β1 Integrin Is Critical for the Maintenance of Antigen-Specific CD4 T Cells in the Bone Marrow but Not Long-Term Immunological Memory

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β1 Integrin Is Critical for the Maintenance of Antigen-Specific CD4 T Cells in the Bone Marrow but Not Long-Term Immunological Memory

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The long-term maintenance of memory CD4 T cells promotes protective immunity against future pathogen reinfection. As a site rich in survival cytokines, the bone marrow is proposed to be a critical niche for the survival of memory CD4 T cells. We demonstrate that endogenous, polyclonal Ag-specific CD4 T cells rapidly enter and are recovered long-term from the bone marrow following i.v. infection with Listeria monocytogenes. β1 integrin-deficient CD4 T cells also populate the bone marrow early following an infection, but their numbers in this site rapidly decline. This decline was not caused by increased death of T cells lacking β1 integrin but rather by reduced retention in the bone marrow after the primary immune response. The loss of memory CD4 T cells from the bone marrow does not lead to a loss of the predominant source of memory CD4 T cells in the spleen or the ability to mount a memory response. Thus, β1 integrin-dependent maintenance of memory CD4 T cells in the bone marrow is not required for long-term CD4 T cell memory. The Journal of Immunology, 2011, 186: 000–000.

The maintenance of Ag-specific T cell memory against previously encountered pathogens is a defining aspect of the adaptive immune system. Following Ag challenge, activated T cells distribute to lymphoid (spleen and lymph nodes) and nonlymphoid peripheral tissues in the body (1, 2). Peripheral tissues that are in direct contact with the environment, such as the lungs and skin, serve as the initial barrier against invading pathogens. T cells at these peripheral sites are predicted to enact their protective functions and provide surveillance against pathogen invasion. Accumulation of T cells in the bone marrow is also observed following infection. However, the function of these cells and the benefits they may receive from residing in this location are not completely understood.

The bone marrow is a unique site that shares overlapping properties with both primary/secondary lymphoid organs and peripheral nonlymphoid sites (3). T cells in the bone marrow are involved in promoting memory Ab responses (4, 5), hematopoietic development (6), antiviral (7), and antitumor responses (8–10). In addition, the bone marrow microenvironment has been proposed to support memory T cell maintenance (11), because bone marrow-resident memory CD8 T cells demonstrate an active phenotype and a higher basal rate of proliferation than their lymphoid counterparts (12–14). IL-15 and IL-7 present in this site are predicted to promote CD8 T cell longevity by driving homeostatic division. Thus, the bone marrow may function as a “survival niche” where CD8 T cells are maintained in reserve until needed to respond to a secondary encounter with a pathogen (3, 15).

Memory CD4 T cells are also found in the bone marrow and colocalize with IL-7–producing stromal cells that express the α4β1 integrin ligand VCAM-1 (16, 17). Because IL-7 is the main cytokine required for CD4 T cell survival (18, 19), the bone marrow is also predicted to function as a survival niche for memory CD4 T cells (3, 17). The CD4 T cells recovered from the bone marrow express low levels of CD62L and CCR7, an effector memory-like phenotype (5, 17). One group has demonstrated that these cells have a lower proliferation rate (17). Thus, enhanced survival in the bone marrow may be mediated by IL-7–induced survival signaling rather than homeostatic division. In that system, the bone marrow was demonstrated to be the predominant site of memory CD4 T cell residence. In contrast, others have demonstrated that memory CD4 T cells can localize to the bone marrow following an infection but predominantly reside in the lymph nodes and spleen (20). The specific role of the bone marrow as a recirculatory survival pit-stop for the maintenance of systemic CD4 T cell memory was not explored. In addition, after bacterial infection, the kinetics of CD4 T cell migration to the bone marrow and the adhesion molecule requirements for maintenance in this location have not been investigated.

Binding of the α4β1 integrin to VCAM-1 promotes firm arrest and the subsequent entry of T cells into the bone marrow (15, 21). A function for the α4β1 (LFA-1) integrin in bone marrow entry has also been shown, particularly when the α4β1/VCAM-1 interaction is inhibited (21–23). Another β1 integrin family member, α6β1, is induced following T cell activation and is thought to be critical for T cell retention in nonlymphoid tissues such as the lung (24–26). The binding of T cell-expressed α6β1 integrin to tissue collagen may also provide signals that promote T cell survival (27). Because VCAM-1 is expressed on stromal cells that produce IL-7 in the bone marrow (28), α4β1 may also promote CD4 T cell
survival in the bone marrow. However, the exact function that $\beta_1$ integrins play in CD4 T cell maintenance in this unique site remains poorly defined.

In this study, we find that endogenous Ag-specific CD4 T cells rapidly enter the bone marrow following i.v. bacterial infection. We show that memory CD4 T cell maintenance in the bone marrow is dependent on expression of the $\beta_1$ integrin subunit. The reduced number of CD4 T cells in the bone marrow in the absence of $\beta_1$ integrin is not related to altered survival but is primarily driven by reduced retention in this site. In our system, the bone marrow is not required for the systemic maintenance of CD4 T cell memory or for a rapid response to secondary Ag challenge.

