B Cells Negatively Regulate the Establishment of CD49b<sup>+</sup>T-bet<sup>+</sup> Resting Memory T Helper Cells in the Bone Marrow

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During an immune reaction, some antigen-experienced CD4 T cells relocate from secondary lymphoid organs (SLOs) to the bone marrow (BM) in a CD49b-dependent manner and reside and rest there as professional memory CD4 T cells. However, it remains unclear how the precursors of BM memory CD4 T cells are generated in the SLOs. While several studies have so far shown that B cell depletion reduces the persistence of memory CD4 T cells in the spleen, we here show that B cell depletion enhances the establishment of memory CD4 T cells in the BM and that B cell transfer conversely suppresses it. Interestingly, the number of antigen-experienced CD4 T cells in the BM synchronizes the number of CD49b<sup>+</sup>T-bet<sup>+</sup> antigen-experienced CD4 T cells in the spleen. CD49b<sup>+</sup>T-bet<sup>+</sup> antigen-experienced CD4 T cells preferentially localize in the red pulp area of the spleen and the BM in a T-bet-independent manner. We suggest that B cells negatively control the generation of CD49b<sup>+</sup>T-bet<sup>+</sup> precursors of resting memory CD4 T cells in the spleen and may play a role in bifurcation of activated effector and resting memory CD4 T cell lineages.

Keywords: resting memory, CD4 T helper cells, B cells, bone marrow, T-bet

INTRODUCTION

One of the greatest characteristics of the immune system is to memorize previously encountered antigens and mount rapid recall responses. It is well established that CD4 T cells play an essential role in regulating the generation of high-affinity memory B cells and long-lived plasma cells (1–3) as well as the maintenance and expansion of memory CD8 T cells during secondary immune responses (4–7). However, the potential role and whereabouts of memory CD4 T cells were debated in the

Abbreviations: BM, bone marrow; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; PALS, periarterial lymphatic sheaths; SLO, secondary lymphoid organ; Tfh, T follicular helper.
field for a long time. Memory CD4 T cells are generated from antigen-experienced CD4 T cells during the down-sizing of an immune reaction and are maintained in the absence of antigen by survival signals and homeostatic proliferation (8–11). While the bone marrow (BM) has been known to host long-lived plasma cells (12, 13), memory CD4 T cells were thought to constantly circulate throughout the body. Recently, we have uncovered that murine BM is a home of resting memory CD4 T cells (14). Some activated CD4 T cells migrate into the BM in a CD69- and CD49b (integrin α2)-dependent manner and reside and rest as memory CD4 T cells in the survival niches composed of IL-7* collagen-XI* stromal cells (14–17). Interestingly, the repertoire of memory CD4 T cells in human BM is also significantly enriched for systemic pathogens compared to blood memory T cells (18, 19). While the vast majority of activated CD4 T cells undergo apoptosis after clearance of the antigen, it is still poorly understood which subpopulation in antigen-specific activated CD4 T cells has the potential to develop memory Th cells in the BM.

B cells are considered as potent antigen-presenting cells nearly as effective as dendritic cells (DCs) (20). While DCs play an essential role for priming naive CD4 T cells in the perivascular lymphatic sheaths (PALS) of spleen, B cells contribute to the generation of follicular helper T (Tfh) cells (21). Several reports provided direct evidence of the requirement of B cells for the establishment of memory CD4 T cells in the spleen, comparing the expansion and maintenance of antigen-specific CD4 T cells from B cell-depleted or -deficient and control mice (22–26). In case of a lymphocytic choriomeningitis virus (LCMV) infection model, B cell-deficient mice rapidly lost memory CD4 T cells in the spleen during the contraction phase, despite normal expansion after acute infection (26). Thus, B cells are important for the formation of splenic memory CD4 T cells in vaccination and infection models. However, all these reports focused on memory CD4 T cells in the spleen, but not in the BM.

