cells in the BM in dedicated niches with their survival conditional on IL-7 receptor signaling.

Keywords: Bone marrow · CD8 T cell · Gene expression · Interleukin-7 · Memory cells

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Introduction

Memory $CD4^+$ and memory plasma cells residing in the BM are maintained in survival niches organized by dedicated reticular stromal cells expressing IL-7 and CXCL12, respectively [1–5]. They rest in terms of proliferation and activation, with the exception that memory plasma cells secrete large quantities of antibodies. The survival of BM memory cells is conditional on signals from these niches. While the numbers of memory $CD4^+$ T cells in the periphery dwindle, memory $CD4^+$ T cells of the BM survive for long periods of time, months in mice [4] and decades in humans [6].

BM also hosts a prominent population of memory $CD8^+$ T cells [7–11]. In humans, 60% of these cells express CD69, reminiscent of recently activated T cells [9]. It has been claimed that memory $CD8^+$ T cells undergo extensive homeostatic proliferation in BM and spleen [8, 12]. This led to the conclusion that the population of memory $CD8^+$ T cells in the BM is maintained as activated cells, by cytokine-driven "homeostatic" proliferation, with dividing memory cells constantly replacing dying memory cells [13]. Maintenance of memory $CD8^+$ T cells is also dependent on IL-7 [14, 15], but so far it has not been clear, when and where the T cells acquire this IL-7. Here we show that more than 90% of the memory $CD8^+$ T cells of the BM are in direct contact or close to an IL-7^+ reticular stromal cell of the BM, a population of about 0.2–0.3% of all BM cells. More than 90% of the memory $CD8^+$ T cells are resting in G_0 phase of the cell cycle. This frequency may have been underestimated in previous studies because we observe a drastic induction of proliferation of BM $CD8^+$ T cells by BrdU itself, a base analogue used to identify DNA-synthesizing cells among $CD8^+$ T cells. This suggests that homeostatic proliferation, if relevant for the maintenance of $CD8^+$ memory T cells at all, has been overestimated in the previous studies.

Additionally, we show here that BM memory $CD8^+$ T cells are resting in terms of activation and transcription, despite expression of CD69 by up to 30% of them. Our results suggest that memory $CD8^+$ T cells are maintained in BM as resting cells, by IL-7 providing stromal cells, similar to memory $CD4^+$ T cells.

Results

BM is a major compartment for memory $CD8^+$ T-cell maintenance

Murine BM contains a prominent population of $CD44^+CD8^+$ T cells, as large as the population of $CD44^+CD8^+$ T cells in the spleen (Fig. 1A). In order to determine where memory $CD8^+$ T cells are maintained in the long run after a systemic immune response, we tracked Ag-specific $CD8^+$ T cells into the memory phase of a secondary immune response. WT C57BL/6 mice were immunized with cationized OVA and LPS i.p. and challenged 4 weeks later the same way. $CD8^+$ T cells recognizing the OVA-peptide SIINFEKL were detected by $H2K^b$/SIINFEKL-pentamers. On day 7 of the immune response, the spleen contained 6.2×10^4 Ag-specific $CD8^+$ T cells and the BM 1.1×10^4 (Fig. 1B). On day 34, the number of Ag-specific $CD8^+$ T cells dropped to 1×10^4 in spleen and 6.6×10^3 in BM. In BM, the numbers of specific $CD8^+$ T cells remained relatively constant thereafter, whereas in spleen the numbers continued to decline slowly to 9×10^2 on day 188. In a second experiment, numbers of specific $CD8^+$ T cells in spleen and BM remained constant as well, at about 3×10^3, on day 181 (Supporting Information Fig. 1A). In two other independent experiments, the Ag-specific cells were detected in the BM after a year, demonstrating long-term survival of memory $CD8^+$ T cells in the BM, presumably for the lifetime of a mouse (Supporting Information Fig. 1B). A similar distribution was observed for $GP_{33\text{-}41}$- and $NP_{396\text{-}404}$-specific memory $CD8^+$ T cells in an immune response to Armstrong strain of lymphocytic choriomeningitis virus (LCMV, i.p.). On day 60 after infection, BM contained 2.5×10^5 GP_{33}-specific and 2.1×10^5 NP_{396}-specific $CD8^+$ T cells, 1.2×10^5 GP_{33}-specific and 1.5×10^5 NP_{396}-specific cells were detectable in the spleen (Fig. 1C). By day 120 after infection, the number of GP_{33}-specific cells in the spleen dropped to 7.5×10^4, compared to 1.7×10^5 GP_{33}-specific cells in the BM, resulting in a significant difference. Thus, in the immune responses to the systemic Ags, OVA and LCMV, as many, if not more Ag-specific $CD44^+CD8^+$ T cells are maintained in the BM as in the spleen for periods of up to 427 days, the final time point in these experiments.

Ag-specific memory $CD8^+$ T cells of the BM and spleen did not produce the cytokines IL-2 or IFN-γ, unless restimulated with their specific peptide (Fig. 1D and E). Upon in vitro stimulation with $GP_{33\text{-}41}$, $NP_{396\text{-}404}$, and $GP_{276\text{-}286}$ peptides the frequencies of IFN-γ-producing $CD8^+$ T cells increased from <0.1% to 5–10% (Fig. 1D and E). Twenty to thirty percent of the IFN-γ-producing $CD8^+$ T cells also expressed IL-2 (Fig. 1D and E). When restimulated in vitro, IFN-γ^+CD8^+ memory T cells from spleen and BM also expressed CD107a, a marker of degranulation (Fig. 1F). Thus, memory $CD8^+$ T cells generated by defined systemic murine immune responses are maintained in BM as well as in spleen, and they display cytotoxic functions when stimulated with their cognate Ag.

Memory $CD8^+$ T cells are residing on IL-7-producing stromal niches in BM

Memory $CD4^+$ T cells and memory plasma cells are maintained in distinct stromal niches in BM [5, 16]. Memory plasma cells survive in a niche composed of CXCL12-producing stromal cells and eosinophils [1, 17, 18], while memory $CD4^+$ T cells contact IL-7-producing stromal cells [4]. IL-7 is a mandatory survival factor for both memory $CD8^+$ and $CD4^+$ T cells [14, 19]. Therefore, we checked whether memory $CD8^+$ T cells of the BM are also maintained in IL-7^+ stromal niches. In heterozygous IL-7 reporter mice, with a gfp gene introduced into one of their *Il7* genes [20], we analyzed the colocalization of $CD8^+$ memory T cells with stromal cells. In these mice, GFP-expressing cells of the BM are VCAM-1^+, but not $CD31^+$ or $CD45^+$, identifying them as reticular stromal

