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*J Immunol* 2013; 190:1094-1102; Prepublished online 21 December 2012;

doi: 10.4049/jimmunol.1202639

http://www.jimmunol.org/content/190/3/1094

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2012/12/31/jimmunol.1202639.DC1

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Focal Adhesion Kinase Regulates the Localization and Retention of Pro-B Cells in Bone Marrow Microenvironments

Shin-Young Park,* Peter Wolfram,* Kimberly Canty,* Brendan Harley,*†,‡ César Nombela-Arrieta,* Gregory Pivarnik,* John Manis,* Hilary E. Beggs,* and Leslie E. Silberstein*

Progenitor B cells reside in complex bone marrow (BM) microenvironments where they receive signals for growth and maturation. We reported previously that the CXCL12-focal adhesion kinase (FAK)-VLA4 pathway plays an important role in progenitor B cell adhesion and migration. In this study, we have conditionally targeted in B cells FAK, and found that the numbers of progenitor pro-B, pre-B, and immature B cells are reduced by 30–40% in B cell-specific FAK knockout mice. When cultured in methylcellulose with IL-7 ± CXCL12, Fak-deleted pro-B cells yield significantly fewer cells and colonies. Using in situ quantitative imaging cytometry, we establish that in longitudinal femoral BM sections, pro-B cells are preferentially localized in close proximity to the endostem of the metaphyses and the diaphysis. Fak deletion disrupts the nonrandom distribution of pro-B cells and induces the mobilization of pro-B cells to the periphery in vivo. These effects of Fak deletion on pro-B cell mobilization and localization in BM are amplified under inflammatory stress, that is, after immunization with nitrophenol-conjugated chicken \(\gamma\)-globulin in alum. Collectively, these studies suggest the importance of FAK in regulating pro-B cell homeostasis and maintenance of their spatial distribution in BM niches. The Journal of Immunology, 2013, 190: 1094–1102.

The generation of B lineage cells in the bone marrow (BM) is a dynamic process whereby multipotent hematopoietic stem cells differentiate into lineage-restricted progenitors, which then progress through a series of developmental stages culminating in mature B cells (1). Progenitor B cells have been identified near bone-lining osteoblasts or nonhematopoietic stromal cells in BM, or both (2–4). Progenitor B cell growth and maturation are proposed to depend on cues from distinct microenvironments, that is, niches. Earlier studies, limited to transverse sections of the femoral BM, have proposed that progenitor B cells after sublethal irradiation reside close to the endosteal surface of the diaphysis, whereas more mature B cells localized centrally, near the central sinus (5, 6). In addition, the importance of osteoblastic lineage cells in progenitor B cell development has been shown in experimental mouse models (4, 7). More recent data point to the possibility of differentiation-stage-specific niches in B cell development (2, 5, 8).

Signals in BM microenvironments might emanate from cell-cell, for example, VLA4/VCAM-1, cell-extracellular matrix (e.g., CD44/hyaluronate) interactions, as well as cellular responses to cytokines, for example, IL-7, stem cell factor, FLT3 ligand, and chemokines (e.g., CXCL12) (9, 10). Both CXCL12 and its corresponding receptor CXCR4 are essential for progenitor B lymphocyte development (9, 10). CXCL12 is expressed throughout the BM, either in soluble form or immobilized to reticular, endothelial, osteoblast cell types, as well as to components of the extracellular matrix (8, 11–14).

Previously, we showed that the CXCL12-induced focal adhesion kinase (FAK) activation regulates VLA4-mediated cell adhesion to VCAM-1 (CXCL12/CXCR4-FAK-VLA4 pathway) in normal and leukemic progenitor B cells in vitro, suggesting that this pathway might modulate progenitor B cell localization in BM niches in vivo (15, 16). Furthermore, these studies implicated \(G_\alpha\), Src, and Rap1 as intermediary factors (17, 18). FAK, a cytoplasmic tyrosine kinase, has been shown to play an important regulatory function in cell adhesion, motility, growth, and survival in response to environmental cues based on initial studies primarily in fibroblasts (19, 20) and subsequently in hematopoietic cells using lineage-specific knockout (KO) mouse models (21–23). In this study, we investigated the FAK function in the pro-B cells using B cell-specific Fak KO mice, because of its role as an integrator of external cell signaling downstream of \(\mathrm{IL-7, growth factor/chemokines, and integrin receptors (15, 24, 25). Our findings suggest the importance of FAKs in regulating pro-B cell growth and their distinct distribution in the BM microenvironments."

Materials and Methods

**Experimental animals**

Floxed Fak mice (Fak\textsuperscript{floxed}) (26) were crossed to Cd19-Cre mice (Jackson Laboratory) to generate Cd19-Cre\textsuperscript{Fak} mice. Cd19-Cre Fak KO mice with the enhanced GFP reporter gene (EGFP\textsuperscript{+}) Cd19-Cre Fak KO were

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Received for publication September 20, 2012. Accepted for publication November 21, 2012.

This work was supported by the National Institutes of Health (Grants ST32 HL066987, IR21 HL094923, 1R01 HL093139, and U24 HL074355).