Materials and Methods

Mice

Mice with a “floxed” $\beta_1$ integrin gene and the CD4-Cre transgene ($\beta_1^{+/--}$) were produced as described previously (29). $\beta_1$ integrin-deficient mice (The Jackson Laboratory, Bar Harbor, ME) were bred with $\beta_1^{+/--}$ mice to make $\beta_1^{+/--}$ (wild-type (wt)) controls. All mice were on the C57BL/6 background. All experimental protocols involving the use of mice were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Listeria infections, peptide:MHC class II tetramer enrichments, and cell staining

Infection with Listeria monocytogenes and peptide:MHC class II (MHCII) tetramer enrichment were performed as described previously (29). Mice were infected i.v. with $1 \times 10^6$ CFU 2WS-peptide expressing ActA-deficient L. monocytogenes (A Lm-2WS). Single-cell suspensions were made from spleens and bilateral hind limb (femur and tibia) bone marrow. The enriched 2WS-specific population was stained with the previously made tetramer enrichment were performed as described previously (29). Mice were infected i.v. with $1 \times 10^3$ CFU 2WS-peptide expressing ActA-deficient L. monocytogenes (A Lm-2WS). Single-cell suspensions were made from spleens and bilateral hind limb (femur and tibia) bone marrow. The enriched 2WS-specific population was stained with the previously reported mixture of Abs to identify the 2WS1-specific CD4 T cells. Abs were produced as described previously (29).

FLICA-stained samples was performed at 4°C, which decreased back-ground staining. FLICA solution was added with standard Ab mixture for 1 h at 37°C. Samples were then washed twice with FLICA wash buffer and resuspended in 200 μl 0.1 μg/ml DAPI in FLICA staining buffer for 20 min at room temperature. DAPI+ cells were excluded from the analysis.

Fluorochrome inhibitor of caspases staining for active caspases 3 and 7

FAM-fluorochrome inhibitor of caspases (FLICA) reagent was used per manufacturer’s instructions for visualization of active caspases 3 and 7 (Immunochemistry Technologies, Bloomington, MN). Tetramer staining for FLICA-stained samples was performed at 4°C, which decreased back-ground staining. FLICA solution was added with standard Ab mixture for 1 h at 37°C. Samples were then washed twice with FLICA wash buffer and resuspended in 200 μl 0.1 μg/ml DAPI in FLICA staining buffer for 20 min at room temperature. DAPI+ cells were excluded from the analysis.

Short-term in vivo coholing assay

Spleen cells from mice infected 5 or 20 days previously with A Lm-2WS were enriched for CD4 T cells by negative selection using MACS LS columns (Miltenyi Biotec, Auburn, CA). Cells were then labeled with 0.25 mM Cell Tracker Green (CTG) or 2 mM Cell Tracker Orange (CTO) and mixed 1:1 prior to i.v. injection into host mice (5–10 $\times 10^6$ cells/mouse). Host mice were uninfected, day 5 or 20 postinfection with A Lm-2WS. A sample of the input mixture was taken to determine the number of injected cells. Spleens and bone marrow were harvested at 2 and 18 h postcell transfer. Day 5 transfers were processed as indicated above for peptide:MHCII-tetramer enrichment, and day 20 transfers were processed similarly but tetramer staining was not performed.

Statistical analysis

GraphPad Prism software (GraphPad, La Jolla, CA) was used for all statistical analysis.

Results

CD4 T cells rapidly enter the bone marrow following i.v. infection but are not maintained in the absence of $\beta_1$ integrin

We used a recently described peptide:MHCII tetramer approach (30) to track the localization of an endogenous, polyclonal Ag-specific CD4 T cell population in wt mice following an i.v. infection with Act-A-deficient Listeria monocytogenes expressing the 2WS1 peptide variant of peptide 52–68 from the I-E $\alpha$-chain (A Lm-2WS1) (20, 29). This attenuated pathogen is rapidly cleared, thus allowing us to track the endogenous CD4 T cell response in the absence of chronic infection.

In uninfected mice, several hundred 2WS1-specific CD44low CD4 T cells are present in the spleen, but 2WS1-specific CD44low CD4 T cells were rarely recovered from bone marrow (Fig. 1A). This is in agreement with previous studies showing the preferential colonization of the bone marrow by a CD44high T cell population (5, 20). By day 2.5 postinfection, a population of 2WS1-specific CD44high CD4 T cells was observed in the spleen but was essentially absent in the bone marrow. By day 3.5, we observed an expanded population of 2WS1-specific CD44high CD4 T cells in the spleen and also begin to recover these cells from the bone marrow. These findings are consistent with the activation and proliferation of naive CD4 T cells in the spleen and their subsequent migration into the bone marrow. By the peak of the response (day 5), 2WS1-specific CD4 T cells have massively expanded in the spleen, and we recovered a large population from the bone marrow (Fig. 1A). Whole-body mouse perfusion via the left ventricle did not alter the number of 2WS1-specific CD4 T cells recovered from the bone marrow (data not shown). Thus, endogenous Ag-specific CD4 T cells rapidly enter the bone marrow following i.v. bacterial infection.