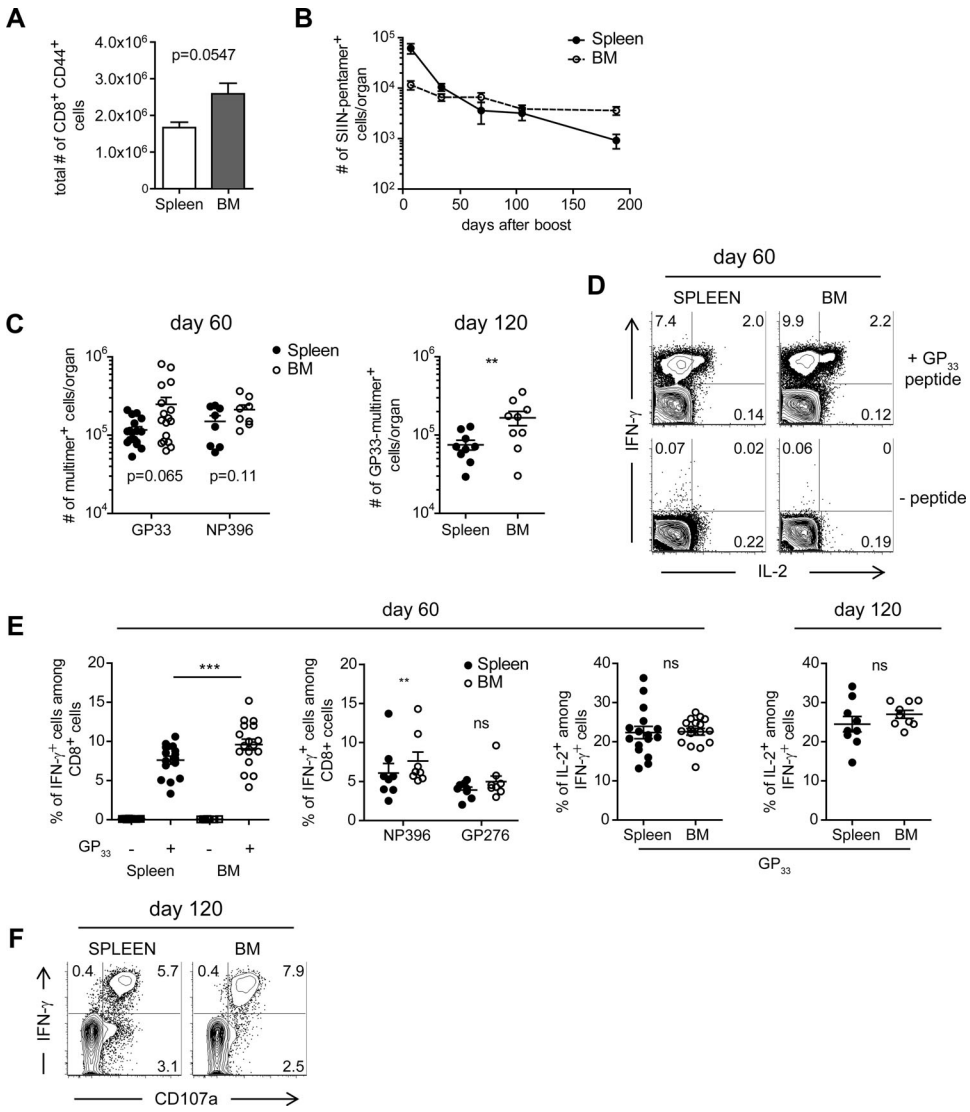

Figure 1. BM is a major compartment for memory CD8$^+$ T-cell maintenance. (A) The total number of CD8$^+$CD44$^+$ T cells in spleen and BM of aged mice were evaluated by flow cytometry analysis. Bars show mean ± SEM from eight mice and are representative of two independent experiments. (B) C57BL/6 mice were immunized subcutaneously twice at 4 week intervals with cationized OVA (catOVA) and LPS. SIINFEKEL-reactive CD8$^+$ T cells were tracked by H2K^b-SIINFEKL pentamer staining in spleen and BM in acute and late phases of the secondary response. CD8$^+$CD44$^+$SIINFEKL-pentamer$^+$ T cells in spleen and BM were analyzed by flow cytometry. Data are shown as mean ± SEM (n = 3–5 mice/time point) and are representative of two independent experiments. (C–F) C57BL/6 mice were infected i.p. with LCMV-Armstrong. Sixty days after infection, GP_{33}- or NP_{396}-reactive CD8$^+$ T cells were tracked by H2D^b-GP_{33} (KAVYNFATM) or H2D^b- NP_{396} (FQPQNGQFI) multimer stainings in spleen and BM. (C) The number of multimer$^+$ cells within the organ 60 and 120 days after infection was detected by flow cytometry. (D) The cells from spleen and BM were either stimulated with LCMV-driven GP_{33-41} peptide or left unstimulated. Representative dot plots show the cells after gating on CD8$^+$CD90$^+$ cells from three independent experiments (see Supporting Information Fig. 6 for gating strategy). (E) The frequencies of IFN-γ$^+$ cells among total CD8$^+$CD3$^+$ or CD8$^+$CD90$^+$ cells and IL-2$^+$ among IFN-γ$^+$CD8$^+$ cells were accessed by flow cytometry upon in vitro stimulation with the indicated peptides. (F) One hundred twenty days after infection, spleen and BM cells were stimulated in vitro with GP_{33-41} peptide and the frequencies of IFN-γ and CD107a in CD8$^+$CD90$^+$ cells were analyzed. Representative dot plots show the cells after gating on CD8$^+$CD90$^+$ cells. (C and E) Each symbol represents an individual mouse. (C, E, and F) Data are shown as mean ± SEM (n = 4–8 mice/time point) and are pooled from two to three independent experiments. (A–F) Statistical analysis was performed with Wilcoxon matched pairs test; ns, nonsignificant; $^{**}p < 0.01$; $^{***}p < 0.001$.

cells (Fig. 2A) and about 50 % of the reticular stromal cells express GFP (Fig. 2B). Of the 268 CD8$^+$CD44$^+$ T cells analyzed, 70.8% directly contacted a GFP$^+$ stromal cell, 23.4% were located within 10 μm range of a GFP$^+$ stromal cell (Fig. 2C and D). A total of 5.8 % were located out of this range. This result probably gives an underestimation of the overall colocalization of T cells and stromal cells, since contacts out of the focal plane of the microscope (above or below the cell) could not be identified. Thus, most if not all memory CD8$^+$ T cells of BM contact an IL-7-expressing stromal cell. Memory CD4 helper T cells also contact IL-7-producing stromal cells [4]. This raises the question whether the very same IL-7-producing stromal cell can sustain CD4$^+$ and a CD8$^+$ memory T-cell survival. To address this, we determined whether CD4$^+$ and CD8$^+$ memory T cells are scattered throughout the BM evenly.

