Immunity
Article

Professional Memory CD4$^+$ T Lymphocytes Preferentially Reside and Rest in the Bone Marrow

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SUMMARY

CD4$^+$ T lymphocytes are key to immunological memory. Here we show that in the memory phase of specific immune responses, most of the memory CD4$^+$ T lymphocytes had relocated into the bone marrow (BM) within 3–8 weeks after their generation—a process involving integrin α2. Antigen-specific memory CD4$^+$ T lymphocytes highly expressed Ly-6C, unlike most splenic CD44^{hi}CD62L$^-$ CD4$^+$ T lymphocytes. In adult mice, more than 80% of Ly-6C^{hi}CD44^{hi}CD62L$^-$ memory CD4$^+$ T lymphocytes were in the BM. In the BM, they associated to IL-7-expressing VCAM-1$^+$ stroma cells. Gene expression and proliferation were downregulated, indicating a resting state. Upon challenge with antigen, they rapidly expressed cytokines and CD154 and efficiently induced the production of high-affinity antibodies by B lymphocytes. Thus, in the memory phase of immunity, memory helper T cells are maintained in BM as resting but highly reactive cells in survival niches defined by IL-7-expressing stroma cells.

INTRODUCTION

CD4$^+$ T lymphocytes are a key component of immunological memory. In their absence, the generation of high-affinity memory B cells and long-lived plasma cells (Gershon and Paul, 1971; Francus et al., 1991; Crotty et al., 2003) and the maintenance and secondary expansion of memory CD8$^+$ T lymphocytes (Shedlock and Shen, 2003; Sun and Bevan, 2003; Janssen et al., 2003; Sun et al., 2004) are impaired. Despite their eminent importance for the regulation of immune reactions and immunological memory, little is known about differentiation, diversity, and homeostasis of memory CD4$^+$ T cells in body. Memory CD4$^+$ T cells have an extended lifespan and rapidly respond to antigen. It is believed that in the absence of antigen, their numbers are maintained by survival signals and homeostatic proliferation. Intentionally antigen specific and unintentionally generated, CD44^{hi}CD62L$^-$ "memory-phenotype" CD4$^+$ T cells isolated from secondary lymphoid organs or blood have been analyzed for signals required for their homeostatic maintenance (Sprent and Surh, 2002; Schluns and Lefrancois, 2003; Jameson, 2005; Robertson et al., 2006; Ma et al., 2006; Seddon et al., 2003). IL-15-deficient mice show decreased homeostatic proliferation of memory CD8$^+$ T cells, but homeostasis of memory CD4$^+$ T cells is not affected (Lodolce et al., 1998; Kennedy et al., 2000; Goldrath et al., 2002; Becker et al., 2002; Judge et al., 2002). IL-7 synergizes with IL-15 to support survival and homeostatic division of memory CD8$^+$ T cells (Tan et al., 2002; Kieper et al., 2002). IL-7 is considered critical for the maintenance of CD4$^+$ T cell memory, because memory CD4$^+$ T cells from spleen and lymph nodes (LN), when adoptively transferred into IL-7-deficient mice, do not persist (Kondrack et al., 2003), although in IL-7 receptor-deficient mice, CD44^{hi}CD62L$^-$ CD4$^+$ T cells are still detectable in the spleen (Seddon et al., 2003).

Memory CD8$^+$ T cells and memory plasma cells reside in BM. After their generation in secondary lymphoid organs, memory CD8$^+$ T cells preferentially locate to BM for homeostatic maintenance (Becker et al., 2005; Mazo et al., 2005) and they are likely to obtain IL-15 there, although this has not been formally demonstrated so far. Memory plasma cells require CXCL12 for survival and reside next to CXCL12-expressing stroma cells in BM (Slifka et al., 1995; Manz et al., 1997; Tokoyoda et al., 2004). Newly generated plasmablasts migrate to BM and there probably compete with resident plasma cells for habitation of survival niches provided by CXCL12-expressing cells (Radbruch et al., 2006). In contrast, memory CD4$^+$ T cells for a long time had been presumed to constantly circulate the body for surveillance (Dutton et al., 1998). However, recent studies have suggested that newly generated memory CD4$^+$ T cells compete with older ones for hypothetical niches defining the homeostatic pool (Hataye et al., 2006; Blair and Lefrançois, 2007). So far, a preferred home tissue of memory CD4$^+$ T cells and their survival niches has not been identified.

Here we show that in mice, antigen-specific memory CD4$^+$ T cells reside in BM. Within 3–8 weeks after their activation in secondary lymphoid organs, most antigen-specific CD4$^+$ T lymphocytes relocated to BM. All of them highly expressed Ly-6C, whereas few splenic CD4$^+$ T cells expressed this marker. The tropism for BM was mediated by integrin α2. In BM the CD4$^+$ T cells resided in contact with IL-7-expressing VCAM-1$^+$ stroma cells and rested in terms of proliferation and gene expression. Memory CD4$^+$ T cells from BM reacted quickly to challenge with antigen and provided efficient help to activated B lymphocytes for the production of high-affinity antibodies.

Their slow turnover, fast reaction, and efficient helper function mark Ly-6C^{hi}CD44^{hi}CD62L^{-} CD4^{+} T cells of BM as professional memory CD4^{+} T cells, which are maintained in the BM as an essential component of immunological memory. More than 80% of the CD4^{+} T cells with this phenotype reside in the BM, as compared to the rest of the body.

RESULTS

Antigen-Specific Memory CD4^{+} T Cells Relocate to BM

To define the location of antigen-specific memory CD4^{+} T cells in the body with respect to their generation and persistence, we have analyzed memory CD4^{+} T cells that had been generated in intentional immune responses to distinct antigens. First, we tracked LCMV-specific CD4^{+}CD44^{hi} T cells in the course of immune responses to LCMV-derived peptide of Smarta T cells in C57BL/6 mice (Figure 1A). 4 days after immunization, activated CD4^{+}CD44^{hi} LCMV-specific T cells were present in spleen and LN but not in BM. From day 28 onward, the number of CD4^{+}CD44^{hi} T cells in spleen and LN decreased, whereas in BM their number increased. From day 60 onward, most LCMV-specific CD4^{+} T cells resided in BM, more than 80% of total. They persisted in BM at constant numbers for more than 134 days, which is the period of observation (Figure 1A). In an immune response to ovalbumin, the antigen-specific memory CD4^{+} T cells translocated to BM with similar kinetics, remaining constant for more than 90 days (Figures S1A and S1B available online). In the immune response of C57BL/6 mice to KLH and complete Freund's adjuvant (CFA), KLH-specific CD4^{+} T cells, identified according to expression of IFN-γ (Figure 1B) as well as CD154 (Figure 1C; Frentsch et al., 2005) upon restimulation with antigen ex vivo, showed again similar kinetics of relocation to BM. We conclude that in the memory phase of an immune response, most antigen-specific CD4^{+} T cells reside in the BM.

Ly-6C Is a Marker of Memory CD4^{+} T Cells

Ly-6C is a marker of memory CD8^{+} T cells (Walunas et al., 1995). Here we show that Ly-6C is also a marker of antigen-specific memory CD4^{+} T cells. Ly-6C was highly expressed on all LCMV-specific memory CD4^{+} T cells in BM 134 days after immunization (mean fluorescence intensity [MFI], 534 ± 76) (Figure 2A). Of all CD44^{hi}CD62L^{-} CD4^{+} T cells from BM of unimmunized mice 40% expressed Ly-6C highly (MFI, 465 ± 35) and the remaining 60% still expressed Ly-6C, but less of it (MFI, 6.4 ± 0.8) (Figure 2B). Most CD44^{hi}CD62L^{-} CD4^{+} T cells of the spleen of unimmunized mice expressed little if any Ly-6C (MFI, 0.9 ± 0.5). In the spleen, CD44^{hi}CD62L^{-} CD4^{+} T cells expressing high amounts of Ly-6C were rare, less than 5% (Figure 2B). Of all Ly-6C^{hi}CD44^{hi}CD62L^{-} CD4^{+} T cells in normal adult C57BL/6 mice, 80% resided in BM and 20% in spleen, mesenteric LN (mLN), peripheral LN (pLN; brachial, axillary, and inguinal LN), thymus, peyer's patches (PP), blood, and liver (Figure 2C). CD44^{hi}CD62L^{-} CD4^{+} T cells of BM are residing in the stroma, not in BM blood, as indicated by the fact that they could not be mobilized by perfusion (Figure S2). Our results suggest that Ly-6C is a marker of memory CD4^{+} T cells in the memory phase of an immune response.