Migration into the bone marrow at steady state is largely dependent on the interaction between T cell expressed $\alpha_4\beta_7$ integrin and its vascular ligand VCAM-1 (15, 21). At day 5, 2WS1-specific CD44high CD4 T cells recovered from the bone marrow were enriched for those expressing the highest levels of $\beta_1$ integrin compared with the 2WS1-specific population in the spleen (Fig. 1B). To determine whether the entry of these cells is dependent on $\beta_1$ integrin, we tracked the 2WS1-specific response in mice lacking $\beta_1$ on their T cells (29). We have previously reported similar numbers of 2WS1-specific CD4 T cells from the spleens of wt and $\beta_1^{+/--}$ mice at all sampled time points following A Lm-2WS infection (29). At day 5 following infection, CD4 T cells were found in the bone marrow of $\beta_1^{+/--}$ mice, and as expected, these cells lacked expression of $\beta_1$ integrin (Fig. 1B). Thus, the CD4 T cells entering the bone marrow are not enriched for those that may have escaped $\beta_1$ integrin deletion as has been previously reported in another system (31). The majority of 2WS1-specific CD4 T cells in the spleen at day 5 are CCR7low in both wt and $\beta_1^{+/--}$ mice compared with naive CD4 T cells (Fig. 1C). CD4 T cells recovered from the bone marrow are even further enriched for the CCR7low phenotype.

Unexpectedly, in the absence of $\beta_1$ integrin, similar numbers of 2WS1-specific CD4 T cells were recovered from the bone marrow at day 5 (Fig. 2). The ability of $\beta_1^{+/--}$ CD4 T cells to enter the bone marrow is not related to their enhanced expression of $\alpha_4\beta_7$ (29), as CD4 T cells lacking both $\beta_1$ and $\beta_2$ integrins ($\beta_1^{+/--}$) also have similar CD4 T cell numbers in the bone marrow at day 5 (Fig. 2). Thus, the entry of recently activated CD4 T cells into the bone marrow can occur in a $\beta_1$ integrin and a $\beta_2$ integrin-independent manner.

Interestingly, $\beta_1^{+/--}$ T cells were lost from the bone marrow during the contraction phase (days 5–20) of the immune response.
and are present in statistically lower numbers in the bone marrow than wt cells out to day 40 postinfection (Fig. 2). Because β1b7 integrin expression on CD44high CD4 T cells is lost from the bone marrow, enhanced homing to the gut of β1−/− cells (29) is unlikely to account for the T cell loss from the bone marrow. In summary, following a bacterial infection, the absence of β1 integrin does not alter the early accumulation of CD4 T cells in the bone marrow but alters their steady-state maintenance at this site.

Cell death does not account for the loss of CD4 T cells from the bone marrow after the peak of the response

Integrins may provide prosurvival signals to memory T cells in nonlymphoid tissue (24, 27) and facilitate T cell localization to IL-7–producing, VCAM-1+ stromal cells in the bone marrow (28). To determine whether the loss of CD4 T cells from the bone marrow of β1−/− mice could be accounted for by increased rates of apoptosis, we assessed activation of the effector caspases 3 and 7 in 2W1S-specific CD4 T cells during the contraction phase. These caspases are downstream of both the intrinsic and extrinsic apoptotic pathways in T cells (32). At day 12, the total 2W1S-specific CD4 T cell numbers in the spleen were identical between wt and β1−/− mice, whereas the bone marrow of β1−/− mice contained significantly less CD4 T cells (Fig. 3A). To assess the percentage of cells undergoing apoptosis, we stained 2W1S-specific CD4 T cells using a fluorescent reagent that specifically binds and inhibits active caspases 3 and 7 (33). As expected, naive 2W1S-specific CD4 T cells from the spleen of uninfected mice have little staining for active caspases 3 and 7 (Fig. 3B). Robust activation of caspases 3 and 7 was observed in ~25% of wt and β1−/− 2W1S-specific CD4 T cells in the spleen at day 12 postinfection (Fig. 3B). Importantly, the percentage of 2W1S-specific CD4 T cells in the bone marrow of β1−/− mice undergoing apoptosis was similar to that of wt mice (Fig. 3B). The percentage of DAPI+ cells was also equivalent between wt and β1−/− mice (data not shown),

![FIGURE 1. CD4 T cells rapidly enter the bone marrow following i.v bacterial infection. A, Tracking the 2W1S-specific population following i.v. infection with A− Lm-2W1S in wt mice. Numbers indicate calculated cell number from gate (day 5 are averages, n = 9). B, Spleen and bone marrow histograms of β1 integrin expression on 2W1S-specific CD44high CD4 T cells day 5 postinfection from wt and β1−/− mice. C, Spleen and bone marrow histograms of CCR7 expression on 2W1S-specific CD44high CD4 T cells day 5 postinfection from wt and β1−/− mice. Gray histogram represents naive CD44low CD4 T cells from the spleen. Numbers indicate percent of cells in the drawn gate (n = 3, mean ± SD).](http://www.jimmunol.org/)