In this study, we investigated whether B cells are involved in the establishment of resting CD4 T cell memory in the BM. We examined the accumulation of antigen-specific activated CD4 T cells in the BM of B cell-depleted or B cell-deficient mice in the early phase of an immune reaction. We here show that B cells make a negative impact on the accumulation of CD49b+T-bet+ resting memory CD4 T cells in the BM, suggesting that B cells contribute to a quantitative balance of the commitment to effector Tfh cell and BM resting memory CD4 T cell lineage.

**MATERIALS AND METHODS**

**Mice**

Lymphocytic choriomeningitis virus–TCR tg [SMARTA (27)], Tbx21-KO (28), JHT (29), T-bet-ZsGreen reporter (30), or Rag1-KO mice were used. In all experiments, the mice were used at 6–16 weeks of age and were maintained under specific pathogen-free conditions. All mouse experiments were performed in accordance with the German law for animal protection and with permission from the local veterinary offices, and in compliance with the guidelines of the Institutional Animal Care and Use Committee. For immunizations, mice were injected intraperitoneally (i.p.) with LCMV GPs1–80 peptide (synthesized by Genecust) plus lipopolysaccharide (LPS, O111:B4).

**Flow Cytometry and Cell Sorting**

Single-cell suspensions were prepared from the spleen, BM, and blood of individual mice. The viability of cells was assessed by trypan blue exclusion. For cell staining, cells were stained for 15 min at 4°C with monoclonal antibodies against CD4 (RM4–5), CD19 (6D5), CD25 (PC61.5), CD44 (IM7), B220 (RA3-6B2), CD49b (HMa2), CD62L (MEL-14), NK1.1 (PK136), Thy1.1 (OX-7), CXCR4 (L276F12), CXCR5 (L138D7), CCR7 (4B12), and T-bet (4B10) and isotype controls. To exclude dead cells, we stained the cells with 1 μg/ml propidium iodide (Sigma). Intracellular staining for transcription factors was performed using Foxp3/transcription factor staining buffer kit (eBioscience) according to the manufacturer's protocol.

**Real-Time PCR Analysis**

The total RNA was extracted from splenocytes, and reverse-transcribed with High Capacity RNA-to-cDNA Kit (Life Technologies). Real-time PCR analysis was performed using the SYBR Green Master Mix (Life Technologies). Samples were normalized to the Hprt expression. Cxcl12 primer sequences: forward 5'-AAA CCA GTC AGC CTG AGC TAC C-3', reverse 5'-GGC TCT GGC GAT GTG GC-3'; Hprt primer sequences: forward 5'-TCC TCC TCA GAC CGC TTT T-3', reverse 5'-CAT AAC CTG GTT CAT CAT CGC-3'.

**Cell Sorting and Adoptive Transfer**

For positive sorting of splenic CD4 T cells, the Fab fragments of anti-CD4 antibody and Streptavidin MicroBeads (Miltenyi Biotech) were used. For negative sorting of splenic B cells, splenocytes were stained with FITC-conjugated anti-Mac1 (M1/70), anti-CD4 (12–20), and anti-CD8a (53–6.7) antibodies and then with anti-FITC and anti-Thy1.2 MicroBeads (Miltenyi Biotech) and were sorted by a magnetic cell separation system (MACS, Miltenyi Biotech). Approximately 0.5–1 × 10⁶ purified LCMV–TCR tg CD4 T cells were transferred intravenously (i.v.) into C57BL/6, JHT, or Rag1-KO mice, if any, with 1 × 10⁷ purified splenic B cells.

**B Cell Depletion**

B cells were depleted by antibody-mediated antigen-receptor cross-linking, as previously described (31). Mice were injected i.p. with either 200 μg of anti-IgD (11.26c) or isotype control followed by injection of 200 μg of mouse anti-rat IgG antibody (MAR18.5). The efficiency of B cell depletion was examined by flow cytometric analysis using anti-B220 (RA3-6B2) and anti-CD19 (6D5) antibodies.