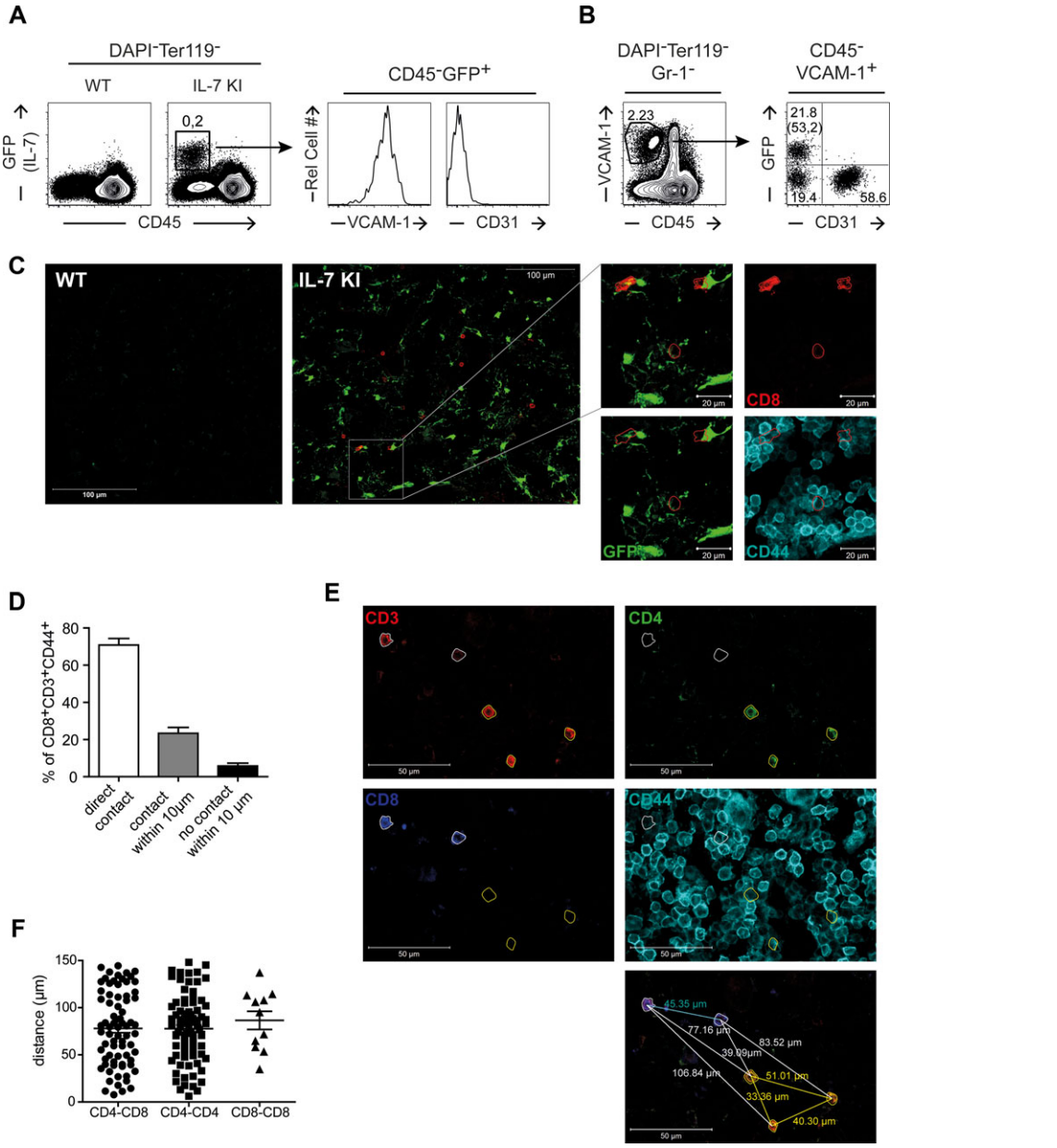

Figure 2. Memory $CD8^+$ T cells colocalize with IL-7-producing stromal cells. (A and B) BM from WT and heterozygous IL-7 knock-in mice ($n = 10$) was digested and cells were analyzed by flow cytometry. Plots depict the expression of (A) GFP with respect to CD45 or (B) GFP and CD31 among $CD45^-VCAM^+$ stromal cells after gating doublets, erythrocytes ($Ter119^+$), and dead cells out. (A) Histograms show the expression of VCAM-1 and CD31 on $CD45^-GFP^+$ cells (see Supporting Information Fig. 7A for the gating strategy). (B) Representative dot plots show the expression of GFP and CD31 among $CD45^-VCAM^+$ stromal cells after gating erythrocytes ($Ter119^+$), $Gr\text{-}1^+$, and dead cells out (see Supporting Information Fig. 7B for the gating strategy). (B) The numbers in the gates of the dot plot on the right indicate the frequencies among $CD45^-VCAM^+$ cells and the number in the parenthesis refers to the frequency of GFP^+ cells among $CD31^-VCAM^+CD45^-$ cells. (A and B) Plots are representative of four independent experiments. (C and D) BM sections from naive or OVA + LPS immunized IL-7 knock-in mice were stained with anti-CD8, anti-CD44, anti-CD3, anti-GFP Abs. (C) Images show GFP/IL-7 (green), CD8α (red), and CD44 (turquoise) expression. As a control for anti-GFP staining, WT (GFP^-) mouse BM sections were stained with anti-GFP Ab. One representative of 16 image acquisitions is shown. Scale bars: 100 µm (left) and 20 µm (right); 20× magnification. (D) $CD3^+CD8^+CD44^+$ cell interactions with GFP^+ ($IL7^+$) cells were quantified manually by counting. The frequencies of $CD3^+CD8^+CD44^+$ cells that are contacting directly are shown, which are located within or further than 10 µm distance to the GFP^+ stromal cells. Data are shown as mean ± SEM (50-180 $CD3^+CD8^+CD44^+$ cells were counted from each mouse) and are pooled results from one naive, two OT-I transferred, and one nontransferred mice immunized with OVA + LPS. (E) Mice expressing GFP ubiquitously were irradiated and T cell depleted BM cells were transferred one day later. Four weeks after reconstitution, BM of these mice were analyzed for $CD8^+$ and $CD4^+$ T-cell interactions with GFP^+ nonhematopoietic cells. BM sections were stained for $CD4^+$ (green), CD8α (blue), CD3 (red), and CD44 (turquoise). $CD44^+CD8^+CD3^+$ cells were circled with white and $CD44^+CD4^+CD3^+$ cells were circled with yellow lines. Numbers indicate the distance between two T cells. Scale bars = 50 µm; 20× magnification. (F) Graph illustrates the distances between a $CD4^+$ and $CD8^+$, two $CD4^+$ and two $CD8^+$ memory T cells. Each symbol represents the distance between two indicated cells (74 CD4-CD8 pairs, 82 CD4-CD4 pairs, 11 CD8-CD8 pairs). Bars depict mean distance ± SEM and data are pooled from four image acquisitions.

For this purpose, we measured the distances between $CD4^+CD44^+$ and $CD8^+CD44^+$, two $CD4^+CD44^+$ and two $CD8^+CD44^+$ T cells in the BM. Only the nearest neighbors of the T cells were taken into account, and cells that were distant to each other more than 150 μm were excluded from the evaluation (Fig. 2E). The average distance between a $CD4^+$ and a $CD8^+$ memory T cell was 78 ± 4.5 μm (SEM), as it was for two $CD4^+$ (77.7 ± 3.9 μm [SEM]) or two $CD8^+$ (86.5 ± 9.6 μm [SEM]) memory T cells (Fig. 2F). In more than 87% of the cases, distances between a $CD4^+$ and a $CD8^+$ memory T cell, as well as two $CD4^+$ and two $CD8^+$ memory cells were larger than 30 μm. These results suggest that the majority of the T cells are not interacting with the very same stromal cell. Thus, $CD4^+$ and $CD8^+$ memory T cells share niches organized by IL-7-expressing stromal cells, but presumably only one memory T cell, either $CD4^+$ or $CD8^+$, inhabits one niche.

A subset of memory $CD8^+$ T cells in BM express CD69

CD69 is expressed on resting memory $CD4^+$ T cells in BM [6, 21] and tissue-resident $CD8^+$ T cells in skin and various other peripheral tissues [22–27]. As we show here, a subset of murine memory $CD8^+$ T cells residing in BM also expresses CD69. Ten to forty percent of both polyclonal SIINFEKL- and GP_{33-41}-specific memory $CD8^+$ T cells and $CD8^+$ T cells from elderly mice expressed CD69 (Fig. 3A–C). In contrast, less than 5% of memory spleen cells and 1% of memory cells in blood were $CD69^+$ (Fig. 3A and B). $CD69^+$ SIINFEKL-specific memory $CD8^+$ T cells of BM were retained in equal numbers over time, between days 30 (684 ± 233 [SEM]) and around 180 (643 ± 80 [SEM]) after immunization, while the numbers of $CD69^-$ cells dropped from 4908 ± 1138 on day 30 to 2716 ± 404 on around day 180 (Fig. 3D). In the LCMV response, the numbers of $CD69^+$ cells also remained stable between days 60 and 120 after immunization (Fig. 3E).

$CD69^+CD8^+$, like $CD69^-CD8^+$ memory T cells expressed CD122 (IL-2Rβ chain) and CD127 (IL-7Rα chain) (Fig. 3F and G, Supporting Information Fig. 2), allowing them to receive signals from IL-7 and IL-15. Sixty days after infection, approximately 80% of the $CD69^+$ cells did not display surface CD62L corresponding to the phenotype of effector memory cells and 15.2% of $CD69^+$ and 42.4% of $CD69^-CD8^+$ memory T cells from BM expressed killer cell lectin-like receptor subfamily G member 1 (KLRG1), a marker of short-lived effector cells (Fig. 3F and G). This implies that the majority of the $CD69^+$ and $CD69^-CD8^+$ T cells of BM are not senescent cells but rather qualify as cells preserving long-term memory [28, 29].

Neither $CD69^+$ nor $CD69^-CD8^+$ T memory T cells from BM expressed the gene encoding for sphingosine-1-phosphate receptor 1 (S1PR1), which is required for cells to egress from tissues [30], as opposed to $CD69^-CD8^+$ cells from the spleen (Fig. 3H). This result suggests that $CD8^+$ memory T cells from BM, in particular those expressing $CD69^+$, an antagonist of S1PR1 expression [31], are resident, like tissue-resident $CD8^+$ memory T cells [24, 27, 32, 33].