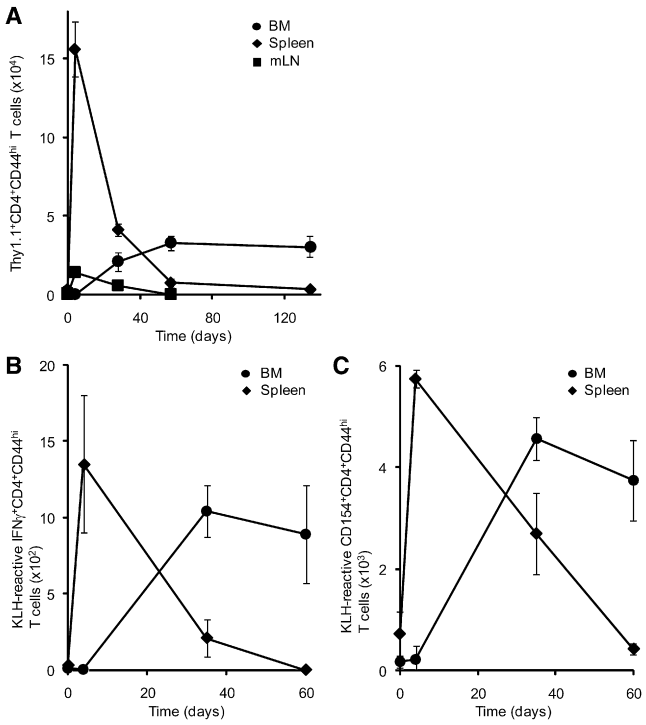


Figure 1. Memory CD4^{+} T Cells Reside in BM

(A) Kinetics of tissue distribution of LCMV-specific memory CD4^{+} T cells in immunocompetent mice. C57BL/6 mice transferred with 10^{6} CD4^{+} T cells from Thy1.1-LCMV-TCR Tg (Smarta) mice were injected with 100 μg of GP_{61-80} peptide plus 10 μg LPS. The numbers of the transferred T cells and their offspring were quantified as Thy1.1^{+}CD4^{+}B220^{-}CD44^{hi} T cells in BM (circles), spleen (diamonds), and mLN (squares) of host mice at 0, 4, 28, 57, and 134 days. Cell numbers in each tissue are shown as the mean ± SD. Data are representative of two independent experiments.

(B and C) Kinetics of appearance of KLH-specific memory CD4^{+} T cells. C57BL/6 mice were immunized with 100 μg of KLH in CFA and analyzed at 0, 4, 35, and 60 days for KLH-reactive IFN-γ^{+} (B) or CD154^{+} (C) CD4^{+}CD44^{hi} T cells in BM (circles) and spleen (diamonds), after stimulation with KLH ex vivo. Cell numbers in BM and spleen are shown as the mean ± SD. Data are representative of three independent experiments.

Memory CD4^{+} T Cells Rest in BM

CD44^{hi}CD62L^{-} CD4^{+} T cells of spleen and BM show no difference in their expression of well-known cell surface molecules. Expression of CD3, CD4, CD28, CD44, CD45RB, CD62L, CXCR4, CXCR5, IL-7Rα, integrin α4, TCR-β, and their TCR Vβ repertoires were comparable (data not shown). For a global assessment of the differences in gene expression between CD44^{hi}CD62L^{-} CD4^{+} T cells from spleen and BM, we compared their transcriptomes. 1756 transcripts were differentially expressed (Figure 3A). Expression of 96% of these transcripts was downregulated in BM cells. This shows that Ly-6C^{+} CD44^{hi}CD62L^{-} memory CD4^{+} T cells from BM are resting as compared to CD44^{hi}CD62L^{-} CD4^{+} T cells from spleen.

Differential expression of some of the genes identified by microarray analysis and encoding cell surface proteins was also analyzed and verified on the level of protein expression of single cells by immunofluorescence (Figure 3B). Expression of CD24 (HSA), CD93, CD119 (IFN-γR1), CD122 (IL-2Rβ), CD186

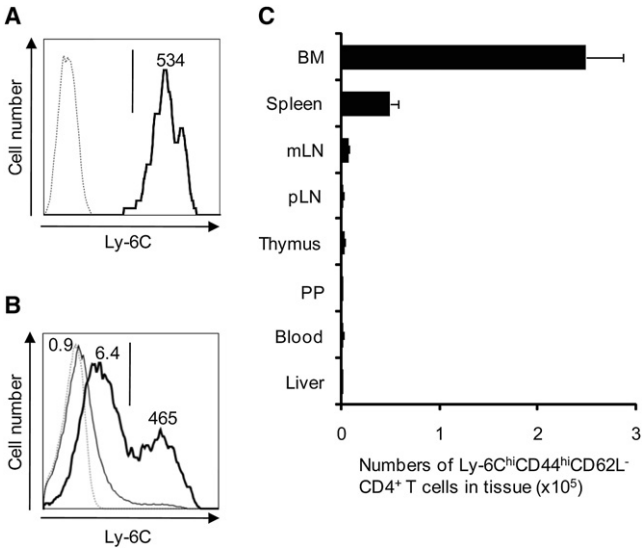


Figure 2. Memory CD4+ T Cells Express Ly-6C

(A) Expression of Ly-6C on antigen-specific memory CD4+ T cells. C57BL/6 mice transferred with 10^6 CD4+ T cells from Thy1.1-LCMV-TCR Tg mice were injected with 100 μg of GP_{61-80} peptide plus 10 μg LPS and the expression of Ly-6C on Thy1.1+CD4+B220−CD44hi T cells in BM at 134 days was analyzed by flow cytometry (bold line; isotype control shows dashed line). The inserted number shows the MFI. Data are representative of two independent experiments.

(B) Expression of Ly-6C on $CD44^{hi}CD62L^-$ CD4+ T cells of spleen and BM. The histograms show their expression by $CD4^+CD44^{hi}CD62L^-CD25^-B220^-$ T cells from BM (bold line) and spleen (solid line), and isotype control (dashed line) and the inserted number shows MFI. Data are representative of four independent experiments.

(C) $Ly\text{-}6C^{hi}CD44^{hi}CD62L^-$ CD4+ T cells in various organs. The number of $Ly\text{-}6C^{hi}CD44^{hi}CD62L^-CD25^-B220^-$ T cells in BM, spleen, mLN, pLN, thymus, PP, blood, and liver of C57BL/6 mice is shown as the mean + SD of three independent experiments.

(CXCR6), and CD192 (CCR2) on $CD44^{hi}CD62L^-$ CD4+ T cells from BM were higher than on those from spleen.

Also with regard to proliferation, memory CD4+ T cells from BM were resting. In steady state, less than 0.4% of the cells were in the $S+G_2/M$ phases of the cell cycle, similar to naive CD4+ T cells (Figures 3C and 3D). Less than 8% synthesized any DNA, i.e., incorporated 5-bromo-2′-deoxyuridine (BrdU), within 48 hr (Figure S3). These frequencies were 3- to 4-fold higher for $CD44^{hi}CD62L^-$ CD4+ T cells from other tissues, indicating that in BM, memory CD4+ T cells are maintained as resting cells. Taken together, memory CD4+ T cells rest in the BM, downregulate overall gene expression, and do not proliferate.