**Immunofluorescent Staining and Confocal Microscopy**

For immunofluorescence staining, samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose. Cryostat sections of adult spleen were stained with monoclonal antibodies against Thy1.1 (HIS51), B220 (RA3-6B2), and CD4 (RM4–5). All histological analyses were carried out with a confocal laser microscope (LSM710, Carl Zeiss).
**Statistical Analyses**

All statistical analyses were performed using two-tailed Student’s *t*-test.

**RESULTS**

**B Cell Depletion Enhances the Accumulation of Antigen-Experienced CD4 T Cells in the BM**

B cell depletion inhibits the generation of memory CD4 T cells in the spleen, while it does not affect the expansion of CD4 T cells (22–26). To examine the effect of B cell depletion on the generation of resting memory CD4 T cells in the BM, we transferred LCMV–TCR tg CD4 T cells into mice pretreated with anti-IgD or isotype control followed by treatment of anti-rat IgG. The depletion protocol reduced more than 80% of B220<sup>+</sup>CD19<sup>+</sup> mature B cells in the spleen and mesenteric lymph nodes (mLN), and around 70% in BM, but did not affect BM B cell precursors, including pro-B cells, which may compete for IL-7-expressing stromal niches with memory CD4 T cells (Figure S1 in Supplementary Material) (32, 33). Next, we immunized mice with LCMV peptide. Although LCMV-specific CD4 T cells in the spleen of B cell-depleted and control mice expanded similarly, there were more CD4 T cells in the BM of B cell-depleted mice compared to the BM of B cell-sufficient mice (Figure 1A). A similar result was obtained in the BM from *Rag1<sup>−/−</sup>* host mice that had received antigen-specific CD4 T cells (Figure 1B). By contrast, when wild-type splenic polyclonal B cells were cotransferred with LCMV-specific CD4 T cells into *Rag1<sup>−/−</sup>* mice followed by immunization with LCMV peptide (Figure S2A in Supplementary Material), the accumulation of antigen-specific CD4 T cells in the BM was dramatically decreased, despite normal expansion in the spleen (Figure 1C). Furthermore, in B cell-deficient JHT mice transferred with LCMV–TCR tg CD4 T cells, despite smaller expansion in the spleen compared to C57BL/6 host mice, the equivalent number of antigen-specific CD4 T cells was detected in the BM, involving in an increased ratio of antigen-specific CD4 T cells in the BM compared to the spleen (Figure S2B in Supplementary Material). Collectively, these results indicate that B cells suppress the accumulation of antigen-experienced CD4 T cells in the BM.

**B Cell Depletion Enhances the Induction of CD49b<sup>+</sup>T-bet<sup>+</sup> Antigen-Specific CD4 T Cells**

How does B cell depletion affect antigen-specific CD4 T cells in the spleen? CD49b<sup>+</sup> antigen-specific activated CD4 T cells in the BM from *B cell-depleted* C57BL/6 Rag1<sup>−/−</sup> mice were transferred into *Rag1<sup>−/−</sup>* host mice that had received antigen-specific CD4 T cells. T-bet<sup>+</sup> antigen-specific activated CD4 T cells in the spleen were analyzed by flow cytometry and enumerated. Data represent the mean ± SD; **p < 0.01; ***p < 0.001; N = 4.

![Figure 1](image_url)
the spleen preferentially migrate into the BM (15). We speculated that B cell depletion affects the expression of CD49b in antigen-specific activated CD4 T cells in the spleen. Notably, splenic antigen-specific CD4 T cells in B cell-depleted mice expressed significantly more CD49b compared to control mice (Figure 2A) and conversely the CD4 T cells in B cell-transferred Rag1−/− mice decreased its expression (Figure 2B), suggesting that B cells negatively regulate the expression of CD49b and consequently reduce the accumulation of antigen-specific CD4 T cells in the BM.