Memory $CD8^+$ T cells are resting in G_0 of cell cycle

$CD8^+$ T cells of BM have been reported to be in the state of heightened activation [9, 34, 35] and pronounced proliferation of BM $CD8^+$ T cells has been observed, as compared to those found in other organs [8, 12]. We also analyzed the proliferation status of Ag-specific memory $CD8^+$ T cells from spleen and BM in murine immune responses against LCMV and OVA by staining for expression of Ki-67. Ki-67 is an Ag that is exclusively expressed by cells in the G_1, S, and G_2/M phases of cell cycle, and is not present in cells resting in the G_0 phase [36]. Ninety-four percent of the LCMV-NP_{396}-specific cells in BM were Ki-67 negative, and therefore in G_0 on day 60 of the immune response, as compared to 88% in spleen. Ninety-three percent of GP_{33}-specific $CD8^+$ T cells in the BM and 91% in the spleen did not express Ki-67. Similarly, 95% of the SIINFEKL-specific T cells of the BM were in G_0 around day 180 of the immune response against OVA, as compared to 88% in the spleen (Fig. 4A and B). Less than 0.5% of the memory $CD8^+$ T cells of BM and spleen were positive for propidium iodide, which is used to detect cells in S and G_2/M phases of cell cycle and on average 0.25% of $CD69^+CD8^+$ memory T cells of BM were PI^+ (Supporting Information Fig. 3).

Finally, we analyzed BM memory $CD8^+$ T cells for their ability to incorporate BrdU into their DNA, that is, to perform DNA synthesis. Mice were fed for 3 days with BrdU containing drinking water. When BM and spleen $CD8^+$ memory T cells were analyzed for BrdU incorporation and counterstained for coexpression of Ki-67, the frequencies of $Ki\text{-}67^+CD8^+CD44^+$ cells in BM massively increased from 5.7 to 6% in untreated mice to 73–78% in BrdU-treated mice. There was a similar, albeit less pronounced, increase in the spleen of those mice with 7.1–7.6% $Ki\text{-}67^+$ cells in untreated versus 27–32% in BrdU-treated individuals (Fig. 4C and D). Ki-67 expression correlated with BrdU incorporation indicating that the increase in Ki-67 expression was due to the active proliferation of the cells (Fig. 4C). Furthermore, the frequencies of cells in S and G_2/M phases increased significantly, as shown by PI staining, that is, from 0.4 to 5.4% in $CD8^+$ memory T cells from BM (Fig. 4E). Thus, BrdU is activating resting memory $CD8^+$ T cells both in spleen and BM to leave the G_0 phase of cell cycle and start to synthesize DNA.

Memory $CD8^+$ T cells of BM rest in terms of gene expression

Tracking of Ag-specific $CD8^+$ T cells into the memory phase of an immune response revealed that those cells express CD127, the IL-7 receptor α chain, in the memory phase (Supporting Information Fig. 4). To compare the transcriptomes of resting memory $CD8^+$ T cells from spleen and BM, we isolated $CD44^+CD127^+CD8^+$ T cells from spleen and BM (Supporting Information Fig. 5), using an approach that we had demonstrated before to conserve the in vivo gene expression [37]. Such memory cells contained very low amounts of total RNA, 0.6 pg/cell on average, which was comparable to naive $CD44^{low}CD127^+CD8^+$ T cells. When