![FIGURE 2. Maintenance, but not entry, of CD4 T cells in the bone marrow is dependent on β1 integrin. 2W1S-specific CD44high CD4 T cells recovered from the bone marrow of wt, β1−/−, and β1b7−/− mice following A− Lm-2W1S infection. Data are from 10 experiments (n = 3–11, mean ± SEM). ns, p > 0.05, *p < 0.05, **p = 0.009 compared with wt, two-tailed unpaired t test.](http://www.jimmunol.org/)

![FIGURE 3. Loss of CD4 T cells from the bone marrow is not related to increased cell death. A, Number of 2W1S-specific CD44high CD4 T cells recovered from the spleen and bone marrow 12 d postinfection with A− Lm-2W1S in wt and β1−/− mice (bars represent mean). *p < 0.05, two-tailed unpaired t test. B, Staining for active caspases 3 and 7 (FLICA 3 and 7) in 2W1S-specific CD44high CD4 T cells from the spleen of uninfected mice or 2W1S-specific CD44high CD4 T cells from the spleen and bone marrow 12 d postinfection. Bottom graphs show replicates (bars represent mean). ns, p > 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, followed by Tukey’s multiple comparison test.](http://www.jimmunol.org/)
indicating our analysis is not missing cells that have already undergone apoptosis.

Interestingly, the percentage of 2W1S-specific CD4 T cells in the bone marrow undergoing apoptosis was significantly less than the apoptotic percentage of 2W1S-specific CD4 T cells from the spleen in both wt and β1−/− mice (Fig. 3B). Overall, the loss of β1−/− CD4 T cells from the bone marrow between days 5 and 20 is not accounted for by increased cell death.

β1 integrin is not required for bone marrow localization at day 5 but is required at day 20

T cells can emigrate out of the bone marrow and enter into other lymphoid organs and bones (34, 35). In addition, connecting the blood circulation (parabiosis) of an immune mouse and an uninfected mouse rapidly results in the equilibration of bone marrow memory T cells between mice (36). These findings predict that memory T cells continually enter and exit the bone marrow rather than take up long-term residence, as is seen in the intestinal epithelium (36, 37). Thus, loss of CD4 T cells from the bone marrow of β1−/− mice may relate to an altered ability of cells lacking β1 integrin to enter into and/or be retained in the bone marrow after the peak of the immune response.

To determine the entry and retention properties of recently activated (day 5) versus early memory T cells (day 20), we isolated 2W1S-specific wt and β1−/− CD4 T cells from infected mice and performed cotransfers into various hosts. First, we transferred recently activated (day 5) wt and β1−/− 2W1S-specific CD4 T cells into recipient mice infected 5 d earlier with A. Lm-2W1S. Infected hosts were used to replicate the inflammatory milieu present during infection and also to provide an Ag source. The bone marrow was harvested at 2 and 18 h post-transfer as relative measures of entry and retention, respectively (15). At 2 and 18 h post-transfer, the percentage of wt and β1−/− 2W1S-specific CD4 T cells localizing to the bone marrow of day 5 hosts was similar (Fig. 4A). As a control, we demonstrate that an equivalent percentage of transferred wt and β1−/− 2W1S-specific CD4 T cells are observed in the spleen of these same mice at 18 h post-transfer (Fig. 4B). These results reproduce the findings from infections of intact mice, where similar numbers of wt and β1−/− 2W1S-specific CD4 T cells are recovered in the spleen and bone marrow at day 5 postinfection (Fig. 2) (29). To separate T cell intrinsic homing capabilities from environmental alterations that may occur during an acute infection, we also cotransferred cells into uninfected hosts. Interestingly, an equivalent percentage of wt and β1−/− 2W1S-specific CD4 T cells were recovered at 2 and 18 h (Fig. 4A). Overall, at day 5 postinfection, wt and β1−/− CD4 T cells have similar entry into and retention in the bone marrow with or without the presence of infection.

By day 20 postinfection, the population of 2W1S-specific CD4 T cells in the spleen of wt and β1−/− mice has contracted to ~20,000 cells (29). At this time point, we observe a significantly decreased number of CD4 T cells in the bone marrow of β1−/− mice (Fig. 2). Because of the small number of 2W1S-specific CD4 T cells at day 20, we were unable to obtain enough Ag-specific early memory CD4 T cells from donor mice to exclusively track the migration of the 2W1S-specific population to the bone marrow following transfer. Thus, we monitored the localization of the bulk population of CD44high CD4 T cells (including 2W1S-specific cells) at day 20 postinfection. At 2 h post-transfer, there was a modest reduction in the percentage of day 20 postinfection β1−/− CD4 T cells recovered from the bone marrow of day 20 hosts that was not statistically significant (Fig. 4C). Similar findings were observed when day 20 cells were transferred into day 5 postinfection hosts (Fig. 4C). These results reveal little to no reduction in the entry of day 20 β1−/− CD4 T cells into the bone marrow.