Integrin α2 Mediates Homing of Memory CD4+ T Cells to BM

Another protein differentially expressed by $CD44^{hi}CD62L^-$ CD4+ T cells from spleen and BM was integrin α2 (Figure 4A). Only 15% of splenic $CD44^{hi}CD62L^-$ CD4+ T cells expressed integrin α2 (MFI, 68 ± 15), whereas about 70% of $CD44^{hi}CD62L^-$ CD4+ T cells from BM expressed integrin α2, and they expressed it at a higher amount (MFI, 106 ± 25). The preferential homing of BM $CD44^{hi}CD62L^-$ CD4+ T cells to BM is dependent on their expression of integrin α2, as shown by the fact that it can be blocked by integrin α2-specific antibodies (Figure 4B; Figure S4). Integrin α2-specific antibodies injected into C57BL/6 mice at day 4, 5, and 6 of an intentional immune reaction to KLH inhibited relocation of antigen-specific memory CD4+ T cells into BM (Figure 4C). Integrin α2 is thus an essential mediator for homing of memory CD4+ T cells into BM.

Memory CD4+ T Cells Reside on IL-7-Expressing Stroma Niches in BM

Persistence of memory CD4+ T cells is likely to depend on signal(s) they receive in survival niches (Hataye et al., 2006). In the BM, about 90% of $CD44^{hi}CD62L^-$ CD4+ T cells individually colocalized with VCAM-1+ stroma cells, which also express IL-7 (Figures 5A and 5B; Tokoyoda et al., 2004), providing a molecular rationale for the presence of memory CD4+ T cells in BM (Kondrack et al., 2003). In a specific immune response to LCMV peptide, more than 95% of the Thy1.1+ LCMV-specific memory CD4+ T cells colocalized with IL-7-expressing cells of BM (Figure 5C). The frequency of VCAM-1+ IL-7-expressing stroma cells in murine BM is about 2%, i.e., a mouse contains about 5×10^6 IL-7+ BM niches for memory CD4+ T cells. Because we never observed more than one CD4+ T cell in contact with one stroma cell, we estimate the capacity of murine BM for memory CD4+ T cells to be about 5×10^6 cells. It remains to be determined to what extent the number of survival niches indeed determines homeostasis of the memory CD4+ T cell pool. Memory CD4+ T cells in BM thus rest next to a stroma cell expressing their mandatory survival factor IL-7.

BM Memory CD4+ T Cells Provide Efficient and Fast Help to B Cells

Do $CD44^{hi}CD62L^-$ CD4+ T cells from BM function as professional helper T cells, in that they provide the signals required for affinity maturation of the antibodies of activated B cells? Naive B cells were transferred together with naive or $CD44^{hi}CD62L^-$ CD4+ T cells from BM or spleen of unimmunized mice into lymphopenic mice, which were then immunized with the T cell-dependent antigen 4-(hydroxy-3-nitro-phenyl) acetyl-coupled KLH (NP-KLH) and monitored for the appearance and quality of NP-specific antibodies in serum (Figure 6A). Both spleen and BM of unimmunized mice contain detectable and comparable numbers of $CD44^{hi}CD62L^-$ CD4+ T cells (cross)-reactive to KLH protein (Figure 1C). As determined by short-term restimulation ex vivo, about 170 of 5×10^5 cells of total BM $CD44^{hi}CD62L^-$ CD4+ T cells (0.034%) and 710 cells of 3×10^6 splenic $CD44^{hi}CD62L^-$ CD4+ T cells (0.024%) reacted to KLH by expression of CD154 (Frentsch et al., 2005). In a primary immune response to NP-KLH, $CD44^{hi}CD62L^-$ CD4+ T cells from BM and spleen provided help to the cotransferred B cells for the production of antigen-specific low-affinity antibodies (anti-NP_{24} IgM and IgG_1), but only $CD44^{hi}CD62L^-$ CD4+ T cells from BM induced the production of high-affinity antibodies (anti-NP_3 IgG_1), reflecting affinity maturation, a hallmark of T helper cell function (Figure 6A). The help provided by $CD44^{hi}CD62L^-$ CD4+ T cells from BM is antigen specific, because it depended on the presence of antigen-specific memory T cells (Figure S5).

In a nonlymphopenic C57BL/6 system, memory CD4+ T cells from BM induced affinity maturation as well (Figures 6B and 6C), both in primary and secondary immune responses, the latter

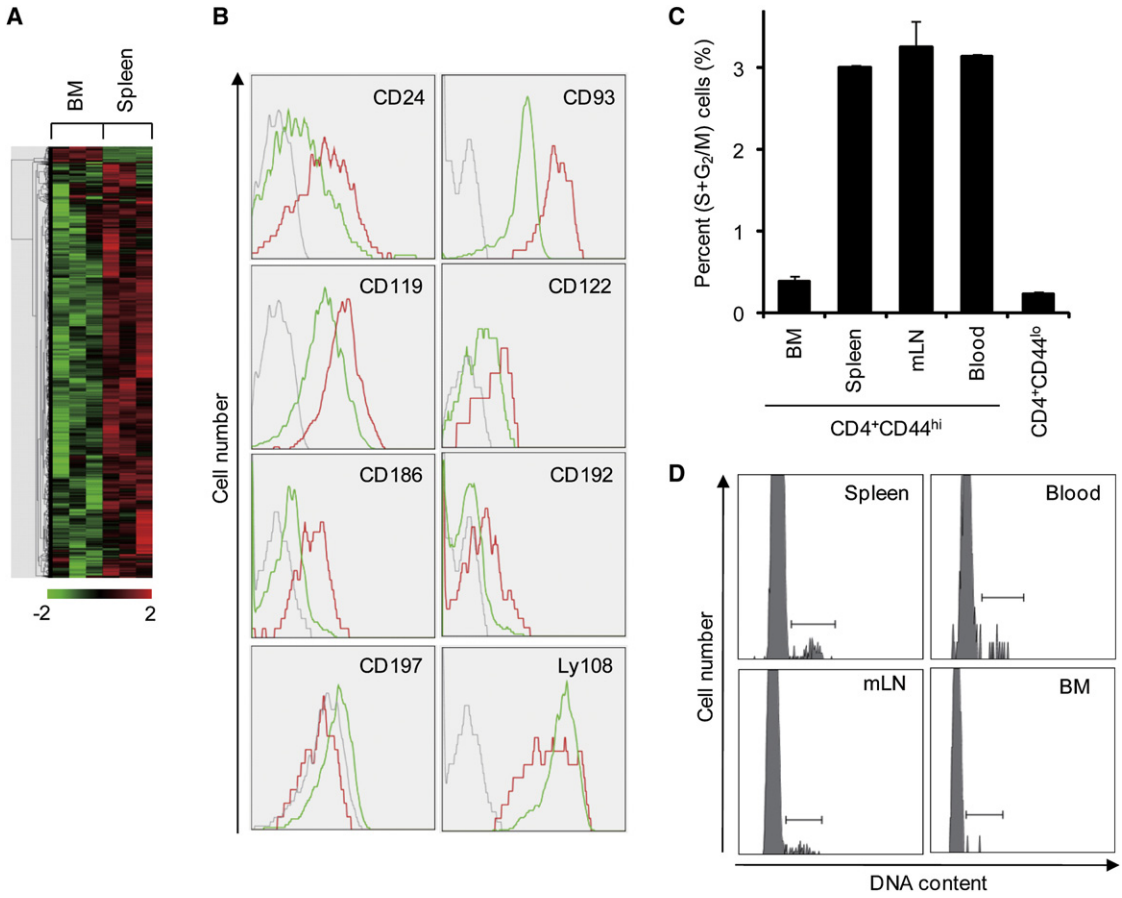


Figure 3. Gene Expression and Cell Cycle Analysis of Memory CD4+ T Cells from BM or Spleen

(A) Hierarchical cluster analysis of all 1756 differentially expressed genes of $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells in BM and spleen. These parameters combined a threshold for gene expression, Present and Marginal detection, and Decrease or Increase change calls, or a Bonferroni corrected t test p value. Upregulated genes are shown in red and downregulated genes are in green. The data represent three individual gene arrays for each of the BM and spleen cells.