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; BM, bone marrow; CFC, colony-forming cell; D-PBS, Dulbecco’s PBS; EGFP, enhanced GFP; FAK, focal adhesion kinase; KO, knockout; LSC, laser-scanning cytometry; NP-CGG-alum, nitrophenol-conjugated chicken \(\gamma\)-globulin in alum; PB, peripheral blood; WT, wild type.

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produced by crossing Cd19-Cre\textsuperscript{fl/fl} Fak\textsuperscript{660} mice with Rosa26-EGFP\textsuperscript{660} mice provided by Dr. Stuart Orkin (Boston Children's Hospital). The compound mice were backcrossed to C57BL/6 mice (Taconic Farm) for more than six generations. Age- and sex-matched CD19-Cre\textsuperscript{660} Fak\textsuperscript{660} mice or littermate CD19-Cre\textsuperscript{660} Fak\textsuperscript{660} mice, which we found to be phenotypically equivalent, at 8–12 wk of age were used as wild type (WT) controls. The mb-1–Cre\textsuperscript{660} Rosa26-EGFP\textsuperscript{660} mice were generated by crossing Fak\textsuperscript{660} Rosa26-EGFP\textsuperscript{660} with mb-1–Cre\textsuperscript{660} mice, a generous gift from Dr. Michael Plückthun (Max-Planck Institute for Immunobiology, Freiburg, Germany). The mb-1–Cre\textsuperscript{660} Fak\textsuperscript{660} mice have higher excision efficiency at the pro-B cell stage, and thus yield significantly higher numbers of Fak-deleted pro-B cells than CD19-Cre\textsuperscript{660} Fak\textsuperscript{660} mice (Supplemental Fig. 1G, 1H). Animal experiments were performed in accordance with the animal protocols that were approved by the Boston Children's Hospital Animal Care and Use Committee and the Harvard Medical School Standing Committee on Animals.

**PCR genotyping**

WT, floxed, and deleted Fak genes were assessed by PCR with primers 1, 2, and 3 as shown in Supplemental Fig. 1A: primer 1: 5'-GAGGCTCAGCTTCTATTTCTC-3' primer 2: 5'-GAATGCTCAGGACAGCAAATCA-3' primer 3: 5'-GAGAATCCGTACGAGGATTTTTG-3'. The amplified PCR products consisted of a WT (1.4 kb by P1 and P2 primers) 290 bp by P2 and P3 primers (1.6 kb by P1 and P2 primers), 400 bp by P2 and P3 primers), and Cre-mediated recombined fragment (327 bp by P1 and P2 primers) (26). Cre genotyping was performed using the PCR primers (forward 5'-CATAAACAGTTAGTACCGG-3', reverse 5'-CCTGATACGCCTAGCTG-3') as previously described (27). Egrfp genotyping was performed using the PCR primers (forward 5'-GAGACAGTGAGAACCAGCA-G-3', reverse 5'-CCATGGGGTTTCTGCTG-3') with the conditions: 33 cycles of 94˚C for 30 s, 53˚C for 15 s, 72˚C for 1 min, resulting in a 456-bp product. For mb-1Cre genotyping, mbCre PCR was used as previously described with the primers hCre dir (5'-CCCTGCTGATGATTCTCCT-3') and hCre reverse (5'-GTCTGTGATCTGCTAG-3'), resulting in a 450-bp product (28). In contrast with Cd19-Cre gene expression, mb-1–Cre gene expression starts earlier in B cell development, leading to almost complete deletion of floxed genes in BM progenitor B cells (28).

**Flow cytometry analysis**

Single-cell BM suspensions were prepared by crushing and gently grinding the femurs and tibias using a mortar and a pestle in washing buffer ( Dulbecco's PBS (D-PBS), Ca\textsuperscript{2+} free, Mg\textsuperscript{2+} free, 2% FBS) followed by hemolysis with ammonium chloride-potassium buffer (Life Technologies BRL). After blocking FcRs with anti-CD16/CD32 (2.4G2) Ab, cells were stained with specific Abs. To sort progenitor B cells, we stained fresh BM cells from colony-forming cell assay in culture. The cells were harvested from colonies for cell counting and Annexin V apoptosis analysis

