nature immunology

Cannabinoid receptor 2 mediates the retention of immature B cells in bone marrow sinusoids

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Immature B cells developing in the bone marrow are found in the parenchyma and sinusoids. The mechanisms that control the positioning of B cells in the sinusoids are not understood. Here we show that the integrin $\alpha_4\beta_1$ (VLA-4) and its ligand VCAM-1 were required, whereas the chemokine receptor CXCR4 was dispensable, for sinusoidal retention of B cells. Instead, cannabinoid receptor 2 (CB2), a $G\alpha_i$ protein–coupled receptor upregulated in immature B cells, was required for sinusoidal retention. Using two-photon microscopy, we found immature B cells entering and crawling in sinusoids; these immature B cells were displaced by CB2 antagonism. Moreover, CB2-deficient mice had a lower frequency of immunoglobulin λ-chain–positive B cells in the peripheral blood and spleen. Our findings identify unique requirements for the retention of B cells in the bone marrow sinusoidal niche and suggest involvement of CB2 in the generation of the B cell repertoire.

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The bone marrow contains specialized yet poorly defined microenvironments that help maintain stem cells and support hematopoiesis. In early studies examining the compartmentalization of developing B cells, B220$^+$ cells and immature immunoglobulin M–positive (IgM$^+$) cells were found scattered throughout the bone marrow parenchyma, and immature IgM$^+$ cells have also been reported in sinusoids[1–4]. Bone marrow sinusoids are specialized thin-walled venous blood vessels that travel through the tissue parenchyma, often joining by anastomosis before connecting to the large central sinusoid that carries the blood and newly produced cells to the venous circulation[5]. All cells produced in the bone marrow, including red blood cells, platelets, granulocytes and lymphocytes, are thought to enter the circulation through the sinusoids[5]. Given this cellular diversity, sinusoids are unexpectedly enriched for lymphocytes, and it has been suggested that there might be 'lymphocyte loading' of sinusoids[2,6]. However, the mechanisms that control the retention of immature B cells in bone marrow sinusoids or their release from these sinusoids have not been defined.

The integrin-ligand pair $\alpha_4\beta_1$ (VLA-4)–VCAM-1 helps retain hematopoietic stem cells in the bone marrow[7–9]. VCAM-1 is expressed by a subset of bone marrow stromal cells and by sinusoidal endothelium[9]. The function of $\alpha_4\beta_1$–VCAM-1 interactions in B cell development or retention in the bone marrow has been unclear, as some gene-ablation studies have suggested minimal involvement[10–13], whereas others have documented fewer immature and mature B cells in the bone marrow after ablation[14,15]. SDF-1 (CXCL12), a chemokine that can activate $\alpha_4\beta_1$ (ref. 16), is produced by bone marrow stromal cells and has also been detected on bone marrow endothelium[17–19]. The SDF-1 receptor CXCR4 helps retain hematopoietic progenitor cells and developing B cells in the bone marrow[20,21] and promotes the homing of progenitor cells, plasma cells and T cells from blood to bone marrow[19,22,23]. However, CXCR4 is partially downregulated between the pre–B cell and immature B cell stages[16,21,24–26], and it is unclear whether CXCR4 continues to function in immature B cells.

The $G\alpha_i$ protein–coupled cannabinoid receptor 2 (CB2) is abundantly expressed in mature B cells and is also present in myeloid cells, natural killer cells and various other cell types[27,28]. The CB2 ligand 2-arachidonoylglycerol (2-AG) is generated from arachidonic acid–containing phospholipids and has been detected in many tissues, including bone[28,29]. Intake of cannabinoid receptor agonists has a variety of effects on the immune system, but the direct *in vivo* actions of such agonists on lymphoid cells remain poorly understood[30].

Here we use an *in vivo* pulse-labeling procedure to distinguish cells in bone marrow sinusoids from those in the parenchyma and to establish that the retention of immature B cell in sinusoids is dependent on $\alpha_4\beta_1$ and VCAM-1. We found that CXCR4 was not critical for retention in sinusoids but that pertussis toxin–mediated inhibition of $G\alpha_i$ displaced sinusoidal cells. The expression and function of CB2 were upregulated in immature B cells, and intrinsic deficiency in CB2 prevented the accumulation of immature B cells in bone marrow sinusoids. Using two-photon microscopy, we found immature B cells entering and crawling in bone marrow sinusoids; these cells were displaced by CB2 antagonism. Finally, CB2 deficiency resulted in a lower percentage of peripheral B cells that expressed immunoglobulin λ-chain. Our findings identify unique requirements for the retention of B cells in the bone marrow sinusoidal niche and suggest a function for CB2 in the formation of the B cell repertoire.