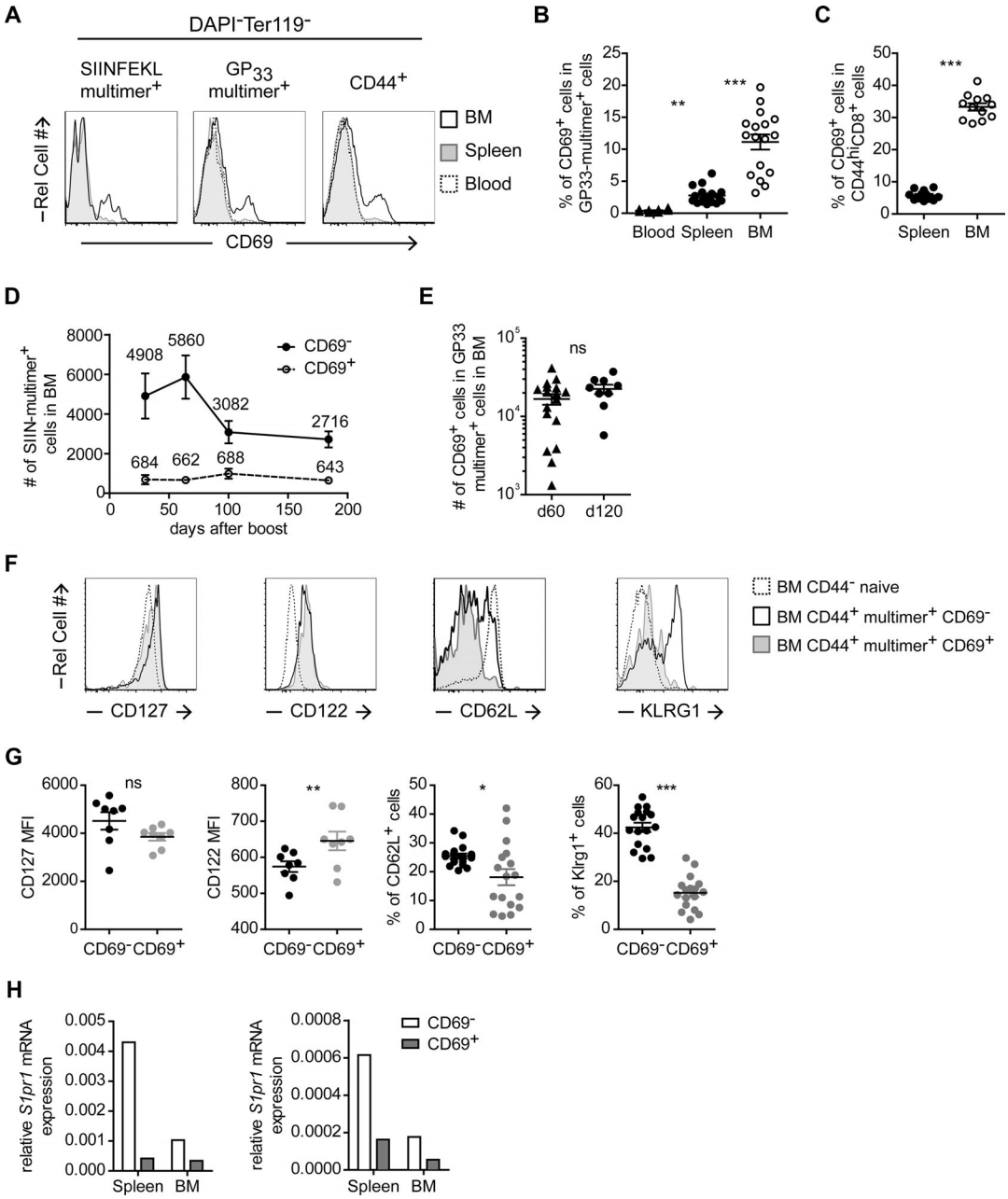

Figure 3. A subset of CD8$^+$ memory T cells in BM expresses CD69. Mice were immunized or infected as described in Fig. 1. (A) The expression of CD69 was evaluated among SIINFEKL-multimer$^+$ cells 181–189 days after catOVA + LPS rechallenge or among GP_{33}-multimer$^+$ and among total CD8$^+$CD44$^+$ cells 60 days after LCMV infection by flow cytometry. Representative histograms of CD69 expression in spleen (gray, filled), BM (black, solid line), and blood (black, dashed line) cells (see Supporting Information Fig. 6 for the gating strategy). (B and C) Graphs depict the frequency of CD69$^+$ cells among GP_{33}-multimer$^+$ cells in (B) LCMV infected (day 60) and (C) among total CD44$^+$CD8$^+$ cells in uninfected aged C57BL/6 mice. (D) Numbers of CD69$^+$ and CD69$^-$ SIINFEKL-multimer$^+$ cells in BM were determined at various time points after catOVA + LPS rechallenge by flow cytometry. (E) The number of CD69$^+$ GP_{33}-multimer$^+$ cells in BM of individual mice 60 and 120 days after LCMV infection were determined by flow cytometry. (F) The expression levels of CD127, CD122, CD62L, and KLRG1 were evaluated in CD69$^+$ and CD69$^-$ GP_{33}-specific memory CD8$^+$ T cells 60 days after LCMV infection. Histograms depict the expression of the indicated molecules on CD69$^+$ (gray, filled), CD69$^-$ (black, solid line), GP_{33}-multimer$^+$CD8$^+$CD44$^+$, and CD8$^+$CD44$^-$ naive (black, dashed line) cells. Histograms are from one representative experiment (n = 4–8) of two (for CD122 and CD127) or three (CD62L and Klrg1) independent experiments performed. (G) Graphs show the MFI of CD127 and CD122 or the frequencies of the CD62L$^+$ and KLRG1$^+$ cells on CD69$^+$ or CD69$^-$ GP_{33}-multimer$^+$CD8$^+$ cells as determined by flow cytometry. For CD127 and CD122 one representative experiment of two is shown (see Supporting Information Fig. 2 for the second experiment). For CD62L and KLRG1, three experiments were pooled. (H) CD69$^+$ and CD69$^-$ CD44$^+$CD8$^+$ T cells were sorted from spleen and BM of aged C57BL/6 mice for the quantification of *S1pr1* expression. Graphs show the expression of *S1pr1* relative to *U6*. Data shown are from two independent experiments. (B, C, E, and G) Each symbol represents an individual mouse. (B–E and G) Data are shown as mean ± SEM of (B, C, E, and G) n = 4–8 or (D) n = 3–5 and (A–E) are pooled from two to three independent experiments. Statistical analysis was performed with Wilcoxon matched pairs test (A–C and G) and Mann–Whitney test (E); ns, nonsignificant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

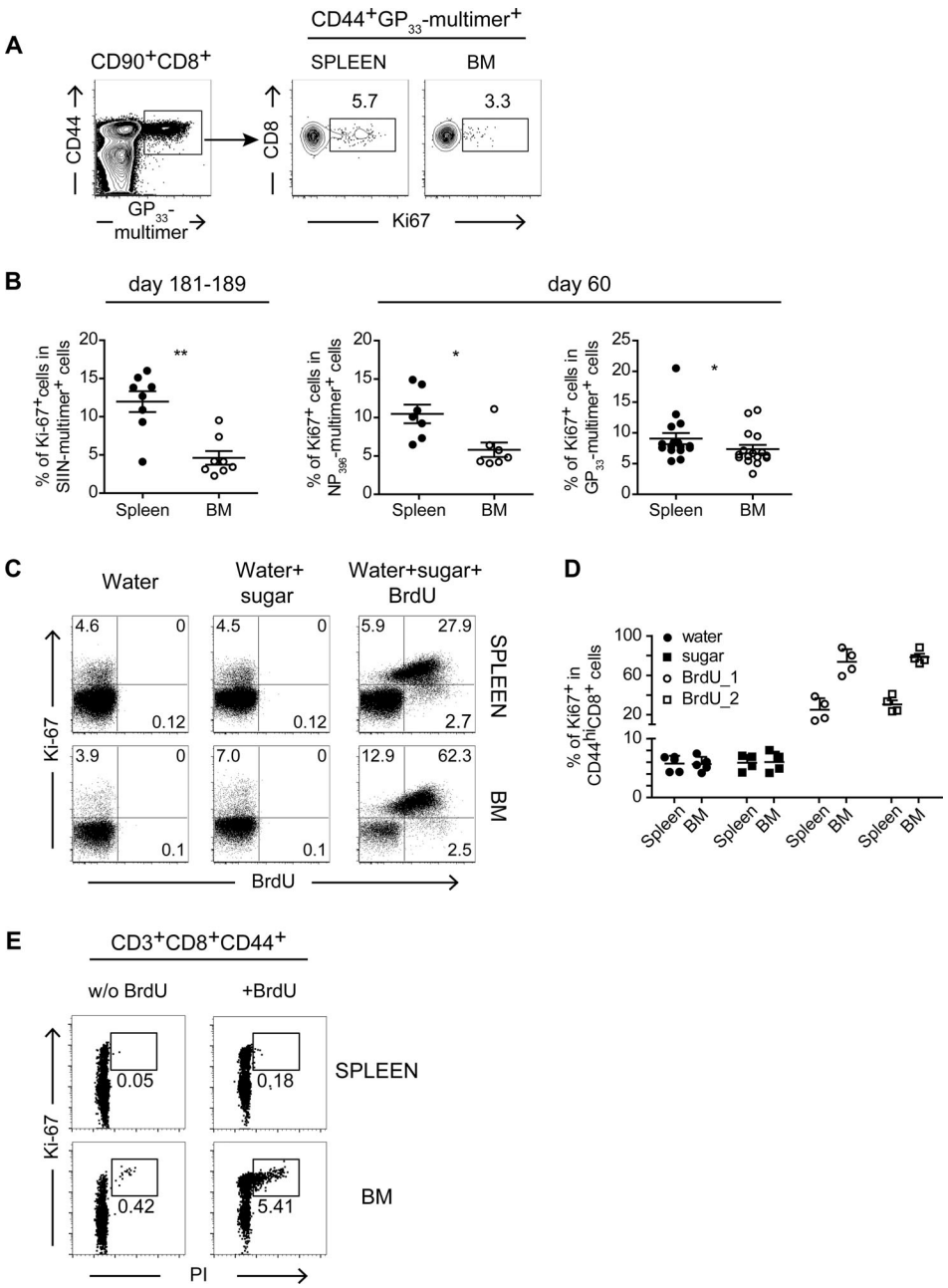

Figure 4. Memory CD8$^+$ T cells from BM are resting in terms of proliferation. (A and B) Mice were immunized with catOVA + LPS or infected with LCMV as described in Fig. 1. The frequency of Ki-67$^+$ cells was evaluated within the multimer$^+$ cells. (A) Representative dot plots of Ki-67 expression after gating on CD44$^+$ GP_{33}-specific CD8$^+$ T cells. The values indicate the frequency of Ki-67$^+$ cells in GP_{33}-multimer$^+$ cells. (B) Graphs show the frequencies of Ki-67$^+$ cells in SIINFEKL-specific (two experiments, $n = 4$), GP_{33}-specific (three experiments, $n = 4$–8), and NP_{396}-specific (one experiment, $n = 8$) T cells. (C–E) Exbreeder mice were treated orally for 3 days with water containing sugar and 1 mg/mL BrdU; sugar alone or water only. (C) Representative dot plots show Ki-67 versus BrdU staining among CD44$^+$ CD3$^+$CD8$^+$ T cells (see Supporting Information Fig. 8 for gating strategy). (D) The frequencies of Ki-67$^+$ cells among CD44$^+$ CD8$^+$ T cells are shown. BrdU_1 and BrdU_2 symbolize two different lots of BrdU. This experiment was performed twice with BrdU_1 ($n = 4$–5) and once with BrdU_2 ($n = 4$). (E) Cell cycle analysis was performed on the spleen and BM cells of BrdU-treated or BrdU-untreated mice ($n = 4$). Representative dot plots show Ki-67 and PI staining after gating on CD3$^+$CD8$^+$CD44$^+$ T cells. (A and C) Plots are representative of (A) three, (C) two, and (E) one independent experiments. (B and D) Each symbol indicates an individual sample. Bars indicate mean ± SEM. Statistical analysis was performed with Wilcoxon matched pairs test; ns, nonsignificant; $^*p < 0.05$; $^{**}p < 0.01$.

activated with anti-CD3/anti-CD28 for 42–44 h, the amount of RNA increased to an average of 15 pg/cell in spleen and 20 pg/cell in BM memory CD8$^+$ T cells, indicating that before activation, the cells had been resting in terms of transcription (Fig. 5A). A global overview on genes transcribed in memory T cells from BM and spleen, as obtained by analysis with Affymetrix MG_U430_2 GeneChips, shows that 11 806 genes were expressed differentially (fold change >2/pg RNA) between naive and memory CD8$^+$ T cells from BM and spleen isolated ex vivo, on the one hand, and those cells reactivated with anti-CD3 and anti-CD28 (Fig. 5B).