**Immunofluorescence staining of cryopreserved sections of femurs**

Whole mouse femurs were fixed in phosphate-buffered 1-lysin with 1% paraformaldehyde/peridate >4 h at 4˚C, followed by washing in 0.1 M phosphate buffer pH 7.5, and cryoprotected for 4 h in 30% sucrose/0.1 M phosphate buffer. The fixed bones were embedded in OCT compound (optical cutting temperature compound; a water-soluble glycol-resin compound; Sakura Finetek), snap frozen in 2-methylbutane/ dry ice mix, and stored at −80˚C. Fresh cryopreserved, nondecalcified femurs were sectioned longitudinally at 4–5-μm thickness to facilitate in situ laser-scanning cytometry (LSC) analysis of a single cell-thick layer using the CryoJane tape-transfer system (Instrumedics) in a cryostat (LEICA CM1800). All sections were prepared from the middle of the femur to include the central sinus. Thawed frozen sections were thoroughly air-dried and rehydrated in D-PBS. The sections were blocked in blocking medium (10% normal donkey serum/0.025% Tween 20 in Ca\textsuperscript{2+}-free, Mg\textsuperscript{2+}-free D-PBS) for 1 h followed by streptavidin/biotin blocking solution (Vector Laboratories). The sections were stained with rat anti-CD43 Ab (S7; BD Biosciences) or rat anti-IgD Ab (11-26c; BioLegend), followed by Dylight 488-labeled donkey anti-rat IgG (Jackson Immunoresearch). After blocking with normal rat serum, the sections were stained with biotinylated anti-CD20 Ab (BD Biosciences) or biotinylated anti-IgM Ab (S7; BD Biosciences), and goat anti-rat IgG, followed by Dylight 549-streptavidin (Jackson Immunoresearch). For nuclear staining, sections were stained with goat anti-osteopontin Ab (R&D Systems) for osteoblasts, rabbit anti-laminin (Chemicon) for vascular tissue, or goat anti-CD105 (endoglin) Ab (R&D Systems) for sinusoids followed by Dylight 649-labeled donkey antirat IgG (Jackson Immunoresearch). For Rosa26-GFP Fak WT or KO mice, we used chicken anti-GFP Ab (Invitrogen), rat anti-CD20 Ab (S7; BD Biosciences) or goat anti-CD105 Ab (R&D Systems), followed by staining with Dylight dye-labeled donkey antirat immunoglobulin Ab (Jackson Immunoresearch). Pro-B cells were identified by GFP\textsuperscript{+} CD43\textsuperscript{+} cells. Dilutions of primary and secondary Abs were optimized for LSC and confocal microscopy imaging. Isotype control slides were stained with Dylight 549–, Dylight 649–, or Dylight 488–labeled secondary Abs after isotype control primary Ab staining. All slides were then labeled with 1 μM DAPI for 3 min (Invitrogen), and coverslips were placed using slow-fade mounting media (Molecular Probes).

**In situ solid-phase LSC analysis**

For all LSC analyses, the iCys Research Imaging Cytometer (Compucyte) with four excitation lasers (405, 488, 561, and 633 μm), four emission filters (425–455, 500–550, 575–625, 650 nm long pass), and four photomultiplier tubes was used. For each fluorescent marker, images are built pixel by pixel from the quantitative photomultiplier tube measurements of laser-specific fluorescent signals (29). The quantitative imaging cytometry control software generated a single “region” image of the entire BM cavity from a sequence of high-magnification (40×/NA 0.95 dry objective) “field” immunofluorescence images (250 × 190 μm field image) that were subjected to automated analysis of contour-based cellular events, their fluorescence levels, and their location within the BM section. Bone lining was identified by autofluorescence of the collagen in bone. Individual cellular events are defined by threshold contouring of DAPI-stained nuclei. In longitudinal BM sections, each femoral scan produces an average of 100,000 cellular events. The total fluorescence intensity from individual cellular events is measured in each channel within the integration contour. The integration contour is set as two pixels out from the threshold contour, a value that allows discrimination of the threshold contour from the fluorescence signal. For all LSC analyses, the iCys Research Imaging Cytometer (Compucyte) with four excitation lasers (405, 488, 561, and 633 μm), four emission filters (425–455, 500–550, 575–625, 650 nm long pass), and four photomultiplier tubes was used. For each fluorescent marker, images are built pixel by pixel from the quantitative photomultiplier tube measurements of laser-specific fluorescent signals (29). The quantitative imaging cytometry control software generated a single “region” image of the entire BM cavity from a sequence of high-magnification (40×/NA 0.95 dry objective) “field” immunofluorescence images (250 × 190 μm field image) that were subjected to automated analysis of contour-based cellular events, their fluorescence levels, and their location within the BM section. Bone lining was identified by autofluorescence of the collagen in bone. Individual cellular events are defined by threshold contouring of DAPI-stained nuclei. In longitudinal BM sections, each femoral scan produces an average of 100,000 cellular events. The total fluorescence intensity from individual cellular events is measured in each channel within the integration contour. The integration contour is set as two pixels out from the threshold contour, a value that allows discrimination of the threshold contour from the fluorescence signal. For all LSC analyses, the iCys Research Imaging Cytometer (Compucyte) with four excitation lasers (405, 488, 561, and 633 μm), four emission filters (425–455, 500–550, 575–625, 650 nm long pass), and four photomultiplier tubes was used. For each fluorescent marker, images are built pixel by pixel from the quantitative photomultiplier tube measurements of laser-specific fluorescent signals (29). The quantitative imaging cytometry control software generated a single “region” image of the entire BM cavity from a sequence of high-magnification (40×/NA 0.95 dry objective) “field” immunofluorescence images (250 × 190 μm field image) that were subjected to automated analysis of contour-based cellular events, their fluorescence levels, and their location within the BM section. Bone lining was identified by autofluorescence of the collagen in bone. Individual cellular events are defined by threshold contouring of DAPI-stained nuclei. In longitudinal BM sections, each femoral scan produces an average of 100,000 cellular events. The total fluorescence intensity from individual cellular events is measured in each channel within the integration contour. The integration contour is set as two pixels out from the threshold contour, a value that allows discrimination of the threshold contour from the fluorescence signal.
longitudinal femur sections demonstrates the integrity of BM tissue architecture (Supplemental Fig. 3B).