(B) Expression analysis of selected cell surface molecules. $CD4^{+}CD44^{hi}CD62L^{-}CD25^{-}B220^{-}$ cells from BM (red) or spleen (green; isotype control shows gray) were stained with antibodies against CD24, CD93, CD119, CD122, CD186, CD192, CD197, and Ly108 and analyzed by flow cytometry. Data are representative of two independent cytometric experiments.

(C and D) $CD44^{hi}CD25^{-}B220^{-}$ $CD4^{+}$ T cells from BM, spleen, mLN, and blood and $CD44^{lo}CD25^{-}B220^{-}$ $CD4^{+}$ T cells from spleen of C57BL/6 mice were fixed and their DNA stained with PI. The figures indicate the frequencies of cells with more than 2n of DNA content, i.e., cells in $S+G_2$-M phases (C) and the flow cytometric analysis (D). Data are shown as mean + SD of two independent experiments.

after adoptive transfer of $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells from BM or spleen into C57BL/6 mice. This professional help was dependent on integrin α2, as shown by the fact that it could be blocked in the primary immune reaction by integrin α2 antibody injected on days 4, 5, and 6 after immunization (Figure 6B). Moreover, $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells from BM but not spleen of immunized mice could transfer help for affinity maturation (Figure 6C). $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells from BM contained about 40% Ly-$6C^{hi}$ and 60% Ly-$6C^{lo}$ cells (Figure 2B). Antigen-specific memory $CD4^{+}$ T cells expressed Ly-6C highly (Figure 2A). Ly-$6C^{hi}$ but not Ly-$6C^{lo}$ $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells did provide help for affinity maturation (Figure 6D).

We analyzed whether memory $CD4^{+}$ T cells from BM are mobilized when reactivated and leave the BM for the secondary lymphoid organs. In contrast to the spleen, in BM we could not detect PNA^{hi} B cells 11 days after immunization (Figure 6E), indicating that the interaction between memory $CD4^{+}$ T cells and B cells occurred in secondary lymphoid organs and not in the BM.

We then examined the molecular basis for the more efficient help provided by BM memory $CD4^{+}$ T cells, as compared to $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells from spleen. When activated by anti-CD3 in vitro, $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells from BM strongly expressed all cytokines of those assayed, at frequencies higher than those of $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells from spleen (Figure 7A), whereas both $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells showed the same degree of vigorous proliferation and activation-induced cell death (data not shown). Expression of IFN-γ (Figure 7B) and CD154 (Figure 7C) was rapidly induced in BM memory $CD4^{+}$ T cells upon CD3 ligation, like in in vitro-differentiated effector $CD4^{+}$ T cells (Löhning et al., 2003), reaching a peak at 2 to 3 hr. IFN-γ- and CD154-expressing $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells from spleen peaked later, at about 4–6 hr, as has been described previously (Figures 7B and 7C;

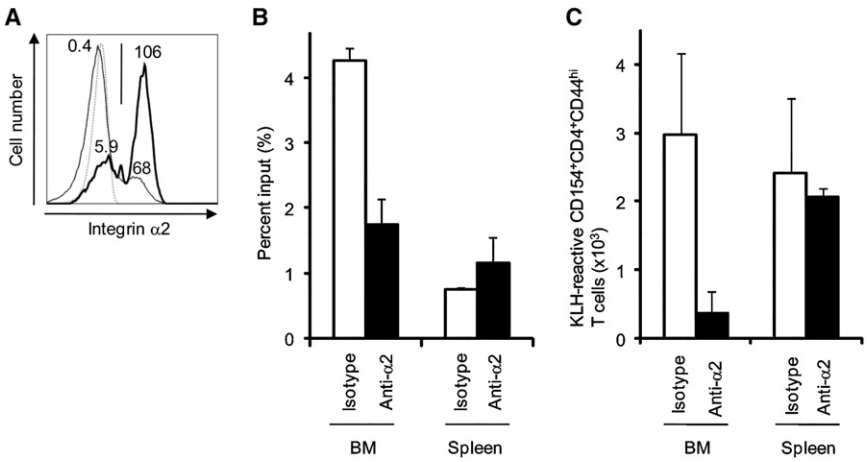


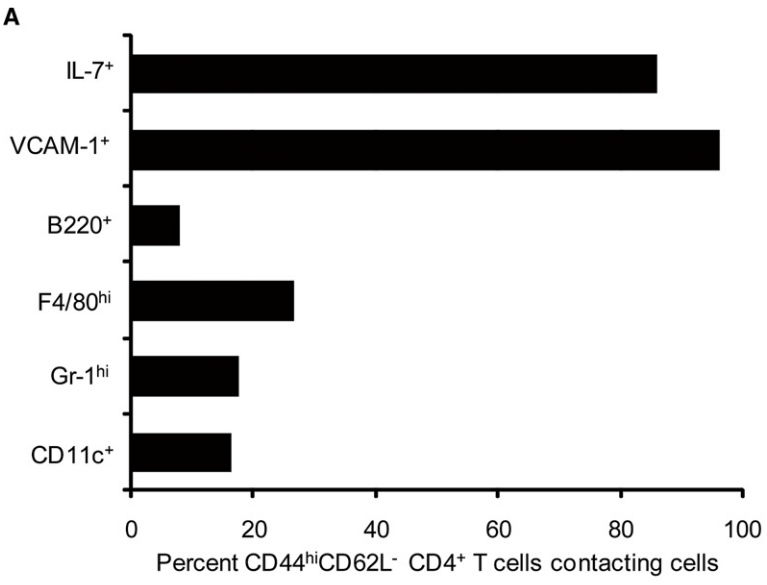
Figure 4. Homing of Memory CD4+ T Cells to the BM

(A) Expression of integrin α2 on CD44^{hi}CD62L^{-} CD4^{+} T cells of spleen and BM. The histograms show the expression of integrin α2 by CD4^{+}CD44^{hi}CD62L^{-}CD25^{-}B220^{-} T cells from BM (bold line) and spleen (solid line), and isotype control (dashed line) and the inserted number shows MFI. Data are representative of four independent experiments.

(B) Homing of CD44^{hi}CD62L^{-} CD4^{+} T cells to the BM is dependent on integrin α2. 2 × 10^{5} CD4^{+} CD44^{hi}CD62L^{-}CD25^{-} T cells from BM of C57BL/6 mice were isolated by FACS. Half of the cells were incubated with Fab fragments of anti-integrin α2 (filled column) or isotype control (open column), labeled with CFSE or PKH-26, and together transferred into RAG1-deficient mice. One day later, CFSE^{+} or PKH-26^{+} CD4^{+}CD44^{hi} T cells were enumerated in BM and spleen. The value indicates the frequencies of total numbers of stained cells in each tissue relative to the numbers of transferred cells. Data indicate the mean + SD of three independent experiments.

(C) Relocation of antigen-specific memory CD4^{+} T cells to the BM is dependent on integrin α2. At day 4, 5, and 6 after immunization of C57BL/6 mice with 100 μg of NP-KLH, the mice were injected with 100 μg of Fab fragments of anti-integrin α2 (filled column) or isotype control (open column) and analyzed for KLH-reactive CD4^{+}CD44^{hi} T cells in BM and spleen at day 10. KLH-reactive cells were enumerated according to expression of CD154 and data indicate the mean + SD of two independent experiments.