We next compared the expression of individual genes expressed by resting versus activated CD8$^+$ memory. Genes reflecting recent activation events, like the genes encoding 4-1BB (*Tnfrsf9*), CD25 (*Il2ra*), cytotoxic T lymphocyte associated protein 4 (*Ctla4*), were barely transcribed in naive and ex vivo isolated memory CD8$^+$ T cells from BM and spleen, whereas they were upregulated 10- to 130-fold per picogram RNA, following in vitro activation (Fig. 5C). Genes encoding cytokines and cytolytic effector molecules, such as IFN-γ (*Ifng*), lymphotoxin α (*Lta*), perforin (*Prf*), and granzyme B (*Gzmb*) were expressed at signal strengths of 600 and below, per picogram RNA, while they were expressed at signal strengths of 2000–10 000 per picogram RNA in activated cells (Fig. 5D). The same result was observed for genes involved in cell cycle progression, namely, the genes encoding the proteins cyclin E1, E2, A2, and B1, which are expressed in S, G_2, and M phases of cell cycle, respectively [38, 39]. These genes were expressed at

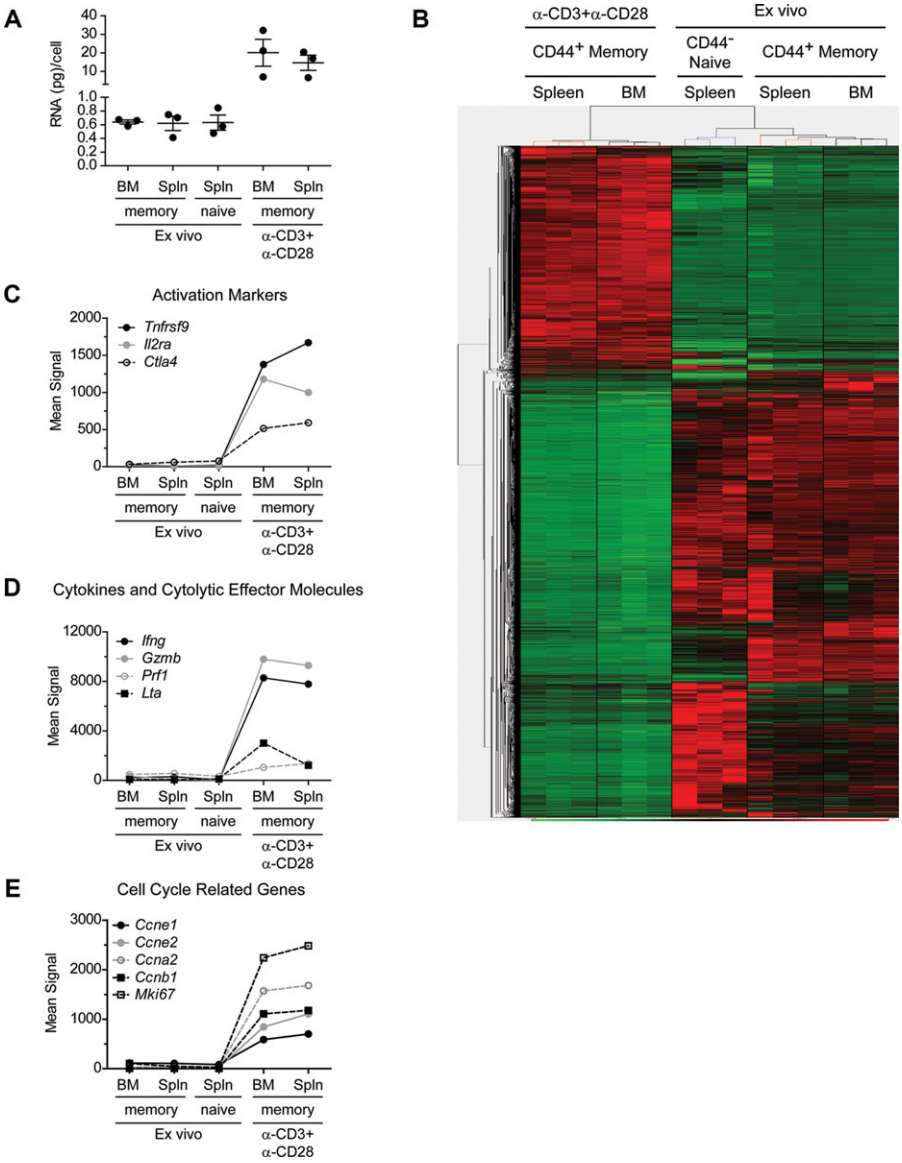

Figure 5. Memory CD8$^+$ T cells from BM are resting in terms of transcription. CD44$^+$CD127$^+$ or CD44$^-$CD127$^+$ CD8$^+$ T cells were sorted from spleen and BM of aged C57BL/6 mice. RNA from memory cells was either prepared immediately or following 2 days of anti-CD3/anti-CD28 stimulation. RNA was then used for microarray analysis. (A) Graph shows the RNA amount/cell. Each symbol represents one experiment. Bars indicate mean ± SEM ($n = 6$–12 mice/experiment) and data pooled from three independent experiments. (B) Hierarchical cluster analysis of all 11 806 differentially expressed genes (fold change >2) of naive, resting memory and in vitro activated memory CD8$^+$ T cells from BM and spleen is shown. The filter parameters for significance of differential expression combined a threshold for gene expression height: a present or marginal detection call, more than 50% decrease or increase change calls, or a Bonferroni corrected *t*-test *p* value for all ten comparisons of three versus three chips. Shown here are the combined signals of all ten gene lists for all 15 chips for genes that are found significant in at least one of ten comparisons. Upregulated genes are shown in red and downregulated genes are in green. (C–E) Graphs show the mean expression level of genes encoding for the indicated activation markers (C), cytokines and cytolytic effector molecules (D), and cyclins (E) as determined by microarray analysis. (C–E) Data are representative of three independent experiments performed.

signal strengths of about 1000 in activated cells and less than 100 in resting memory T cells from BM or spleen, per picogram RNA (Fig. 5E). Likewise, expression of *Mki67*, the gene encoding the protein Ki-67, was induced upon activation of the T cells about 20- to 40-fold per picogram RNA. Thus, in terms of RNA and gene expression, CD8$^+$ memory T cells from spleen and BM are similar and resting.

Discussion

Here we show that CD8$^+$ memory T lymphocytes generated in systemic immune reactions are maintained in the spleen and BM of mice, for long periods of time, most likely extending to the lifetime of the mice. Such cells are resting in terms of proliferation and gene expression, and the ones in BM reside in niches organized by IL-7-expressing mesenchymal stromal cells. Analysis of phenotypic markers revealed that memory CD8$^+$ T cells of BM resemble tissue-resident memory cells, since 30% of them express CD69 and lack expression of S1PR1, KLRG1, and CD62L [24, 27, 32, 33].