**Homing analysis**

B220+ CD43+ EGFP+ pro-B cells were sorted from mb-1–Cre+ Rosa26-EGFP+ KO and mb1-Cre+ Rosa26-EGFP+ WT mice by FACS Aria (Becton Dickinson). Sorted B220+ CD43+ EGFP+ pro-B cells from Fak KO or WT were differentially labeled with chloromethyl derivatives of fluorescein diacetate dye and chloromethyl derivatives of aminocoumarin dye. 5-(and-6)-(4-chloromethyl)benzoyl]aminol tetramethylrhodamine–labeled WT splenic B cells were coinjected as reference cells. A total of 1 × 10^6 of each labeled cells were mixed and i.v. transferred to WT C57BL/6 recipient mice. Some input cells were saved to assess the concentration of transferred Fak KO cells and WT cells.

**FIGURE 1.** Selective decrease of progenitor B cell number in B cell–specific FAK KO mice. (A) BM cells from Cd19-Cre Fak KO or WT control mice, (B) spleen cells from Cd19-Cre Fak KO or WT control mice, or (C) BM cells from mb1-Cre Fak KO or littermate WT control mice were prepared and stained with mAbs. Total numbers of each population per limb (one femur and one tibia) or per spleen were calculated using flow cytometry analysis and automated complete blood count. Student t tests were performed as shown: *p < 0.05, **p < 0.01 (unpaired two-tailed). n = 8. Dots indicate individual mice; bar indicates the mean. BM cell gates: total B (B220+ CD19+), pro-B (B220lo CD19+ IgM+ CD43+), pre-B (B220lo CD19+ IgM+ CD43+), immature B (Imm B; B220hi CD19+ IgM+ CD43+), and mature B cells (Mat B; B220hi CD19+ IgM+ AA4.1+); spleen cell gates: total B (CD19+), Imm B (CD19+CD23+CD21/35+), follicular B (FB; CD19+CD23+CD21/35mid), and marginal zone B cells (MZB; CD19+CD23-CD21/35+). Data are pooled from four independent experiments.

**FIGURE 2.** Fak deletion affects pro-B cell growth. (A and B) B220lo IgM+ CD34+ EGFP+ sorted BM pro-B cells (2 × 10^3 per plate) were sorted from CD19-Cre Fak KO or WT mice and plated in CFC pre-B (IL-7) media in the presence or absence of CXCL12 (500 ng/ml). (A) Colony count on day 7, followed by total live cell count after staining with 7-AAD. (B) A representative flow cytometry analysis of 7-d CFC colonies is shown; percentage of IgM+ cells and IgM+ c-Kit+ cells is marked under each gate as mean ± SEM. Data are pooled from 10 experiments. (C) B220lo IgM+ CD34+ EGFP+ BM cells (2 × 10^3 per plate) were plated in CFC pre-B (IL-7) media in the presence or absence of CXCL12 (500 ng/ml). Colonies were recovered after 7 d and replated under the same culture conditions (2 × 10^3 per plate). The number of secondary colonies was counted on day 7 (n = 10). Data are pooled from 10 experiments. (D) Cells from the CFC colonies were analyzed by flow cytometry. The fraction of apoptotic (Annexin V+ 7-AAD+) and dead (Annexin V+ 7-AAD+) cells were assessed among the CD19+ IgM+ cell population. Percentage Annexin V+ (apoptotic and dead) cells were plotted (mean ± SEM). Data are pooled from four experiments. Student t test, *p < 0.05, **p < 0.01.
cytometry analysis. (red), CD43 or IgD (green), and DAPI (blue) followed by iCys imaging.

Tudoral femur sections from C57BL/6 WT mice were stained for B220 cells in the endosteal region of the metaphyses and the diaphysis. Longitudinal, **comparative distance of B220+ CD43+ and B220+ IgD+ cells to the bone

quencies (mean ± SD) of fluorescently stained B220+ IgD+ and B220+ CD43+ cells, can be visualized in a tissue map with

formation so that the distribution of discrete cell populations, that is, CD19-Cre deletion affects progenitor B cell growth (31). Intravascular B cells were determined by FACS. For the BM, the number of homed cells was calculated by multiplying the number of cells harvested from a hind leg by 14.3, assuming that one limb contains ~7% of all BM cells (31). Intravascular B cells were determined by in vivo PE-Cy7 labeling.

Results

Fak deletion causes a selective decrease in BM progenitor B and immature B cells

Fak was conditionally deleted in B cells by breeding FakKO mice with CD19-Cre- mice to generate CD19-CreKO FakKO mice, that is, CD19-Cre Fak KO (from now on termed Fak KO). PCR and immunoblotting analysis of sorted BM or spleen cells showed that Fak deletion was evident beginning at the pro-B cell stage and was associated with absence of FAK protein (Supplemental Fig. 1A–E). Progenitor B cell populations, for example, CD19+ B220+ CD43+ IgM+, CD19+ B220+ CD43+ IgM+, and CD19+ B220+ AA4.1+ IgM+ immature B cells, were reduced by 30–40% compared with WT control mice (Fig. 1A). Similarly, newly emigrated immature B cells from BM, for example, T1 B cells in spleen, also were reduced by 30–40% (Supplemental Fig. 2B, 2C), whereas the follicular and marginal zone B cell compartments were unaffected (Supplemental Fig. 2A). The total number of BM B220+ B cells in CD19-Cre Fak KO was reduced by 30% compared with WT control mice. In contrast, the numbers of recirculating mature B cells in BM and mature B cells in peripheral blood (PB) and spleen (CD19+ AA4.1+ IgM+/IgD- cells) were similar in CD19-Cre Fak KO and WT control mice (Fig. 1A, 1B), even though these mature B cell populations exhibited efficient Fak deletion (Supplemental Fig. 1C, 1D). Subsequently, the selective decrease of progenitor B cells and immature B cells in the BM was confirmed in mb1-Cre FakKO mice (Fig. 1C). Fak deletion did not cause a change in CXCR4 and VLA4 surface expression on B cells from BM and spleen (data not shown). Moreover, expression of the FAK-related tyrosine kinase Pyk-2 was not increased in Fak-deleted progenitor and mature B cells (Supplemental Fig. 1E); similarly, Pyk-2 compensation was not observed in Fak-deleted megakaryocytes, neuronal cells, and embryonic endothelial cells (26, 32).