Frentsch et al., 2005; Sander et al., 1993). The rapid kinetics and high frequencies of re-expression of cytokine and the essential costimulator CD154 qualify BM memory CD4^{+} T cells as efficient helpers of B cells. Taken together, our results show that in the memory phase of immune responses, memory T helper cells reside in BM as resting cells but can quickly react



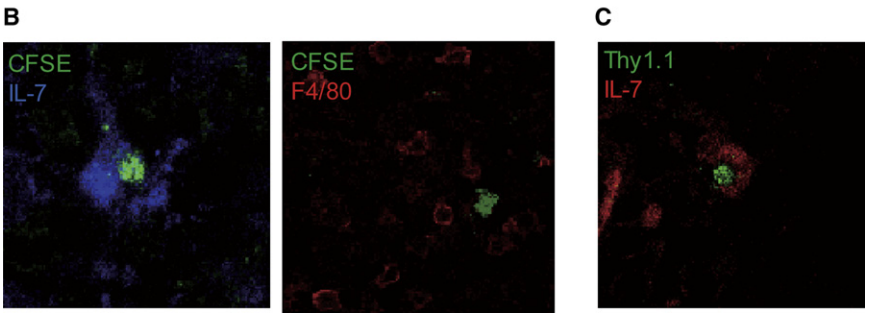


Figure 5. Memory CD4+ T Cells Attach to IL-7+VCAM-1+ Stroma Cells in the BM

(A) Sorted CD4^{+}B220^{-}CD62L^{-}CD25^{-} T cells from BM were labeled with CFSE and transferred into RAG-1-deficient mice, and 16 hr later the location of CFSE-labeled cells in the host BM was inspected microscopically. CFSE-labeled cells next to IL-7^{+}, VCAM-1^{+}, B220^{+}, F4/80^{hi}, Gr-1^{hi}, or CD11c^{+} cells were enumerated and are shown as frequencies of labeled cells in contact with any such cell types in BM. 150 CD44^{hi}CD62L^{-} CD4^{+} T cells were counted to determine the frequency of contacts with IL-7^{+} cells: 166 for VCAM-1^{+}, 214 for B220^{+}, 275 for F4/80^{hi}, 212 for Gr-1^{hi}, 37 for CD11c^{+} in the transferred bone of three independent experiments.

(B) The photos show a representative picture of an IL-7 (blue, left) or F4/80 (red, right) staining versus CFSE (green).

(C) The photo shows a representative colocalization of an IL-7 staining (red) stroma cell versus a Thy1.1 staining (green) T cell. 78 of 82 Thy1.1^{+} cells were in contact with an IL-7^{+} cell.

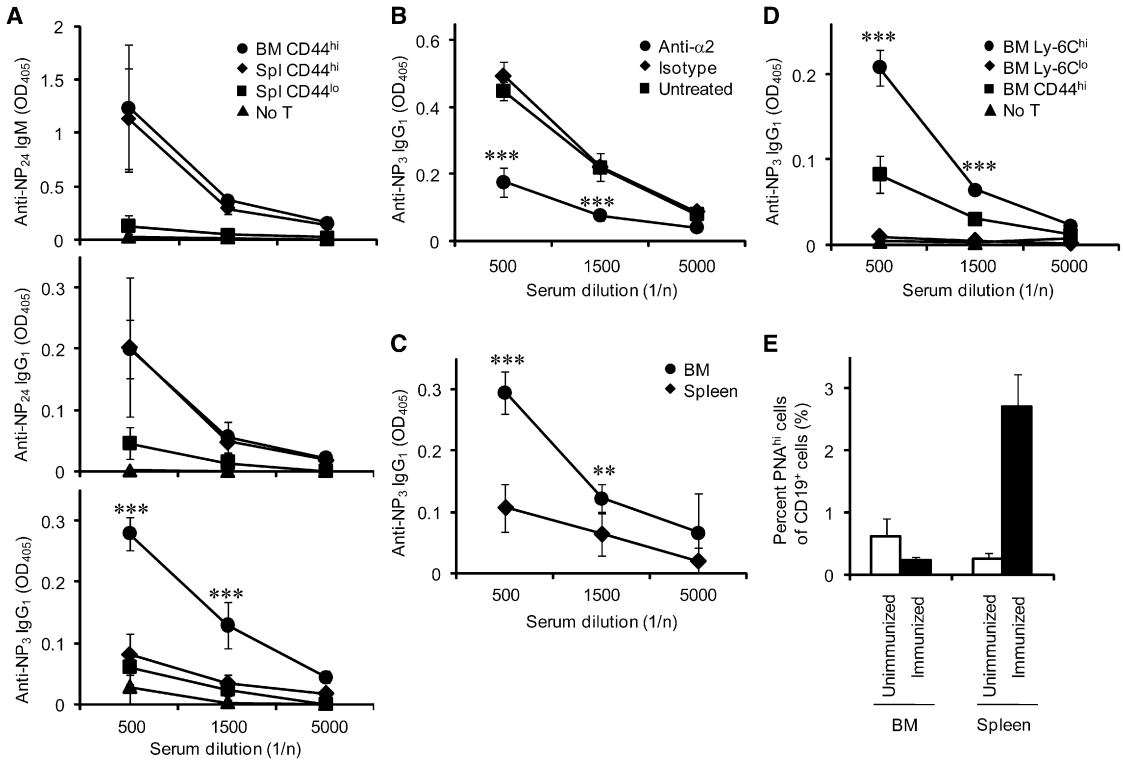


Figure 6. BM Memory CD4+ T Cells Provide Help for Affinity Maturation of Antibodies In Vivo

(A) Transferred $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells from BM provide help for affinity maturation of antibodies in vivo. 4×10^6 $CD19^{+}CD138^{-}CD4^{-}$ cells from spleen were transferred with 10^5 $CD4^{+}CD44^{lo}(CD62L^{+})CD25^{-}$ or $CD4^{+}(CD44^{hi})CD62L^{-}CD25^{-}$ T cells from BM or spleen of C57BL/6 mice, or without T cells, into RAG1-deficient mice. One day later, recipients were immunized with 100 μg NP-KLH in CFA. Blood taken from transferred mice 25 days later was analyzed for anti-NP_{24}-IgM, anti-NP_{24}-IgG1, and anti-NP_3-IgG_1 by ELISA. Data are the mean + SD of three independent experiments.

(B) Relocation of antigen-specific $CD44^{hi}CD62L^{-}$ $CD4^{+}$ T cells into the BM is essential to induce affinity maturation of antibodies in normal mice. At day 4, 5, and 6 after immunization of C57BL/6 mice with 100 μg of NP-KLH, mice were injected with 100 μg of Fab fragments of anti-integrin α2, isotype control, or not injected at all, as indicated. At day 15, blood was taken from immunized mice and analyzed by ELISA. Data indicate the mean + SD of two independent experiments.

(C) Antigen-specific memory $CD4^{+}$ T cells from BM induce efficient production of high-affinity antibodies upon restimulation. 10^5 $CD4^{+}(CD44^{hi})CD62L^{-}CD25^{-}$ T cells from BM and spleen of NP-KLH-immunized mice at day 25 were transferred together with 2×10^6 $CD19^{+}CD138^{-}CD4^{-}$ cells from the immunized mice into naive C57BL/6 mice, followed by injection of NP-KLH. At day 7 after transfer and secondary immunization, the blood of the recipient mice was analyzed by ELISA for the presence of high-affinity antibodies. Data indicate the mean + SD of three independent experiments.