It has been previously shown that the BM is a preferred residency of memory plasma cells [2, 40] and CD4$^+$ memory T cells [16] that are generated in systemic immune responses, and are maintained in the apparent absence of Ag [41, 42]. Plasmablasts generated in secondary immune reactions migrate from secondary lymphoid organs into BM within a week [43–45] and dock on to reticular stromal cells expressing the chemokine CXCL12 [1]. About 10–20% of the plasmablasts generated in the immune reaction develop into memory plasma cells and survive in their niches presumably for a lifetime [3]. CD4$^+$ memory T-cell precursors translocate from secondary lymphoid organs into the BM in the contraction phase of an immune reaction. Likewise, 10–20% of the cells generated in that immune reaction develop into memory CD4$^+$ T cells in the BM, in niches organized by IL-7-expressing reticular stromal cells [4]. Here we confirm that the BM is also a major compartment for the maintenance of memory CD8$^+$ T cells,

as published previously [7, 8]. We show that in the BM most if not all of the $CD8^+$ memory T cells contact IL-7-expressing stromal cells as previously demonstrated for adoptively transferred, in vitro differentiated central memory type $CD8^+$ T cells in IL-7 reporter mice [46]. IL-7 has been shown to be essential for the survival of $CD8^+$ T cells [14, 15]. IL-7-producing stromal or epithelial cells have been found in the thymus, peripheral LNs, spleen, gut, and BM, but their relevance for the maintenance of CD8 memory is not clear [20, 46–49]. The colocalization of resting memory $CD8^+$ T cells and IL-7-expressing stromal cells in the BM strongly suggests that the BM is a privileged site for the maintenance of $CD8^+$ T-cell memory. As memory $CD4^+$ T cells are also located close to IL-7-producing stromal cells [4], it remains to be shown whether memory $CD4^+$ and $CD8^+$ T cells compete for the same survival niches or whether they occupy distinct niches specialized for memory $CD4^+$ and $CD8^+$ T-cell maintenance.

Like $CD4^+$ memory T cells, $CD8^+$ memory T cells of the BM are resting in terms of gene expression. First of all, the amount of RNA per cell is equal in $CD8^+$ memory T cells from spleen and BM, about 0.6 pg per cell and comparable to naive $CD8^+$ T cells. Upon reactivation, the amount of RNA per cell increases to 15–20 pg. In relation to RNA content per cell, differences in gene expression are thus even more pronounced, when calculated on a per cell basis, than when calculated on a per picogram basis. With respect to expression of distinct genes indicative of "activation," $CD8^+$ memory T cells from spleen and BM do not express *Ctla4*, *Tnfrsf9* (gene encoding for 4-1BB), *Il2ra*, nor do they express cytokine genes, although they can readily be induced to do so by cognate reactivation.

As we show here, about 30% of murine $CD8^+$ memory T cells in BM are $CD69^+$ and their numbers remain constant for months after priming. In humans, about 60% of the BM $CD8^+$ memory T cells express CD69 [6], which has been interpreted as a hallmark of "activation" [9]. It has been reported before that also resting $CD4^+$ human memory T cells from BM express CD69 [6, 9], and that this is required for murine $CD4^+$ memory T cell entry into the BM [21]. CD69 also has been identified as a retention marker, in conjunction with S1PR1, which allows cells to enter blood [30]. Taken together, CD69 expression by memory T cells of BM is most likely not an indicator of activation but suggests that these cells are resident within BM as demonstrated previously for other organs [24, 27, 32, 33].

$CD8^+$ memory T cells of the BM are also resting in terms of proliferation. Here we used Ki-67 to discriminate between cells in the G_0 phase of cell cycle, which do not express Ki-67, and cells in the G_1 to M phase of cell cycle, which do express Ki-67 [36]. About 95% of the $CD8^+$ memory T cells of BM, and 90% in the spleen, were in G_0,that is, they were not proliferating. This is similar to the situation in human BM, where more than 95% of the $CD4^+$ and $CD8^+$ memory T cells were Ki-67^- [6]. Of the few cells that were not in G_0, about 0.5–5% were in the S/G_2/M phase of cell cycle, that is, were actively proliferating at the time of analysis. This corresponds to less than 0.05–0.5% of all $CD8^+$ memory T cells. This finding contrasts sharply with previous analyses, in which the frequencies of proliferating $CD8^+$ memory T cells in the BM were calculated according to BrdU incorporation into cellular DNA, in cells undergoing active DNA synthesis [8, 12, 13]. These studies had suggested that over a time period of 40 days about 80% of the memory $CD8^+$ T cells proliferate in the BM [13]. As we show here, these previous results can be explained by the ability of BrdU to induce resting $CD8^+$ memory T cells from BM and spleen to proliferate. Oral administration of BrdU induced 40% of $CD8^+$ T memory cells in spleen and 75% in the BM to leave the G_0 phase of cell cycle, express Ki-67 and incorporate BrdU. The frequencies of $CD8^+$ memory T cells in the S/G_2/M phases of cell cycle, according to staining with propidium iodide, increased from 0.05 to 0.18% in the spleen and from 0.42 to 5.41% in the BM. This massive induction of proliferation of resting $CD8^+$ memory T cells, in particular in the BM, was caused by BrdU itself, since it was not observed upon feeding of the mice with sugar water alone. A similar effect of BrdU on hematopoietic stem cell proliferation has been documented before [50]. The present results demonstrate that the extent of (homeostatic) proliferation of $CD8^+$ memory T cells in the BM has been vastly overestimated. This raises the question whether memory $CD8^+$ T cells are maintained at all by a balance between cytokine-driven homeostatic proliferation and cell death, or whether they are maintained as resting cells in dedicated niches, surviving conditionally on IL-7 receptor signaling, as we show here for the majority of $CD8^+$ memory T cells from BM. It should be noted that in BM, most $CD8^+$ memory T cells do not contact cells producing IL-15 [9, 51], which has been shown to induce "homeostatic" proliferation of $CD8^+$ memory T cells [15, 52–54].

Our findings indicate that BM is a major site for the maintenance of memory $CD8^+$ T cells, which rest in terms of proliferation and transcription in niches involving IL-7-producing stromal cells. It remains a challenge to understand the relationship between tissue-resident, BM-resident, and circulating $CD8^+$ memory T cells and their relative contributions to reactive immunological memory against the diversity of pathogenic challenges.

Materials and methods

Mice

C57BL/6J mice (B6) and OT-I mice were purchased from Charles River (Sulzfeld, Germany) and Jackson Laboratories, respectively. IL-7 knock-in mice were kindly provided by Koichi Ikuta. Mice expressing GFP ubiquitously (Ubq:GFP) were bred in DRFZ. All mice were housed under specific pathogen-free conditions. All animal experiments were performed according to institutional guidelines and Japanese and German Federal laws on animal protection.

Immunization, LCMV infection, and adoptive T-cell transfer

Mice aged 8–12 weeks were immunized twice at 4 week intervals with 100 μg cationized OVA and 10 μg LPS (*Salmonella minnesota*,

Invivogen). In order to generate LCMV-specific memory T cells, mice were infected i.p. with 2×10^5 PFU of the Armstrong strain of LCMV. Untreated mice or mice immunized with 100 μg KLH and 10 μg LPS served as controls for the quantification of Ag-specific cells. For the adoptive transfer of T cells, CD90.1$^+$ CD8$^+$ cells from OT-I mice were enriched by streptavidin-coupled MACS microbeads after the cells were labeled with biotin-coupled anti-CD8 Fab fragment. A total of 1×10^6 CD8$^+$ T cells were then transferred into each IL-7 GFP knock-in mouse. One day later, mice were immunized i.p. with 100 μg OVA and 10 μg LPS. Four months later, the femurs and spleen from the mice were fixed with 4% PFA.