Fak deletion affects progenitor B cell growth

Given the selective decrease in progenitor B cell populations in BM of Fak KO mice (Fig. 1A), we theorized that Fak deletion might influence pro-B cell growth in response to progenitor B cell cytokines/chemokines. To explore this possibility, we generated CD19-Cre+ FakKO Rosa26-EGFP+ mice in which EGFP ex-
pression correlates with efficient Fak gene deletion (Supplemental Fig. 1F). CFC assays with IL-7 were performed in the presence and absence of CXCL12 to examine the effect of CXCL12 signaling on pro-B cell growth. The number of CFC colonies and total cell number from Fak-deleted B220<sup>lo</sup> IgM<sup>-</sup> CD43<sup>+</sup> EGFP<sup>+</sup> pro-B cells cultured in IL-7 ± CXCL12 were 40–50% lower compared with the number of colonies and cells from WT B220<sup>lo</sup> IgM<sup>-</sup> CD43<sup>+</sup> EGFP<sup>+</sup> pro-B cells (Fig. 2A). Of interest, B220<sup>lo</sup> IgM<sup>-</sup> CD43<sup>+</sup> pro-B cells did not generate colonies in IL-7 ± CXCL12 methylcellulose (data not shown). CXCL12 acted synergistically with IL-7 to promote colony formation and cell growth in WT mice and Fak KO mice (Fig. 2A). Flow cytometry performed on CFCs from both WT and Fak KO mice retrieved on day 7 of culture showed that >80% were IgM<sup>-</sup> progenitor B cells, of which 20 and 14% are c-Kit<sup>+</sup> pro-B cells, respectively (Fig. 2B).

The colony-replacing assay is an in vitro surrogate assay in which replating capacity of CFCs reflects the limited self-renewal capacity of hematopoietic precursor cells, including pro-B cells (33, 34). As shown in Fig. 2C, the number of secondary colonies derived from Fak-deleted pro-B cells was significantly reduced, indicating that Fak deletion reduces the replating activity of pro-B cells. Finally, further investigation of the in vitro cultures showed that Fak deletion also promoted cell apoptosis; the fraction of apoptotic cells was significantly higher (ranging from 50–70%) in Fak-deleted progenitor B cells compared with WT pro-B cells. 20 and 14% are c-Kit<sup>+</sup> pro-B cells, respectively (Fig. 2B).

**FIGURE 4.** FAK regulates the distribution of pro-B cells in femoral BM microenvironments. Based on their frequency within specific BM regions of CD19-Cre Fak KO and WT control mice, B220<sup>lo</sup> CD43<sup>+</sup> pro-B cells were graphed in the (A) metaphyses versus diaphysis of BM and in the endosteal region (ER) versus central medullary region (CMR) of diaphysis. Columns and error bars represent mean ± SEM, Student’s t test: **p < 0.01, ***p < 0.001. n = 4. (B) The distances of CD19-Cre Fak KO and WT control B220<sup>lo</sup> CD43<sup>+</sup> pro-B cells to endosteum in endosteal areas were measured and graphed (n = 4 mice). Dots indicate individual cells, n = 262 (WT) and n = 300 (KO). A scatter dot plot is shown with median ± interquartile range indicated as broader and narrower horizontal lines, respectively. Two-tailed Mann–Whitney U test: ***p < 0.001. Accumulated percentage of B220<sup>lo</sup> CD43<sup>+</sup> pro-B cells from WT or Fak KO mice is shown in endosteal areas. Numbers of cells analyzed for the distance to endosteum, n = 262 (WT) and n = 300 (KO). Broken lines indicate the accumulated percentage of target cells within 100-μm distance to endosteum. (C) Longitudinal femur sections were stained with Abs against B220 (red), CD43 (green), and osteopontin (white), and DAPI (blue) followed by iCys imaging cytometry analysis. Representative field images show B220<sup>lo</sup> CD43<sup>+</sup> pro-B cell (white arrowhead) on the osteopontin <i>n</i>nich. Cellular events in osteopontin <i>n</i> integration contour (within 5 μm outside of osteopontin signals; Cyan) are identified as cells contacting Osn cells. Percentages (mean ± SEM) of total cells, B220<sup>lo</sup> B cells, and B220<sup>lo</sup> CD43<sup>+</sup> pro-B cells contacting osteopontin cells are shown in the metaphyses of CD19-Cre Fak WT and KO. Average of 39,683 (WT) and 38,354 (KO) cells in the metaphyses of femur sections were analyzed from 4 mice each. Student’s t test, *p < 0.05, n = 4. Data are pooled from four independent experiments. (D) The frequency of B220<sup>lo</sup> CD43<sup>+</sup> pro-B cells and in specific BM regions of mb1-Cre Fak KO and WT control mice were graphed in the metaphyses versus diaphysis of BM and in the endosteal region (ER) versus central medullary region (CMR) of diaphysis as shown in (A). Student’s t test: *p < 0.05, **p < 0.01, n = 3.
the metaphyses, whereas mature B cells were preferentially in the diaphysis (Fig. 3C). Moreover, within the diaphysis, pro-B cells localized predominantly in the endosteal region (<100 μm from the endosteum), whereas mature B cells tended to localize outside the endosteal region (Fig. 3D). In addition, the distance to endosteal surface was measured for individual pro-B cells and mature B cells in longitudinal BM sections. The collective data illustrate a distinctive, nonrandom gradient distribution of pro-B cells with a high predilection for the endosteum (Fig. 3E, 3F).