By 18 h post-transfer, there is a notable reduction in the percentage of β1−/− CD4 T cells recovered from the bone marrow (Fig. 4C). To determine whether the reduced presence of β1−/− CD4 T cells in the bone marrow was related to changes in the bone marrow versus T cell intrinsic changes, we transferred day 20 cells into mice infected 5 d prior. These experiments also demonstrate a reduced percentage of β1−/− CD4 T cells localized to the bone marrow by 18 h post-transfer (Fig. 4C). Of note, equivalent percentages of wt and β1−/− CD44high CD4 T cells were recovered from the spleen after cotransfer into day 20 hosts, even when the number of β1−/− CD44high CD4 T cells in the bone marrow was significantly reduced (Fig. 4D). These findings replicate the loss of β1−/− 2W1S-specific CD4 T cells observed in the bone marrow 20 d following infection (Fig. 2). Overall, our results suggest that the loss of CD4 T cells from the bone marrow of β1−/− mice after an acute infection is due primarily to reduced retention of β1−/− CD4 T cells during the memory phase.

Altered homing molecule expression on CD4 T cells early and late postinfection

T cell activation quickly results in altered cell surface expression of multiple costimulatory and homing receptors (38). The interaction of appropriately glycosylated, functional PSGL-1 with P- and E-selectin results in the initial slowing and rolling of T cells on the vessel wall, which is mediated primarily by α4β1/VCAM-1 at a steady state. α4β1 (LFA-1) integrin binding to vascular ICAM-1 can also play a secondary role for entry into the bone

![figure](http://www.jimmunol.org/)

**Figure 4.** Loss of CD4 T cells from the bone marrow is due to reduced retention. A, Bar graphs showing the percentage of transferred 2W1S-specific CD44high CD4 T cells recovered from the bone marrow of host mice at 2 and 18 h post-transfer. Percentage of input was calculated by dividing recovered cell number by transferred cell number (mean ± SEM, n = 4–6). B, Representative dot plots of spleen and bone marrow of mice infected 5 d prior to cotransfer of day 5 postinfection wt and β1−/− 2W1S-specific CD44high CD4 T cells. Transferred cells are distinguished from the host population by labeling with CTO (wt) and CTO (β1−/−). C, Bar graphs showing the percentage of transferred CD44high CD4 T cells recovered from the bone marrow of host mice at 2 and 18 h post-transfer (n = 4–5, mean ± SEM). *p = 0.02, **p = 0.008, two-tailed unpaired t test. D, Representative dot plots of spleen and bone marrow of host mice infected 20 d prior to cotransfer of day 20 postinfection wt and β1−/− CD44high CD4 T cells. Transferred cells are distinguished from the host population by labeling with CTO (wt) and CTO (β1−/−). The 2W1S-specific host population (negative for CTO and CTG) has been excluded from all dot plots.
marrow when $\alpha_4\beta_1$ or VCAM-1 function has been compromised (21–23). We show that $\beta_1$ integrin expression is upregulated following activation, and this is maintained on the majority of wt 2W1S-specific CD4 T cells (Fig. 1B) (29). At day 5, the responding wt and $\beta_1^{-/-}$ CD4 T cells in the spleen have also all upregulated expression of LFA-1 compared with naive CD4 T cells (Fig. 5A). All of the CD4 T cells recovered from the bone marrow of wt and $\beta_1^{-/-}$ mice also have similar high levels of LFA-1. On CD4 T cells in the spleen, LFA-1 expression was significantly decreased by day 20 (Fig. 5A, 5C). However, CD4 T cells recovered from the bone marrow at day 20 remain enriched for the highest LFA-1 expression in both wt and $\beta_1^{-/-}$ mice (Fig. 5A, 5C). Early after an infection, $\sim55\%$ of the cells in the spleen are also high for functional PSGL-1 (Fig. 5B, 5D). The population of T cells recovered from the bone marrow of wt and $\beta_1^{-/-}$ mice are enriched for high expression of functional PSGL-1 (Fig. 5B). By day 20, only $\sim30\%$ of the cells remaining in the spleen display this phenotype, yet the majority of cells recovered from the bone marrow remain high for functional PSGL-1 (Fig. 5B, 5D). Thus, early after an infection, $\beta_1$ integrin, LFA-1, and functional PSGL-1 are all seen on a large population of activated cells in the spleen, and the cells recovered from the bone marrow are enriched for this population. By day 20, cells from the spleen express lower amounts of both LFA-1 and functional PSGL-1 and in the absence of $\beta_1$ integrin, these same cells have reduced localization to the bone marrow. Taken together, these findings suggest a compensatory redundancy in homing molecule expression early following an infection but a reduced level of redundancy by day 20.

Memory CD4 T cell retention in the bone marrow is not required for global memory survival or rapid memory response

As the bone marrow is considered a survival niche for memory CD4 T cells (17), we hypothesized that decreased localization to the bone marrow during the memory phase would result in a systemic loss of CD4 T cell memory. Surprisingly, this general loss of 2W1S-specific CD44$^{high}$ CD4 T cells was not observed in our system, because cell numbers in the spleen are equivalent in wt and $\beta_1^{-/-}$ mice out to day 325 (Fig. 6A). In addition, the number of 2W1S-specific CD4 T cells recovered from the spleen was greater at all time points than those recovered from the bone marrow (Fig. 6A, 6B). At the latest time point collected, no 2W1S-specific CD4 T cells could be recovered from the bone marrow (Fig. 6A, 6B). Of note, in one wt mouse, no 2W1S-specific CD4 T cells were recovered from the bone marrow, but the spleen contained normal numbers of memory cells. Thus, even in the absence of memory CD4 T cells in the bone marrow, memory CD4 T cells are maintained in the spleen.