Flow cytometry, intracellular cytokine staining, cell digestion

Single-cell suspensions were prepared from spleen, BM, and where indicated, blood of individual mice. To determine the absolute number of cells in spleen and BM, viable cells, which are negative for DAPI (Sigma-Aldrich) were counted with MACSQuant (Miltenyi Biotech). For cell staining, cells were preincubated in a 0.1% BSA-PBS-5 μM EDTA solution with 10 μg/mL anti-FcγRII/III (2.4G2) (BD Pharmingen) for 10 min at 4°C. When indicated, cells were stained with H-2K^b-SIINFEKL or H-2D^b-FQPQNGQFI (LCMV NP$_{396\text{-}404}$) pentamer (Proimmune) for 10 min at room temperature or with H-2D^b-KAVYNFATM (LCMV GP$_{33\text{-}41}$) streptamer (IBA GmbH) for 45 min at 4°C. The following Abs were used for surface staining: anti-CD8α (53-6.7), anti-CD90.2 (53-2.1), anti-CD3 (145-2C11), anti-B220 (RA3.6B2; DRFZ), anti-CD44 (IM7), anti-CD62L (MEL14), anti-CD69 (H1.2F3), anti-IL-7Rα (A7R34), anti-CD122 (5H4 and TM-β1), anti-KLRG1 (2F1), anti-CD45 (30-F11), anti-Gr-1 (RB6-8C5), anti-VCAM-1 (429), anti-CD31 (390), anti-Ter119 (Ter119). All Abs were purchased from BD Pharmingen, Biolegend, and eBioscience, or were produced in DRFZ. Dead cells were excluded either with DAPI or fixable live-dead staining (eBioscience), which was performed prior to cell surface staining. For Ki-67 staining, cells were fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set (eBioscience) or Lysis Solution and Permeabilizing Solution 2 (both BD FACS^{TM}) according to manufacturer's recommendations and stained with anti-Ki-67 (B56, BD Biosciences) for 30 min at room temperature. To detect cytokines by intracellular cytokine staining, cells were stimulated with 1 μg peptide/mL (GP$_{33\text{-}41}$, NP$_{396\text{-}404}$, and GP$_{276\text{-}286}$) in the presence of Brefeldin A (5 μg/mL; Biolegend) or Monensin A (5 μg/mL; Sigma) for 6 h. CD107a staining was achieved by adding anti-CD107a (1D4B, Biolegend) together with the peptide. Cells were washed, fixed, and permeabilized with Lysis Solution and Permeabilizing Solution 2. Cells were then stained for IL-2 (JES6-5H4; Biolegend) and IFN-γ (XMG1.2; eBioscience). When indicated, BM cells were digested with 0.5 mg/mL Collagenase type IV (Sigma-Aldrich), 1 mg/mL DNase I (Sigma-Aldrich), 0.25 mg/mL Dispase II (Roche), 5 μg/mL Latrunculin B (Sigma-Aldrich), and 2.5 μg/mL Cytochalasin D (Sigma Aldrich) for 30 min at 37°C. Stained samples were analyzed in BD LSR II, BD LSRFortessa, BD FACSCanto II (BD Biosciences), and MACSQuant (Miltenyi Biotech) flow cytometers. Flow cytometric data were analyzed with FlowJo (Tree Star, Inc.) software.

Transcriptome analysis and real-time PCR

Single-cell suspensions from spleen and BM of aged ex-breeder mice were prepared. CD8$^+$ cells were enriched by magnetic cell sorting using anti-CD8α microbeads (Miltenyi Biotech). Afterwards, CD3$^+$ CD8$^+$ CD127$^+$ CD44$^+$ memory-phenotype or CD44$^-$ naive cells were sorted with a BD FACSAria cell sorter or BD Influx (BD Biosciences). Cells were either processed immediately for RNA preparation or after being stimulated with plate-bound anti-CD3 (145-2C11, Miltenyi Biotech; 2.5 μg/mL) and anti-CD28 (37.51, DRFZ; 2.5 μg/mL) for 42–44 h. RNA was prepared with NucleoSpin RNA kit (Macherey-Nagel). DNA microarray analysis of gene expression was performed at the gene array facility in the DRFZ as described before [4]. The chip data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE62691 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE62691). To analyze the gene expression of *S1pr1*, CD8$^+$ CD3$^+$ CD44$^+$, and CD69$^+$ or CD69$^-$ cells from spleen and BM of ex-breeder mice were sorted. Total RNA was isolated with ZR RNA Miniprep kit (Zymo Research). *S1pr1* mRNA was reverse transcribed using the Reverse Transcription kit (Applied Biosystems) and *U6* small nuclear RNA (snRNA) using Taqman MicroRNA Reverse Transcription kit (Applied Biosystems). Respective cDNAs were quantified by quantitative PCR with TaqMan Gene Expression Assay for *S1pr1* (Mm02619656_s1) and TaqMan MicroRNA Assay for *U6* snRNA (001973) (Applied Biosystems) according to manufacturer's recommendations. For normalization the expression values were compared to values of *U6* snRNA by the change in threshold method ($2-\Delta CT$).

BrdU staining and cell cycle analysis

Ex-breeder mice were fed with 1 mg/mL BrdU in drinking water containing 0.1 g/mL sugar for 3 days. As controls, some mice were fed with water containing sugar only. A total of 5×10^6 spleen and BM cells were stained for cell surface markers and were fixed and permeabilized according to a modified BrdU staining protocol provided with BrdU Flow Kit (BD Pharmingen, BD Cytoperm Permeabilization Buffer Plus was replaced with 0.01% Triton X-100 [Sigma-Aldrich]/1%BSA/PBS). Cells were then stained with anti-Ki-67 and anti BrdU (3D4, BD Pharmingen) for 30 min at room temperature. For cell cycle analysis, $2–3 \times 10^6$ cells were stained with cell surface molecules and Ki-67 as explained above. Cells were then treated with 200 μg/mL RNase A (Qiagen) and 20 μg/mL propidium iodide (Invitrogen) in PBS for 30 min at room temperature and directly measured at a MACSQuant flow cytometer.

Preparation of BM histological sections and confocal microscopy

Femurs were fixed in 4% PFA (Electron Microscopy Sciences) for 4 h at 4°C, equilibrated in 30% sucrose/PBS. Bones were frozen and cryosectioned using Kawamoto's film method [55]. Six micrometer sections were stained with Abs in 0.1% Tween-20 (Sigma-Aldrich)/5% FCS/PBS after blocking with 5% FCS/PBS for 30 min. The following primary and secondary reagents were used: anti-CD8α (53-6.7, DRFZ), anti-CD3 (eBio500A2, eBioscience), anti-CD4 (GK1.5, DRFZ), anti-CD44 (IM7, DRFZ), anti-GFP (polyclonal rabbit, Life Technologies), digoxygenin-coupled anti-mouse/human fibronectin (polyclonal rabbit, Sigma-Aldrich, coupled in DRFZ), anti-rat-Alexa 555/Alexa 647 (polyclonal goat, Life Technologies), anti-rabbit A488 (polyclonal donkey, Life Technologies), anti-digoxygenin-Alexa 594 (DRFZ), streptavidin-Alexa 594 (Life Technologies). For the nuclear staining, sections were stained with 1 μg/mL DAPI in PBS. Sections were mounted with Fluorescent Mounting Medium (DAKO). All confocal microscopy was carried out using a Zeiss LSM710 with a 20×/0.8 numerical aperture objective lens and all images were generated by maximum intensity projection of 3–5 Z-stacks each with 1 μm thickness. Image acquisition was performed using Zen 2010 Version 6.0 and images were analyzed by Zen 2009 Light Edition software (Carl Zeiss MicroImaging).

Generation of chimeric mice

BM chimeras were generated from mice that are expressing GFP ubiquitously. The mice were irradiated twice (within 3 h) with 3.75 Gy and reconstituted 24 h later with 3×10^6 total BM cells from C57BL/6J mice, which had been depleted of $CD90^+$ cells by MACS CD90.2 MicroBeads (Miltenyi Biotec) before transfer. Mice were treated with 1 mg/mL Neomycin (Sigma-Aldrich) and vitamins (Ursovit, Serumwerke Bernburg) for 16 days, starting 2 days before irradiation. Four weeks after reconstitution, mice were sacrificed and femurs were frozen. Chimerism was confirmed by flow cytometry.

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Abbreviations: **KLRG1**: killer cell lectin-like receptor subfamily G member 1 · **LCMV**: lymphocytic choriomeningitis virus · **S1PR1**: sphingosine-1-phosphate receptor 1

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