FAK regulates the distribution of pro-B cells in femoral BM microenvironments

Because FAK phosphorylation can be triggered via chemokine, Ag, and integrin receptors on B cells (15, 24, 25), we hypothesized that FAK also might regulate the distribution of B220+ CD43+ pro-B cells in the BM cavity. By quantitative imaging analyses of longitudinal sections of femurs, we found that in comparison with WT mice, the lodgment of pro-B cells is significantly altered in Fak KO mice, resulting in an even distribution in the metaphyses and diaphyses (Fig. 4A). Moreover, their striking close proximity to the endosteum is no longer evident as the accumulated fraction of pro-B cells in the endosteal region is reduced by 50% (Fig. 4B).

We explored this observation more specifically and determined that Fak deletion impairs the close proximity of pro-B cells to osteopontin* bone-lining osteoblasts in the metaphyses (Fig. 4C). We also confirmed the impairment of pro-B cell distribution in the BM of mb1-Cre* FakK0/
K0 mice (Fig. 4D). Thus, we conclude that FAK influences the localization of pro-B cells in BM microenvironments.

Fak deletion leads to mobilization of pro-B cells to the periphery

We considered that Fak deletion might also cause a defect in pro-B cell retention in BM, and thus enhance the egress of pro-B cells

FIGURE 5. Fak deletion leads to mobilization of pro-B cells to the periphery. (A–C) Appearance of colony-forming pro-B cells in the periphery of Fak KO mice under steady-state condition. Representative data of four experiments are shown (n = 8). (A) CFC assays were performed to detect pro-B cells from hemolyzed PB (0.25 ml/dish) in methylcellulose-based IL-7–containing CFC media. Colonies were counted on day 7 and plotted as colony number per 1 ml blood. Data are expressed as the mean ± SEM (n = 12; *p < 0.05). (B) Representative flow cytometry plot of B220+ gated cells is shown. Mean percentages of B220+ IgM− and B220+ IgM− c-Kit+ subpopulations are shown in the indicated gates. (C) The CFC cells from Fak KO mice were subjected to Fak genotyping as shown in Supplemental Fig. 1. (D–F) Appearance of B220+ CD19+ IgM− IgD− CD93+ progenitor B cells in the spleen and PB of immunized WT and Fak KO mice at days 0–14 were characterized and enumerated by flow cytometry (n = 4). (D) Representative flow cytometry profiles of B220+ CD19+ IgM− IgD− CD93+ progenitor B cells in WT spleen from naive and immunized on day 4 are shown. The frequencies of CD93+ B220+ gated cells are indicated as percentage of total cells. The frequency of c-Kit+ progenitor B cells is indicated as percentage of CD93+ B220+ cells. Kinetics of B220+ CD19+ IgM− IgD− CD93+ progenitor B cell numbers in spleen (E) and PB (F) after immunization. (G and H) Localization of B220+ CD43+ pro-B cells was examined in the BM from naive and NPCGG/alum-immunized femurs (n = 4) by LSC as described in Fig. 4. The frequency of B220+ CD43+ pro-B cells in the endosteal region (within 100-μm distance of endosteal surface) is graphed (G) in the metaphyses and (H) in the diaphysis. Data are from four independent experiments. Asterisks indicate significant differences from controls: *p < 0.05, **p < 0.01, ***p < 0.001.
to the periphery. An increase in the number of CD19<sup>+</sup> B220<sup>+</sup> IgM<sup>-</sup> progenitor B cells could not be discerned with confidence by flow cytometry in PB or spleen of Fak KO (not shown). Thus, the methyl cellulose-based CFC assay was applied because this method has been used to quantitate low numbers of mobilized hematopoietic progenitor cells in PB (35). By the CFC assay, significantly increased numbers of pro-B cells were detected in PB of Fak KO compared with control WT mice (Fig. 5A). The 7-d CFCs were then analyzed by flow cytometry and shown to be mainly CD19<sup>+</sup> B220<sup>+</sup> IgM<sup>-</sup> IgD<sup>-</sup> CD93<sup>+</sup> progenitor B cells, of which 22% were c-KIt<sup>-</sup> pro-B cells (Fig. 5B); we assessed for c-Kit surface expression rather than CD43 expression because the latter marker remains high during differentiation of pro-B cells grown in IL-7–containing culture conditions (36). We confirmed that these progenitor B cells were Fak deleted as determined by PCR (Fig. 5C).