To test the ability of day 325 memory mice to respond to Ag rechallenge, we immunized them with 2W1S-peptide and LPS

**FIGURE 5.** Expression of LFA-1 and functional PSGL-1 is decreased between day 5 and 20 post-infection. 2W1S-specific CD44$^{high}$ CD4 T cells from the spleen and bone marrow of wt and $\beta_1^{-/-}$ mice following i.v. infection with A$Lm$-2W1S stained for LFA-1 (A) or functional PSGL-1 (B). Dark gray histograms represent staining of CD44$^{low}$ CD4 T cells (naive) from the spleen. Numbers indicate percent of cells in the drawn gate ($n=3$–4, mean $\pm$ SD). C, Median fluorescence intensity (MFI) of LFA-1 staining on PSGL-1$^{hi}$ 2W1S-specific CD44$^{high}$ CD4 T cells from the spleen and bone marrow of wt and $\beta_1^{-/-}$ mice following i.v. infection with A$Lm$-2W1S. D, Percentage of LFA-1$^{+}$PSGL-1$^{hi}$ 2W1S-specific CD44$^{high}$ CD4 T cells from the spleen and bone marrow of wt and $\beta_1^{-/-}$ mice following i.v. infection with A$Lm$-2W1S. Bar represents the mean. *$p<0.02$, **$p<0.0002$, two-tailed unpaired $t$ test.
T cells lacking of endogenous, polyclonal Ag-specific CD4 T cells in the bone marrow. In this study, we use a peptide:MHCII tetramer enrichment (15, 17). However, it is unknown whether the entry of memory CD4 T cells into the bone marrow is required for the long-term maintenance of CD4 T cell memory.

Retention of CD4 T cells in the bone marrow is not required for survival or response of memory CD4 T cells. 2W1S-specific CD44\textsuperscript{high} CD4 T cells recovered from the spleen (A) and bone marrow (B) of wt and $\beta_1^{-/-}$ mice following i.v. infection with A. Lm-2W1S. At day 325 postinfection, mice were rechallenged with 2W1S-LPS. Spleen and bone marrow were harvested 3 d following rechallenge ($n = 5$, mean $\pm$ SEM). The primary (1\textsuperscript{st}) response of naive (uninfected) mice to 2W1S-LPS was also characterized 3 d following challenge. Horizontal dotted lines on graphs indicate the average number of 2W1S-specific CD4\textsuperscript{44low} CD4 T cells in naive mice. ns, $p > 0.05$, two-tailed unpaired $t$ test.

(2W1S-LPS). The secondary proliferative response in the spleen at day 3 postimmunization was 150 times larger than the primary response from naive mice challenged with 2W1S-LPS (Fig. 6A). This indicates that the observed 2W1S-specific CD4 T cell proliferation is a memory recall response. Furthermore, there were comparable numbers of 2W1S-specific CD4 T cells in the spleen following secondary challenge of wt and $\beta_1^{-/-}$ mice (Fig. 6A). Interestingly, 3 d postimmunization, 2W1S-specific CD4 T cells in the bone marrow equilibrate between wt and $\beta_1^{-/-}$ mice (Fig. 6A). As observed in the primary response, this is consistent with the activation of 2W1S-specific CD4 T cells in the spleen and subsequent migration of these cells into the bone marrow. As seen in the spleen, there was a much larger number of CD4 T cells recovered from the bone marrow following a memory response than primary response (Fig. 6B). These results indicate that the bone marrow is not required to maintain long-term, Ag-responsive CD4 T cell memory following i.v. bacterial infection.

Discussion

The long-term survival of Ag-specific memory CD4 T cells provides enhanced protection against previously encountered pathogens (40). As a site rich in survival cytokines, the bone marrow is predicted to be a critical survival niche for memory T cells (3, 11, 15, 17). However, it is unknown whether the entry of memory CD4 T cells into the bone marrow is required for their long-term survival. In this study, we use a peptide:MHCII tetramer enrichment technique to examine the role of $\beta_1$ integrin in the maintenance of endogenous, polyclonal Ag-specific CD4 T cells in the bone marrow following bacterial infection. We demonstrate that CD4 T cells lacking $\beta_1$ integrin enter the bone marrow early following an infection but are not maintained there long-term. This lack of Ag-specific CD4 T cell maintenance in the bone marrow does not result in a systemic loss of CD4 T cell memory or a reduced ability to mount a secondary response. These findings support the idea that the localization of CD4 T cells to the bone marrow is not required for the long-term maintenance of CD4 T cell memory.