Misumi and Whitmire have reported that B cell depletion affects the expression of Ly-6C and T-bet in antigen-specific CD4 T cells (23). Therefore, in order to examine the expression of Ly-6C and T-bet in antigen-specific CD4 T cells, we used anti-Ly-6C antibodies and T-bet ZsGreen BAC tg mice that express ZsGreen fluorescence protein under control of Tbx21 regulatory elements (30). Ly-6C+ cells were slightly but significantly increased in splenic antigen-specific CD4 T cells from B cell-depleted mice (Figure 3A). T-bet+ antigen-specific CD4 T cells were significantly increased in the spleen and blood after B cell depletion, while the population in the BM was likely saturated (Figure 3B). We also detected three times more T-bet+ cells among antigen-specific CD4 T cells in the spleen of B cell-depleted mice using a T-bet-specific antibody (Figure S3 in Supplementary Material). Intriguingly, CD49b+T-bet+ antigen-specific CD4 T cells in immunized host mice were detected at the lowest percentage in the spleen (14%), at the midst in the blood (34%), and at the highest in the BM (53%) (Figure 3C), suggesting that CD49b+T-bet+ antigen-specific CD4 T cells selectively migrate from the spleen into the BM via blood. These results argue that CD49b+T-bet+ antigen-specific CD4 T cells are the potential precursors of BM resting memory CD4 T cells.

**T-bet**+ Antigen-Specific CD4 T Cells Preferentially Localize in Red Pulp of Spleen

The precursors of long-lived plasma cells egress from splenic B cell follicles toward the BM via splenic red pulp and blood (34). To examine the localization of T-bet+ antigen-specific CD4 T cells in the spleen, we performed a histological analysis and assessed the localization of T-bet+ and T-bet− antigen-specific CD4 T cells (Figure 4). On day 6 after immunization, while most of T-bet− antigen-specific CD4 T cells remained in the white pulp, including B cell follicles and PALS, T-bet+ cells significantly stayed in the red pulp. These data suggest that T-bet+ resting memory CD4 T cell precursors preferentially localize in splenic red pulp and blood. It is well known that the precursors of long-lived plasma cells migrate into the BM in a CXCL12-dependent manner (33, 35). To examine the involvement of some chemokines in the localization of the resting memory CD4 T cell precursors, the expression of Cxc12 mRNA in the spleen tissue and Cxcr4, Cxcr5, and Ccr7 proteins on antigen-specific CD4 T cells in the spleen and BM from B-cell
depleted and control mice were analyzed. However, their expression profiles were not influenced by B cell depletion (Figure S4 in Supplementary Material).

**T-bet Is Not Required for the Accumulation of Antigen-Specific CD4 T Cells in the BM**

Finally, to examine the physiological impact of T-bet on the accumulation of antigen-specific CD4 T cells in the BM, we compared the accumulation of T-bet<sup>+</sup> or T-bet<sup>−/−</sup> LCMV–TCR tg CD4 T cells in the BM. On day 6 after immunization, T-bet-deficient CD4 T cells accumulated in the BM equally as well as T-bet-sufficient cells (Figure 5A). Furthermore, T-bet deficiency did not affect surface CD49b expression (Figure 5B). Consistent with these observations, when we compared the number of CD44<sup>hi</sup> memory-phenotype CD4 T cells in T-bet-sufficient and -deficient backgrounds, both groups showed similar numbers of CD44<sup>hi</sup> CD4 T cells in the spleen and BM (Figure S5 in Supplementary Material). Taken together, T-bet is dispensable for the expression of CD49b to facilitate the accumulation of antigen-specific CD4 T cells in the BM.