We next reasoned that FAK function might be further manifested under stress conditions, such as immunization with the inflammatory Ag nitrophenol-conjugated chicken γ-globulin in alum (NP-CGG-alum), which induces significant progenitor B cell mobilization to the periphery (37). To this end, the presence of B220<sup>lo</sup> CD19<sup>+</sup> IgM<sup>-</sup> IgD<sup>-</sup> CD93<sup>+</sup> progenitor B cells, of which 42% are c-KIt<sup>-</sup> pro-B cells, was analyzed in the spleen and PB after immunization (Fig. 5D). FACS analyses showed that the percentages of B220<sup>lo</sup> CD19<sup>+</sup> IgM<sup>-</sup> IgD<sup>-</sup> CD93<sup>+</sup> progenitor B cells gradually increased from day 4 up to day 14 in the spleen and blood. However, the percentages of progenitor B cells increased substantially (>2.5–3 times) more in Fak KO mice (Fig. 5E, 5F). Immunization of alum alone produced similar results as immunization with NP-CGG-alum (data not shown), indicating that the egress of pro-B cells is induced by inflammatory immunization, and thus is not Ag dependent. Concomitantly by LSC analysis of femoral sections, immunization with NP-CGG-alum led to a reduction in the percentage of B220<sup>lo</sup> CD19<sup>+</sup> IgM<sup>-</sup> IgD<sup>-</sup> CD93<sup>+</sup> progenitor B cells in the endosteal zones (<5 μm osteopontin<sup>+</sup> bone-lining osteoblasts), which also was significantly more noticeable in Fak KO mice (Fig. 5G, 5H). Taken together, these data suggest that FAK plays an important role in the retention of progenitor B cells in BM.

**Pro-B cell homing to the BM cavity is regulated by FAK**

Because homing and engraftment of hematopoietic stem and progenitor cells are dependent on the coordinated function of cytokines/chemokines, for example, CXCL12 (38), and adhesion molecules, for example, VCAM-1 (39), we hypothesized that FAK also might be important in the engraftment of i.v. infused pro-B cells (40). Homing experiments showed that the number of homed pro-B cells was significantly reduced for Fak-deleted compared with WT pro-B cells (Fig. 6A). Noteworthy was the striking reduction in number of homed Fak-deleted pro-B cells that had lodged in the extravascular compartment of BM. In contrast, the number of homed pro-B cells localized intravascularly was equivalent for Fak-deleted and WT pro-B cells (Fig. 6A, 6B).

**Discussion**

Niches defined within disparate anatomical regions of the BM cavity are thought to provide distinct cues for the growth, differentiation, and survival of hematopoietic stem and progenitor cells. Prior studies (15, 17, 18) on the role of the CXCR4-FAK-VLA4 pathway in progenitor B cells led us to examine FAK function in vivo and to test the hypothesis that FAK, by coordinating signaling via chemokine/chemokine and adhesion receptors, might play an important role in progenitor B cell lodgment in BM. Cre-mediated Fak deletion results in a decrease in pro-B-, pre-B-, and immature B cell populations in the BM. In contrast, the mature B cell compartments in the BM and spleen are unaffected. This likely occurs because in Fak KO mice, adequate numbers of immature B cells are generated, which subsequently fill the mature B cell compartment in peripheral lymphoid organs similar to what has been observed in normal B cell development and other gene-targeted mouse models (4, 41). To further explore the underlying reasons accounting for the increase in progenitor B cells in the BM of Fak KO mice, we first considered that FAK function might play an important role in pro-B cell growth. To explore this possibility, we used the cytokine-supplemented CFC methylcellulose assay because it is a well-established assay for growth studies on hematopoietic stem/progenitor cells (42, 43). Although IL-7 is the standard cytokine used for the CFU-B assay (44), we explored the addition of CXCL12 because it is considered essential for early stages of B cell development (10, 45). CXCL12 acted synergistically with IL-7 to promote colony formation and cell growth of WT and Fak-deleted pro-B cells (Fig. 2A). The exact molecular mechanism for this observation is unclear but is likely related to the multiple cytoplasmic and nuclear FAK pathways, which promote cell growth and survival. We find that Fak-deleted cells generate a significantly lower number of progenitor B cells and colonies. Moreover, Fak deleted cells have impaired colony-replating potential, a feature that has been ascribed to the distinct ex vivo clonability of pro-B cells (46). Fak-deleted pro-B cells undergo increased apoptosis in culture, which contributes to their impaired growth. One mechanism for the prosurvival function of FAK is its regulation of p53 (47), which mediates activation of effector caspases during apoptosis (48). In this regard, evidence for caspase-3–mediated apoptosis in Fak-deleted pro-B cell lines was previously reported (49).