We observed comparable numbers of wt and $\beta_1^{-/-}$ Ag-specific CD4 T cells in the bone marrow at early time points after a primary Listeria infection. Cohoming assays of day 5 postinfection cells into day 5 postinfection hosts confirmed that localization of recently activated CD4 T cells to the bone marrow was independent of $\beta_1$ integrin expression. Similar findings were also obtained when day 5 cells were transferred into uninfected hosts. This revealed that potential infection-induced alterations in the bone marrow microenvironment or the presence of Ag in the bone marrow is not required for $\beta_1$ integrin-independent localization of recently activated CD4 T cells to the bone marrow. Our results are consistent with the previously reported promiscuous distribution of recently activated CD4 T cells to normal and inflamed tissue without the presence of Ag (1). Thus, the initial entry of $\beta_1^{-/-}$ T cells into the bone marrow is mediated by cell intrinsic factors rather than systemic changes that occur during acute infection.

Our observation that $\beta_1$ integrin expression is not required for the entry of recently activated CD4 T cells into the bone marrow is surprising, as previous studies have reported that the binding of the $\alpha_b\beta_1$ integrin to VCAM-1 promotes firm arrest and subsequent entry of T cells into the bone marrow (21). However, T cell migration into the bone marrow also involves the function of other adhesion molecules, such as PSGL-1, which promotes T cell rolling (15, 21). In addition, LFA-1 integrin plays a minor role in bone marrow entry that becomes more prominent when the $\alpha_b\beta_1$ VCAM-1 interaction is compromised (21–23). Of note, the vast majority of systemic Ag-specific CD4 T cells responding to Listeria infection express high amounts of LFA-1 and functional PSGL-1. Furthermore, the CD4 T cells recovered from the bone marrow are enriched for this population. Thus, the high-level expression of functional PSGL-1 and LFA-1 on Ag-specific CD4 T cells early postinfection may allow for efficient bone marrow entry in the absence of $\beta_1$ integrin expression. Alternatively, the enhanced expression of $\alpha_b\beta_1$ on $\beta_1^{-/-}$ CD4 T cells might compensate for the absence of $\beta_1$ integrin (29). $\alpha_b\beta_1$ integrin-mediated compensation could occur either through direct binding to VCAM-1 (41) or binding to the $\alpha_b\beta_1$ ligand MadCAM-1, which can be unregulated on the bone marrow vasculature during inflammation (42). Our data argue against either of these possibilities, because $\beta_1^{-/-}$ CD4 T cells additionally lacking $\beta_1$ integrin ($\beta_1\beta_7^{-/-}$) enter the bone marrow in similar numbers as wt cells at day 5.

Previous work has demonstrated that T cells emigrate from and recirculate back to the bone marrow (34, 35). Parabiosis studies also predict that T cells recirculate through the bone marrow (36) rather than take up long-term residence, as can be seen in the intestine (37). Steady-state maintenance of CD4 T cells in the bone marrow is thus highly related to the rates of cell entry and exit. In our system, by day 12 postinfection, the number of 2W1S-specific CD4 T cells in the bone marrow of $\beta_1^{-/-}$ mice is ~60% reduced compared with wt controls. This decreased number of Ag-specific CD4 T cells in the bone marrow of $\beta_1^{-/-}$ mice continues to decline and becomes ~90% reduced compared with wt by day 120. In an attempt to determine the relative contributions of entry and retention to the gradual loss of $\beta_1^{-/-}$ CD4 T cells from the bone marrow, we cotransferred wt and $\beta_1^{-/-}$ CD4 T cells 20 d following an infection into day 20 hosts. There was a modest reduction in the localization of $\beta_1^{-/-}$ CD4 T cells in the bone marrow at 2 h post-transfer, but this finding did not reach statistical significance ($p > 0.05$). Although these cells
express lower levels of LFA-1 and functional PSGL-1 than at day 5 postinfection, high-level expression of these molecules is still observed and may provide some functional redundancy. These findings predict that 20 d after an infection, CD4 T cells lacking $\beta_1^{-/-}$ would only be minimally impaired in their ability to enter into the bone marrow. However, by 18 h post-transfer, there is a significant reduction in the maintenance of $\beta_1^{-/-}$ CD4 T cells in the bone marrow. This is also observed when day 20 cells are transferred into day 5 hosts. Thus, this effect is likely cell intrinsic because it is not overcome by potential alterations in the bone marrow vasculature or stroma that may occur with an acute infection. Taken together, our results suggest that the rapid decline of $\beta_1^{-/-}$ CD4 T cells in the bone marrow following bacterial infection is due predominantly to impaired retention. However, as transferred T cells are continuously entering and exiting the bone marrow, the modest reduction in the entry of $\beta_1^{-/-}$ CD4 T cells observed at 2 h may, over time, also contribute to the loss of T cell accumulation in the bone marrow.