**DISCUSSION**

B cells have been described as a positive regulator to generate CD4 T cell memory in the spleen (22–26). However, we here showed that the depletion or deficiency of B cells actually promotes the accumulation of antigen-specific activated CD4 T cells in the BM, and in contrast the pretransfer of B cells suppresses their accumulation, suggesting that B cells are a negative regulator to generate CD4 T cell memory in the BM. We also found that B cell depletion facilitates the upregulation of the expression of CD49b, a homing receptor of CD4 T cells to the BM, and T-bet, the lineage-specifying transcription factor of Th1 cell differentiation, in the activated CD4 T cells in the spleen. Moreover, the T-bet<sup>+</sup> cell population preferentially localizes in the red pulp of the spleen and in the BM during an immune reaction. We suggest that CD49b<sup>+</sup>T-bet<sup>+</sup> antigen-specific CD4 T cells in the spleen are the precursors of BM resting memory CD4 T cells.

We demonstrated that B cells negatively control the expression of T-bet and CD49b in antigen-experienced CD4 T cells in the spleen. It remains unclear at the molecular level how B cells downregulate the expression of these molecules and whether a T-bet-inducing signal is directly involved in CD49b expression in activated CD4 T cells. Intriguingly, a loss of T-bet did not alter the expression level of CD49b in activated CD4 T cells and hardly affected their accumulation in the BM. The result suggests that T-bet itself plays a dispensable role in the migration of activated CD4 T cells. Rather, the upstream signaling pathway (T-bet-inducing signal) might be critical in this process in parallel with the control of CD49b expression. Naive T cells do not express T-bet and CD49b. Once they are stimulated via the TCR, while T-bet expression is induced via IFNγR and IL-12R signaling pathways (28, 30, 36, 37), CD49b expression is induced as well as VLA-2 on Th1 cells in vitro in an IFNγ- and IL-12-independent manner (38). Hence, opposite to the upregulation of T-bet and CD49b by DCs, B cell-mediated signals may commonly downregulate the expression of these molecules. This notion is supported by recent findings (39, 40). They have described that ICOS costimulation via B cells is crucial to maintain the Thf phenotype through the downregulation of transcription factor KLF2 and that the blockade of ICOS ligand on B cells increases Tbx21 transcripts in antigen-specific CD4 T cells. In a KLF2-transduced HUVEC cell line, CD49b is upregulated compared to a control cell line at the transcriptional level (41). It remains to be further clarified whether B cells suppress T-bet and CD49b in activated CD4 T cells in a KLF2-dependent manner.

Our data suggest that DCs may activate CD4 T cells and license them to differentiate into resting memory cells in the BM during primary immune response, while some activated CD4 T cells contact bystander B cells as follow-up antigen-presenting cells through cognate interaction and further differentiate into effector Thf cells (42–45). Thus, additional activation by B cells following DCs may induce the differentiation of effector Thf cell lineage (21), suppressing the differentiation program for a BM resting memory cell lineage. Resting memory CD4 T cells in the BM are more functional in vitro and in vivo compared to spleen-resident memory cells (14). Adoptively transferred memory CD4 T cells from the BM can efficiently help B cells...
to produce high-affinity antibodies, suggesting that BM memory CD4 T cells can differentiate into Tfh cells during recall response. We have previously shown that a persistent antigen with adjuvants of oil and aluminum hydroxide enhances the expansion of antigen-specific CD4 T cells in the secondary lymphoid organs (SLOs), but not the BM (46). BM resting memory CD4 T cells are unaffected by persistence of antigen (46), while splenic Tfh cells, probably also memory Tfh cells, are sustained by persistence of antigen (47). Thus, the ratio of splenic Tfh cells and BM resting memory cells is markedly affected by antigen persistence. We suggest that the length of antigen persistence may define the quantitative balance of effector and resting memory, i.e., whether the CD4 T cells should contribute to the long-lasting reaction to exclude the persistent antigen or store their ability for recall response.

**AUTHOR CONTRIBUTIONS**

SH, JS, and KT designed the research; SH, JS, CM, MM, and DZ performed the research; AH, JZ, WP, SF, and ML contributed new reagents/analytic tools; and SH, ML, AR, and KT wrote the manuscript.
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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