(D) Ly-$6C^{hi}$ antigen-specific memory $CD4^{+}$ T cells induce efficient production of high-affinity antibodies. 10^5 Ly-$6C^{lo}$ or Ly-$6C^{hi}$ $CD4^{+}(CD44^{hi})CD62L^{-}CD25^{-}$ T cells from BM of C57BL/6 mice were transferred together with 4×10^6 $CD19^{+}CD138^{-}$ $CD4^{-}$ cells from C57BL/6 mice, followed by immunization with 100 μg NP-KLH in CFA. At day 25 after transfer and immunization, blood of recipient mice was analyzed by ELISA for the presence of high-affinity antibodies. Data indicate the mean + SD of two independent experiments.

(E) Germinal center B cells in BM and spleen. Cells from BM and spleen of unimmunized (open column) or immunized C57BL/6 mice 11 days after immunization with NP-KLH in CFA (filled column) were stained with PNA and CD19. The value indicates the percentage + SD of PNA^{hi} cells within $CD19^{+}$ cells. Data are representative of two independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$.

to antigenic challenges to provide professional immunological help (Figure S6).

DISCUSSION

We here show that in the memory phase after an immune response, more than 80% of the antigen-specific T helper cells reside in BM. These cells are characterized by expression of Ly-6C. In BM they persist in stable numbers over long time periods, resting in terms of gene expression and cell division. Integrin α2 plays a critical role in the homing process. In secondary immune reactions, when reactivated, presumably by surveilling antigen-presenting cells, they provide efficient and fast help to activated B cells in secondary lymphoid organs.

Memory T helper cells are an essential component of the immunological memory. They control secondary immune reactions and provide critical signals to B cells and cytotoxic T cells reacting to the same antigen. In order to study the transition of memory T helper cells from the acute immune reaction to the memory phase and their persistence in the memory phase, we have analyzed memory T helper cells generated in defined immune reactions to distinct antigens. Antigens and adjuvants were selected to generate immune reactions limited in time and not persisting, as to allow an unbiased analysis of the memory phase. In long-lasting immune reactions, e.g., those provoked by virus or antigen combined with adjuvants like alum or Freund's adjuvant, $CD44^{hi}CD62L^{-}$ "memory phenotype" T helper cells are found in the spleen and LN over long

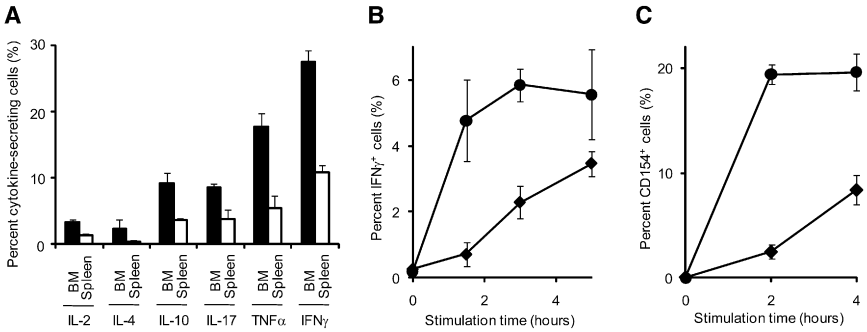


Figure 7. BM Memory CD4+ T Cells Express Cytokines Fast and at High Frequencies

(A) Cytokine expression of CD44^{hi}CD62L^{-} CD4^{+} T cells from BM and spleen ex vivo. CD4^{+} CD44^{hi}CD62L^{-}B220^{-} T cells from BM (filled column) or spleen (open column) from C57BL/6 mice were isolated, stimulated with plate-bound anti-CD3 in the presence of Brefeldin A for 5 hr, fixed, and stained with antibodies for the intracellular cytokines indicated. Data (mean + SD) are representative of two independent experiments.

(B) Kinetics of cytokine expression by CD44^{hi}CD62L^{-} CD4^{+} T cells ex vivo. CD4^{+}CD44^{hi} CD62L^{-}B220^{-} T cells from BM (circles) or spleen (diamonds) from C57BL/6 mice were isolated and stimulated with plate-bound anti-CD3 in the absence of Brefeldin A. At the time points indicated, cells were fixed and stained with anti-IFNγ. Data (mean ± SD) are representative of four independent experiments.

(C) Kinetics of CD154 expression by CD44^{hi}CD62L^{-} CD4^{+} T cells ex vivo. CD4^{+}CD44^{hi}CD62L^{-}B220^{-} T cells from BM (circles) or spleen (diamonds) from C57BL/6 mice were isolated and stimulated with plate-bound anti-CD3 in the presence of Brefeldin A. At the time points indicated, cells were fixed and stained with anti-CD154. Data (mean ± SD) are representative of two independent experiments.

time periods (Homann et al., 2001; MacLeod et al., 2006). However, in case of adjuvants like LPS or Monophosphoryl lipid A, numbers of memory phenotype CD4^{+} T cells in spleen or LN substantially decrease already 1 week after immunization (Mata-Haro et al., 2007). Here we have analyzed the immune responses of DO11.10 and OTII T helper cells to ovalbumin, with LPS as adjuvant, of Smarta T helper cells to LCMV-derived peptide, with LPS as adjuvant, and of wild-type T helper cells to KLH, with CFA. In all these immune responses, more than 80% of the surviving antigen-specific T helper cells translocate to the BM within 3 to 8 weeks after initiation of the immune response, demonstrating that BM is not just another site of location of memory CD4^{+} T cells but the primordial site. These 80% refer to about 20% of the CD44^{hi}CD62L^{-} CD4^{+} T cells generated at the peak of the response on day 4 in spleen and LN. The recruitment of approximately 20% of the cells generated in an immune reaction to the memory phase is remarkably similar to the recruitment of 10%–20% of plasmablasts generated in acute immune reactions to the compartment of long-lived memory plasma cells (Radbruch et al., 2006).

Immigration of memory T helper cells into the BM is dependent on integrin α2, as we have shown here, by blocking immigration with integrin α2-specific antibodies in vivo. Integrin α2β1 (also known as VLA-2) binds with high affinity to collagen type I (Kern et al., 1993), which is one of the major collagen types and the dominant collagen of bone and skin. Expression of integrin α2 on CD4^{+} T cells had been described to correlate with the capability to reexpress effector cytokines (Kassiotis et al., 2006). Not only do memory T helper cells of distinct immune responses depend on integrin α2 for their translocation to BM, but memory CD4^{+} T cells from BM can relocate to BM, by means of integrin α2. We could show this by adoptive transfer of CD44^{hi}CD62L^{-} T helper cells from BM. Within 1 day after transfer, 4% of CD44^{hi}CD62L^{-} CD4^{+} T cells from BM, 70% of which express integrin α2, did home to BM, in an integrin α2-dependent way, less than 1% to the spleen, LN, or blood. Interestingly, less than 1% of splenic CD44^{hi}CD62L^{-} CD4^{+} T cells, 10% of which express integrin α2, homed to BM, spleen, LN, or blood. Neither did splenic naive CD4^{+} T cells, not expressing integrin α2, nor in vitro-generated effector CD4^{+} T cells, home preferentially to BM. It remains to be shown whether apart from the adhesion molecule integrin α2, chemokine attraction is required to lure memory T helper cells into the BM. The induction of integrin α2 expression, by signals so far not defined, is thus apparently a critical step in the development of a stable T helper memory compartment in the BM.