In addition to a reduction in retention of cells, another possible mechanism for the reduced maintenance of $\beta_1^{-/-}$ CD4 T cells in the bone marrow is increased cell death. Signals from $\beta_1$ integrins have been proposed to promote the survival of memory T cells in nonlymphoid tissue (24, 26, 27). In addition, $\beta_1$ integrin may be important for the localization of CD4 T cells to VCAM-1+ stromal cells producing IL-7 in the bone marrow (16, 17). Staining for effector caspases 3 and 7 revealed no differences between wt and $\beta_1^{-/-}$ CD4 T cells in either the bone marrow or the spleen. In both wt and $\beta_1^{-/-}$ mice, 2W1S-specific CD4 T cells did demonstrate significantly reduced caspase activity in bone marrow compared with the spleen. This reduced caspase activity is consistent with the idea of the bone marrow as a T cell survival niche. As both wt and $\beta_1^{-/-}$ CD4 T cells demonstrate higher viability in the bone marrow, this suggests that access to survival factors in the bone marrow is independent of $\beta_1$ integrin expression. This is a surprising result, because the IL-7–producing stromal cells express the $\beta_1$ integrin ligand VCAM-1 (28). Another possibility is that the most viable population of 2W1S-specific CD4 T cells from the spleen preferentially migrates to the bone marrow. Our experimental results do not allow us to distinguish between these two possibilities. Overall, our results suggest that cell death is not a major factor reducing the abundance of $\beta_1^{-/-}$ CD4 T cells in the bone marrow.

In many systems, $\beta_1$ integrins function as T cell retention molecules in peripheral tissue (38). Maintenance of memory T cells in peripheral tissue is predicted to provide rapid protection against pathogen reinfection. The collagen binding integrin $\alpha_1\beta_1$ is critical for T cell retention in the lung following influenza infection (24, 25). In this system, lung resident $\alpha_1\beta_1^{+\ast}$ effector memory CD4 cells are the major IFN-γ–secreting, rapid responders during a secondary infection (26). In humans, the collagen-binding integrin $\alpha_1\beta_1$ is a marker of similar Th1 phenotype CCR7low effector-memory CD4 T cells (43). In addition, skin-resident effector memory CD4 T cells specifically express $\alpha_1\beta_1$, which is involved in their retention in the epidermis (44). In our system, the majority of the CCR7low effector-memory like CD4 T cells are maintained in the spleen and lymphoid organs (20). The CD4 T cells we recover from the bone marrow are enriched for this CCR7low effector-memory-like population compared with the spleen. This CCR7low effector-memory-like population expresses high levels of both $\beta_1$ and LFA-1, consistent with the characterization of effector-memory T cells in humans (45). By day 20, CD4 T cells expressing a CCR7low effector memory-like phenotype remain the predominant population in the bone marrow (data not shown), suggesting that this population is particularly efficient at localizing to this site. This also corresponds to the population of CD4 T cells reported to localize to the bone marrow in other systems (5, 17). Bone marrow-resident CD4 T cells express high levels of the collagen binding integrin $\alpha_2\beta_1$ (17), but a role for $\alpha_2\beta_1$ as a retention molecule in the bone marrow has not been excluded.

For naive CD4 T cell homeostasis, stromal components of the lymph nodes and spleen are thought to be the major source of the survival cytokine IL-7 (46). In contrast, the bone marrow has recently been proposed to be the major site for memory CD4 T cell maintenance and IL-7 survival signaling (17). The bone marrow has unique properties that situate it somewhere between peripheral tissue and lymphoid tissue (3). Unlike peripheral tissue, where the presence of Ag is thought to drive retention (47), localization to the bone marrow can occur with or without local Ag (3, 16). Thus, local immunosurveillance may not completely explain the presence of CD4 T cells in the bone marrow, and its function as a survival reservoir has been gaining support (3, 15). If the bone marrow is the major source of IL-7 for memory CD4 T cells, we expected to observe a global loss of CD4 T cell memory over time in the $\beta_1^{-/-}$ mice. Surprisingly, the decreased ability of $\beta_1^{-/-}$ CD4 T cells to be maintained in the bone marrow does not result in a systemic decrease in CD4 T cell memory or loss of a rapid proliferative response to Ag rechallenge. At nearly 1 yr postinfection, when we are unable to detect $\beta_1^{-/-}$ memory CD4 T cells in the bone marrow using our peptide:MHCII tetramer enrichment technique, the secondary response in the spleen and bone marrow is comparable to wt mice. In our system, the spleen remains the predominant site of memory CD4 T cell localization, although the total percentage of CD4 T cells maintained in the bone marrow compared with the spleen in wt mice does increase over time. These results are consistent with other recently published work using this system (20). The ability to maintain long-term CD4 T cell memory in the presence of decreased bone marrow localization is seemingly in contrast to other work using adoptive transfer systems (17). The reasons for this discrepancy are unclear but may relate to the analysis of polyclonal versus TCR transgenic T cells or the route/method of Ag challenge.

In summary, we have demonstrated an important function for $\beta_1$ integrin in the long-term maintenance of memory CD4 T cells in the bone marrow. Our findings reveal that $\beta_1$ integrin expression is critical for steady-state maintenance of Ag-specific CD4 T cells in the bone marrow during the memory phase. Although we demonstrate significantly reduced numbers of CD4 T cells maintained in the bone marrow of $\beta_1^{-/-}$ mice during the memory phase, this does not result in a global decrease in CD4 T cell memory survival or proliferative response upon rechallenge. Our results suggest that survival signals produced by sources other than the bone marrow, such as the stromal compartment of the spleen and lymph nodes (46), may be sufficient for long-term memory CD4 T cell survival.

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Disclosures

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References