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Received 10 December 2008; accepted 26 January 2009; published online 1 March 2009; doi:10.1038/ni.1710

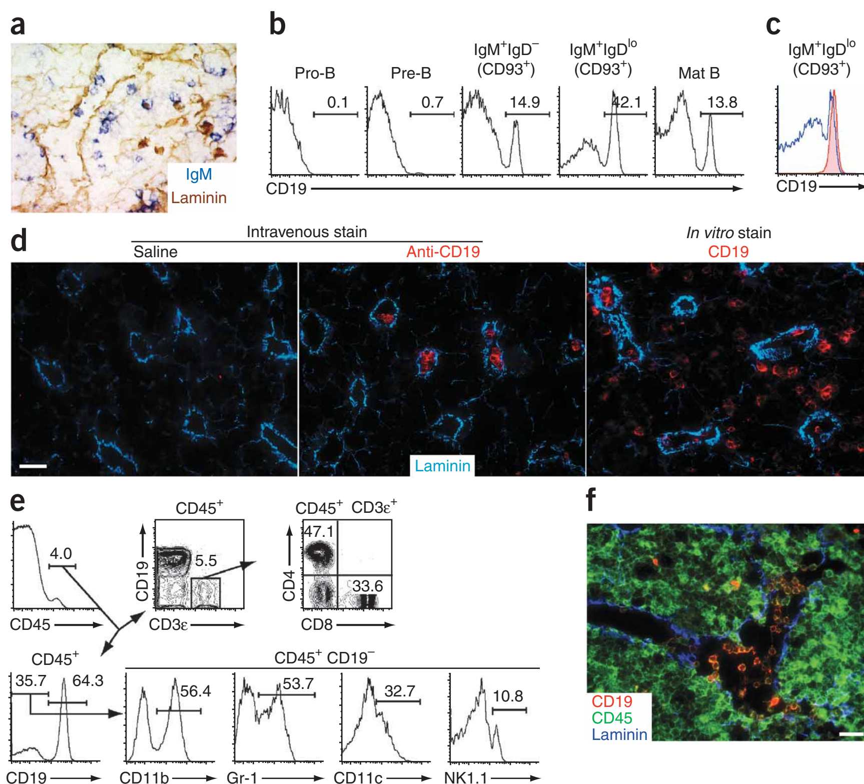

Figure 1 *In vivo* labeling of B lymphocytes in bone marrow sinusoids. (**a**) Femur section immunohistochemically stained with anti-IgM and anti-laminin. Original magnification, ×40. (**b**,**c**) Flow cytometry of bone marrow cells from a mouse injected for 2 min with 1 μg anti-CD19–PE. (**b**) Labeling of bone marrow B cell subsets. Numbers above bracketed lines indicate percent cells in each gate. Mat, mature. (**c**) Labeling of immature IgD^{lo} B cells in bone marrow (blue) and peripheral blood (red). Data in **a**–**c** are from one experiment representative of more than ten experiments with ten mice. (**d**) Femur or tibia sections from mice treated with saline or anti-CD19–PE, analyzed by immunofluorescence microscopy after staining with anti-laminin alone (left and middle) or after further *in vitro* staining with anti-CD19–PE (right). Scale bar, 20 μm. Results are from one experiment representative of more than ten. (**e**) Flow cytometry of bone marrow cells isolated from mice injected for 2 min with anti-CD45–PE, then stained *in vitro* with antibodies specific for various markers (along axes). Numbers above bracketed lines or outlined areas or in quadrants indicate percent cells in each area. Data are from one experiment representative of two. (**f**) Immunofluorescence microscopy of a femur from a mouse injected with anti-CD19–PE; cells are stained with anti-CD45 and anti-laminin. Scale bar, 20 μm. Data are representative of two independent experiments with two mice.