Phenotypic and functional analysis of splenic CD44^{hi}CD62L^{-} CD4^{+} T cells has indicated a considerable heterogeneity (Stockinger et al., 2006). The present analysis shows that memory T helper cells generated in defined immune reactions persist in BM and express two distinct markers, integrin α2 (as discussed above) and Ly-6C, at high amounts. Thus, in mice, Ly-6C is a marker of memory T helper cells and discriminates them from other CD44^{hi}CD62L^{-} T helper cells of unknown etiology and function. In BM, all CD44^{hi}CD62L^{-} T helper cells express Ly-6C, 40% at high amounts and 60% at intermediate amounts. In the spleen, less than 5% of the CD44^{hi}CD62L^{-} T helper cells express Ly-6C. More than 80% of all Ly-6C-expressing CD44^{hi}CD62L^{-} T helper cells counted in the tissues analyzed are in the BM, less than 20% in all of the other organs. We cannot rule out yet that additional alternative homing sites for Ly-6C^{hi}CD44^{hi}CD62L^{-} CD4^{+} T cells exist, e.g., in skin or intestine, although few cells generated in the immune responses analyzed here enhanced the skin-homing molecule CLA or the gut-homing molecules integrin α4β7.

The role of peripheral CD4^{+} T cells expressing CD44^{hi}CD62L^{-}, i.e., the classical memory-phenotype murine CD4^{+} T cells, will have to be redefined. Moreover, central memory T cells expressing CCR7 and CD62L were not found in the BM. The mechanisms of maintenance of these cells, which are even more abundant in humans than in mice, remain to be clarified as well.

In functional terms, memory CD4^{+} T cells from BM are efficient helper cells. They efficiently induce antibody affinity maturation of B cells activated by the same antigen. These immune reactions apparently take place in secondary lymphoid organs, implying that the memory T helper cells have to be reactivated and mobilized from the BM, presumably by surveilling antigen-presenting cells. Our analysis shows that BM memory T helper cells are reactive to antigen substantially faster than other T helper cells, although in terms of gene expression and

proliferation, they rest in BM. The concept that memory T helper cells reside and rest in the BM until they are reactivated by surveilling antigen-presenting cells, is in contrast to previous concepts assuming that memory $CD4^+$ T cells constantly themselves circulate the body for surveillance of cells presenting the appropriate antigen (Dutton et al., 1998).

The apparent molecular rationale for memory $CD4^+$ T cells to home to BM is their quest for IL-7. In BM, $CD44^{hi}CD62L^-$ $CD4^+$ T cells are located next to IL-7-expressing stroma cells. They are resting in terms of both proliferation and gene expression, as compared to $CD44^{hi}CD62L^-$ $CD4^+$ T cells from other organs. IL-7 has been reported to be required for survival of T cells and upregulation of Bcl-2 expression, in the absence of proliferation (Kondrack et al., 2003). It also has been reported that IL-7 mediates the transition of effector into memory $CD4^+$ T cells (Kondrack et al., 2003; Li et al., 2003), an event that in light of the present results takes place in the BM. In the immune response to ovalbumin analyzed here, the specific $CD4^+CD44^{hi}$ T cells translocate to BM between days 6 and 21 of the response, and the transition of ovalbumin-specific effector T cells generated in the spleen and LN to professional memory T cells could well have happened in the BM. We could not detect a colocalization of $CD44^{hi}CD62L^-$ $CD4^+$ T cells with MHC class II-expressing cells in BM (data not shown), arguing against tonic TCR stimulation of memory T cells there and the induction of homeostatic proliferation in a TCR-dependent way (Seddon et al., 2003), a finding in accordance with the low overall proliferation rate we observe here. Our data suggest that homeostatic proliferation may play little if any role in the maintenance of $CD4^+$ T cell memory, as compared to the survival signal provided by IL-7 in the BM.

The observation described here, namely that professional memory $CD4^+$ T cells in the mouse express integrin $\alpha 2$ and Ly-6C and persist in BM as resting cells, until they are reactivated by appropriate antigen-presenting cells, adds a new dimension to immunological memory. Reactive memory to vaccines and pathogens can now be evaluated as resting versus mobilized memory, the latter being the one that had been addressed by research so far. The rules governing settling and mobilization of $CD4^+$ T cell memory have to be (re)investigated, as well as the concepts of homeostatic proliferation versus IL-7-mediated survival. Furthermore, markers of true memory T helper cells, like murine integrin $\alpha 2$ and Ly-6C, may serve as molecular targets to interfere with the persistence of pathogenic T cell memory (Chatenoud, 2003; Taylor, 2003).

EXPERIMENTAL PROCEDURES

Mice

All mice were bred and maintained under SPF conditions at the "Bundesinstitut für Risikobewertung" (BfR, Berlin, Germany), and experiments were performed according to institutional guidelines and German Federal laws on animal protection. Mice were used at 12–16 weeks of age. For immunization, mice were injected i.p. with 100 μg of KLH, ovalbumin (Sigma), NP_{23}-KLH (Biosearch Technologies), $ovalbumin_{323-339}$ peptide (R. Volkmer-Engert, Humboldt University of Berlin, Berlin, Germany), or LCMV GP_{61-80} peptide (synthesized by Neosystems) with CFA (Sigma), LPS (Invivogen), or PBS. For in vivo blocking, mice were injected i.p. with 100 μg of the Fab fragment of integrin $\alpha 2$ antibody or isotype control at day 4, 5, and 6 after immunization. To discriminate between cells resident in BM versus those in blood of BM, blood was removed from BM by perfusion with PBS via the right ventricle of the heart (Bertolino et al., 2005).

Flow Cytometry and Sorting

Single-cell suspensions were prepared from spleen, mLN, pLN, PP, BM, thymus, and blood of individual mice, and liver were perfused, minced, and incubated for 30 min in culture media containing 1 mg/ml collagenase type I (Worthington) and 0.1% DNase I (Roche). Viability of cells was assessed by trypan blue exclusion. Leukocytes were counted in a defined volume of blood by using Türk's solution (Merck) and total blood volume was calculated from body weight, assuming that mice contain 72 ml blood per kg body weight (Diehl et al., 2001). The frequencies of $CD4^+$ lymphocytes among $CD45^+$ leukocytes were then determined by flow cytometry, and their absolute numbers per mouse were calculated with respect to the total blood volume of that mouse. For cell staining, cells were preincubated in a 0.1% BSA-PBS solution of 10 μg/ml anti-FcγRII/III (2.4G2) (BD PharMingen) for 10 min at 4°C. The cells were then stained for 20 min at 4°C with anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B220 (RA3.6B2), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD62L (MEL14), anti-CD19 (1D3), anti-CD28 (37.51), anti-CD3 (2C11), anti-ovalbumin-specific TCR (KJ1.26), anti-NK1.1 (PK136), anti-IL-7Rα (A7R34), anti-CD90.1 (OX-7), anti-CXCR4 (2B11), integrin α4 (R1-2), anti-Ly-6C (AL-21), anti-CD45RB (16A), anti-CXCR5 (2G8), anti-TCRβ (H57.597), anti-CD24 (M1/69), anti-CD119 (GR20), TCR Vβ Screening Panel, PerCP/APC-Cy7-streptavidin (BD PharMingen), anti-CD197 (CCR7, 4B12), anti-CD93 (AA4.1), anti-CD122 (5H4), anti-Ly108 (13G3-19D) (eBioscience), anti-integrin α2 (Sam.G4) (emfret Analytics), anti-CD186 (CXCR6, 221002) (R&D Systems), anti-CD192 (CCR2, E68) (Abcam), biotin-PNA (Vector Laboratories), Alexa Fluor 488-streptavidin (Invitrogen), and Cy5-streptavidin (Jackson Immunoresearch). To exclude dead cells, we stained 1 μg/ml propidium iodide (PI, Sigma) or 0.4 μM 4′,6-diamino-2-phenylindole (DAPI, Roche). For cell sorting, a BD FACSAria cell sorter (BD Biosciences) was used. For intracellular staining, sorted cells were stimulated with 10 μg/ml CD3 antibody in presence of Brefeldin A (5 μg/ml; Sigma) for 5 hr, or in case of analyzing kinetics of IFN-γ expression, in absence of Brefeldin A. Cells were washed, fixed with 2% formaldehyde (Sigma) in PBS for 15 min at room temperature, and stained with anti-IL-2 (S4B6), anti-TNF-α (MP6-XT22), anti-IFN-γ (XMG1.2), anti-IL-10 (JES5-16E3), anti-IL-17 (TC11-18H10), anti-IL-4 (11B11) (BD Biosciences), and anti-CD154 (MR1) (Miltenyi Biotec) as described (Löhning et al., 2003). Stained samples were analyzed in a BD LSR II flow cytometer (BD Biosciences). Flow cytometric data were analyzed with FlowJo (Tree Star, Inc.) software.

Detection of Antigen-Specific $CD4^+$ T Cells

We detected antigen-specific $CD4^+$ T cells according to CD154 expression (Frentsch et al., 2005). Cells from spleen and BM of KLH-immunized mice were stimulated in medium with 10 μg/ml KLH for 6 hr. We added Brefeldin A (5 μg/ml) for the last 4 hr of stimulation. After staining with antibodies against CD4, CD44, and B220 and fixation with 2% formaldehyde, we permeabilized the cells with 0.5% saponin (Sigma) in 0.5% BSA/PBS and stained intracellular IFN-γ and CD154.

Cell Labeling and Adoptive Transfer

For adoptive transfer, $CD44^{hi}CD62L^-$ $CD4^+$ T, naive $CD44^{lo}CD62L^+$ $CD4^+$ T, and naive B cells from C57BL/6, Thy1.1-LCMV-TCR Tg (Smarta), or ovalbumin-TCR Tg (DO11.10 or OTII) mice were sorted with MACS (Miltenyi Biotec) and FACSAria as $CD4^+CD62L^-CD25^-B220^-$, $CD4^+CD44^{lo}CD25^-B220^-$, and $CD4^-CD138^-CD19^+$ cells, respectively. For positive selection and antibody blocking, we used anti-CD4 Fab fragment, anti-CD19 Fab fragment, anti-integrin α2 Fab fragment, anti-Ly-6C Fab fragment, and streptavidin-MACS beads (Miltenyi Biotec). We transferred 1×10^5 to 5×10^6 cells i.v. into C57BL/6, Ly5.1-C57BL/6, Balb/c-scid, or RAG1-deficient mice, as indicated. To monitor transferred cells in host mice, cells were labeled before transfer with 1 μM of Carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) in PBS at room temperature for 3 min or with PKH-26 (Sigma) according to the manufacturer's manual.

Enzyme-Linked Immunosorbent Assay

Serum samples were collected after NP_{23}-KLH immunization and NP-specific antibodies were measured by using plastic surfaces coated with

NP_3- or NP_{24}-BSA (Biosearch Technologies). Hapten-specific antibodies were detected with alkaline phosphatase-conjugated antibodies to mouse IgG_1 and IgM (Southern Biotechnology).

Cell Cycle, BrdU Incorporation, Cell Division, and Apoptosis Analysis

For cell cycle analysis, 2–4 × 10^6 cells were washed twice in PBS and fixed in ice-cold 70% ethanol at 4°C overnight. After washing, cells were treated with 100 U/ml of RNase A in PBS at 37°C for 30 min and incubated in PI (50 μg/ml in PBS) for 30 min at room temperature. For in vivo proliferation, mice were injected i.p. with 1 mg of BrdU four times over 48 hr. Cells from BM, spleen, mLN, and blood were harvested 12 hr after the last injection. BrdU staining of $CD4^+$ T cells was analyzed by flow cytometry according to manufacturer's specification (BD PharMingen). For analysis of proliferation, sorted $CD44^{hi}CD62L^-$ $CD4^+$ T cells were incubated with 1 μM CFSE, then washed thoroughly with 10% FCS-RPMI and stimulated with plate-bound anti-CD3 (2C11, 10 μg/ml) at 37°C in a CO_2 incubator for 3 days before cytometric analysis. For enumeration of apoptotic cells, sorted $CD44^{hi}CD62L^-$ $CD4^+$ T cells from BM and spleen were stimulated with plate-bound anti-CD3 for 24 hr and then stained with AnnexinV (BD PharMingen) and PI according to the manufacturer's protocol.

GeneChip Hybridization of Microarrays and Data Analysis

Total RNA from $CD44^{hi}CD62L^-CD25^-B220^-$ $CD4^+$ T cells isolated from spleen or BM of C57BL/6 mice was prepared with QIAGEN RNeasy Mini kit (QIAGEN). All experiments were repeated three times with individually sorted cells purified to more than 96% homogeneity. DNA microarray analysis of gene expression was performed at the gene array facility in the DRFZ. Hybridization was done in a hybridization oven 640 (Affymetrix). Washing and staining of the MOE-430 2.0 chips were done in the Fluidics Station 400 (Affymetrix). Fluorescent images of hybridized microarrays were obtained with an Affymetrix GeneChip Scanner 3000. All relevant microarray data of the GCOS software (Affymetrix) were imported into the Bioretis database (http://www.bioretis-analysis.de/). All data are publicly available in the Bioretis and GEO databases. The total list of 1756 differentially regulated genes was obtained by combining the two lists with default parameters for increased and decreased expression. These parameters combine a threshold for gene expression, Present and Marginal detection and Decrease or Increase change calls (i.e., >50%, here ≥5 of all 9 calls in either D or I direction) or a very stringent t test p value (i.e., Bonferroni corrected; e.g., p value for this data set was ≤5.279E-08) for 9 versus 12 logged fold change values, all data obtained during chip versus chip comparison or absolute analysis with GCOS software. The unpublished exact filter parameters of high-performance chip data analysis (HPCDA) can be found on the Bioretis website (http://www.bioretis-analysis.de/, click login in the public content, choose "view single results" and then one data set, choose an existing parameter set and see each default parameter; e.g., all 3 versus 3 chips analyses with Affymetrix Latin Square data set are referred to as SGU; database description and a comparison with RMA/SAM or dChip) (Menssen et al., 2009). For hierarchical cluster analysis, we used the program Genes@Work (Califano et al., 2000) with gene vectors as normalization, Pearson correlation with mean as similarity measure, and center of mass.

Generation of Effector $CD4^+$ T Cells

Naive $CD4^+$ T cells from DO11.10 Tg mice were isolated as described previously (Löhning et al., 2003). Irradiated (30 Gy) Balb/c splenocytes were used as antigen-presenting cells at a ratio of 5:1. The $ovalbumin_{323-339}$ peptide was added at 0.5 μM. For Th1 differentiation, cells were stimulated in the presence of 5 ng/ml recombinant murine IL-12 (R&D Systems) and 5 μg/ml anti-IL-4. For Th2 differentiation, cells were stimulated in the presence of murine IL-4 (100 ng/ml, culture supernatant of HEK293T cells transfected with an expression plasmid, encoding murine IL-4), 5 μg/ml anti-IL-12 (C17.8.6), and anti-IFN-γ. Dead cells were removed by Ficoll-Hypaque separation.

Immunofluorescent Staining and Confocal Microscopy

For staining, histological samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS (Tokoyoda et al., 2004). Cryostat sections of adult femurs were stained and mounted with Fluorescent Mounting Medium (DakoCytomation). All confocal microscopy was carried out on a DM IRE2 (Leica). Monoclonal antibodies against VCAM-1 (429), B220, F4/80 (A3-1), Gr-1 (RB6-8C5), CD11c (HL3), and Thy1.1 (HIS51) (BD PharMingen) and a polyclonal antibody against IL-7 (Genzyme) were used. For secondary antibodies, Cy3 or Cy5 donkey anti-goat IgG and Cy3 donkey anti-rat IgG were purchased from Jackson Laboratories.

ACCESSION NUMBERS

All microarray data are publicly available in the Bioretis (http://www.bioretis-analysis.de/) and GEO (GEO accession number GSE15733) databases.

SUPPLEMENTAL DATA

Supplemental Data include five figures and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00187-3.

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