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RESULTS

Labeling of cells in bone marrow sinusoids

To examine the distribution of IgM$^+$ B cells in bone marrow parenchyma and sinusoids, we stained bone marrow sections for endothelial and basement membrane markers. Antibodies to laminin, a protein abundant in basement membranes, were effective in labeling bone marrow vessels; we identified sinusoids among laminin-expressing vessels on the basis of their large lumens and thin walls (**Fig. 1a**). We detected IgM$^+$ B cells in the femur and tibia both in the parenchyma and inside sinusoids (**Fig. 1a**), consistent with published studies[1,4].

To facilitate the quantification and phenotyping of sinusoidal B cells, we developed an *in vivo* labeling procedure. Studies have shown that injected antibodies rapidly equilibrated throughout the bone marrow[1,4,5], and we found that biotin-conjugated CD19-specific antibodies labeled all bone marrow B cells within a few minutes of injection (**Supplementary Fig. 1** online). To test the possibility that larger protein complexes may have more limited access to the parenchyma, we treated mice for 2 min with antibody to CD19 (anti-CD19; approximately 150 kilodaltons) coupled to phycoerytrhin (PE; 240 kilodaltons). In this case, there was a bimodal staining pattern for immature IgM$^+$IgD$^-$ and IgM$^+$IgD^{lo} B cells and mature B cells; in contrast, pro– and pre–B cells were unlabeled (**Fig. 1b**). Among the IgM$^+$ immature B cells, the IgD^{lo} subset was most enriched for cells labeled with anti-CD19–PE (**Fig. 1b**). Injection of PE-conjugated antibodies for longer periods (5–10 min) eventually stained all bone marrow cells targeted by the antibodies (**Supplementary Fig. 1**). When we analyzed immature B cells using a single gate (encompassing IgM$^+$IgD$^-$ and IgM$^+$IgD^{lo} cells; **Supplementary Fig. 1**), we found that 25.4% ± 5.9% (± s.d.; n = 18 mice) were labeled with anti-CD19–PE. The extent of staining on the brightly labeled bone marrow B cell subsets approached the labeling intensity of cells from blood of the same mice (**Fig. 1c**). Immunofluorescence analysis of bone marrow sections from mice treated with anti-CD19–PE, after staining with anti-laminin, showed that the cells labeled with anti-CD19–PE were in sinusoids (**Fig. 1d** and **Supplementary Fig. 2** online). However, *in vitro* staining with anti-CD19–PE showed the expected distribution of CD19$^+$ cells in both the parenchyma and sinusoids (**Fig. 1d** and **Supplementary Fig. 2**). In some cases, the *in vivo*–labeled cells were located in regions of sinusoids adjoining the central collecting sinusoid (**Supplementary Fig. 2**).

Using injection of anti-CD45–PE as an approach for labeling all hematopoietic cell types present in sinusoids, we found that cells of the B lineage constituted approximately two thirds of all sinusoidal cells and the remaining cells were mostly of the myeloid lineage (CD11b$^+$, CD11c$^+$ and/or Gr-1$^+$), together with smaller numbers of natural killer cells (NK1.1$^+$) and CD4$^+$ and CD8$^+$ T cells (**Fig. 1e**). The proportion of immature B cells labeled after injection of anti-CD45–PE was similar to that in mice treated with anti-CD19–PE (data not shown). Consistent with the flow cytometry data, analysis of bone marrow sections from mice treated with anti-CD19–PE and stained *in vitro* with anti-CD45 showed that B cells were the predominant CD45$^+$ cell type in the sinsuoids (**Fig. 1f**). In summary, treatment with PE-conjugated antibodies for 2 min *in vivo* allowed the selective labeling of cells present in bone marrow sinusoids, and this approach showed that about one quarter of immature B cells in the bone marrow are located in this bone marrow niche.

Retention of sinusoidal B cells by α4β1 and VCAM-1

To assess whether $\alpha_4\beta_1$ and VCAM-1 are involved in retaining immature B cells in bone marrow sinusoids, we treated mice for 3 h with blocking antibodies to α_4 or VCAM-1 or with saline and injected anti-CD19–PE 2 min before isolating tissues. We treated a separate group of mice with anti-α_L to block the integrin $\alpha_L\beta_2$ (LFA-1). By counting parenchymal (anti-CD19–PE$^-$) B lineage cells, we found that