Although the association of pro-B cells with specific BM niche cells, such as CXCL12<sup>+</sup> abundant reticulocytes or IL-7<sup>+</sup> stromal cells, has been reported (8), the global distribution of pro-B cells in the entire femur cavity remains unknown to date. Using quantitative imaging cytometry technique LSC, we report a distinctive nonrandom gradient distribution of pro-B cells with a high predilection for the endosteum (Fig. 3E, 3F). The observed gradient in pro-B cell distribution may be the consequence of several
processes. It is conceivable that even the most primitive committed lymphoid progenitor cells already have a gradient distribution. Alternatively, differentiating progenitor B cells migrate within the BM compartment, and their ultimate location is influenced by differences in composition and/or availability of B cell–specific niches in the endosteal region versus the central medullary region, which contains the central sinus.

The disruption of the nonrandom, gradient distribution of pro-B cells in the BM (Fig. 4) by Fak deletion suggests that the distribution of pro-B cells is regulated by a niche-induced FAK signaling pathway, supporting the previously proposed function of FAK in the CXCL12-induced cell adhesion to VCAM-1 in vitro studies (15, 17, 18). Further studies are needed to understand the physiological significance of the distinctive nonrandom gradient distribution of pro-B cells in the BM.

Interestingly, Fak deletion also leads to the egress of pro-B cells into the peripheral circulation. This mobilization was further augmented by immunization with the inflammatory immunogen NP-CGG-alum, which causes a reduction of CXCL12 expression in the BM under inflammatory conditions coincident with egress of the BM under inflammatory conditions coincident with egress of pro-B cells and impaired B lymphopoiesis (37). It is thus reasonable to speculate that in Fak deleted pro-B cells, the CXCR4–FAK- VLA4 pathway is impaired because inhibition of CXCR4/CXCL12 and/or VLA4/VCAM-1 axes leads to the mobilization of hematopoietic stem and progenitor cells (50–53). Together, the data suggest that disruption of the interaction between pro-B cells and niche cells contributes to impaired B lymphopoiesis and possibly in leukemia development because FAK also plays an important function in malignancy, including acute myelogenous leukemia (54–56). Moreover, FAK silencing inhibits leukemogenesis of BCA/ABL-transformed pro-B (BaF3) cells (49). Thus, further definition of the complexity of niche components, for example, vascular and diverse stromal cell types, and the signaling pathways they trigger may provide further insight into B cell development and potentially offer therapeutic targets of leukemia.

Acknowledgments
We thank Drs. C. Walkley and S. Orkin (Boston Children’s Hospital) for Rosa26-EGFP
mic, Drs. Michael Reth (Max-Planck Institute for Immunobiology, Freiburg, Germany) and Klaus Rajewsky (Harvard Medical School, Boston, MA) for Mb-1–Cre mice; N. Calonder (Brigham and Women’s Hospital) for cryosectioning of femoral bones, and CompuCyte (Westwood, MA) for LSC technical help.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Information

FAK Regulates the Localization and Retention of Pro-B Cells in BM Microenvironments.
Shin-Young Park, Peter Wolfram, Kimberly Canty, Brendan Harley, César Nombela-Arrieta, Gregory Pivarnik, John Manis, Hilary E Beggs, and Leslie E Silberstein

Figure S1. Analysis of Cre-mediated Fak deletion. (A) Schematic representation of the wild type, floxed Fak allele, and excised locus. (B) PCR and immunoblot (IB) analysis of BM CD19+ B cells (purity > 97%) and whole BM (WBM) from CD19-Cre+/− Fak0/0 knock out mice (KO) and CD19-Cre−/− Fak0/0 control mice (WT; 8 week old). Fak gene assessed by PCR with primer 1, 2 and 3. FAK protein expression by WB with a monoclonal anti-FAK antibody (Upstate Biotech.) (C) Fak gene deletion by PCR in sorted B cells of BM: Pro-B (CD19+ B220lo CD43+ IgM−), Pre-B (CD19+ B220lo CD43− IgM−), Immature B cells (Imm B, CD19+ B220lo IgM+), Mature B cells (Mat B, CD19+ B220hi IgM+) of Fak KO mice. (D) PCR and immunoblot analysis of splenic CD19+ B cells of Fak KO and WT control mice. Representative data from four independent experiments are shown. (E) Immunoblot (IB) analysis of Pyk2 protein level with monoclonal anti-Pyk2 (Transduction Laboratory) antibodies in sorted B cells of BM: Pro-B, Pre-B, Immature B cells (Imm B), Mature B cells (Mat B) of WT and Fak KO mice. After secondary horseradish peroxidase-conjugated antibodies (Bio-Rad) were treated, immunoblots were analyzed by enhanced chemiluminescence (Amersham Biosciences). (F) Cre-mediated excision of floxed stop codon leads to EGFP gene expression. PCR analysis of Cre-mediated Fak gene deletion were performed with primer P1, P2, and P3 in EGFP+ cells (G2 gate) and EGFP− control cells (G1 gate) of CD19-Cre+/− Fak0/0 ROSA26-EGFP+ (KO) mice. ROSA26-EGFP− mice were used as controls for flow cytometry. (G) Fak gene deletion was analyzed by PCR in sorted B cell subpopulations from mb-1-Cre+/− Fak0/0 ROSA26-EGFP+ or mb-1-Cre−/− Fakwt/wt ROSA26-EGFP+ mice. BM CD3+ T cell from mb-1-Cre+/−
**Figure S2. Fak deletion does not affect the number of follicular and marginal zone B cells in spleen.**

(A) Single cell suspensions from spleen were analyzed by flow cytometry. Gate: Imm – CD19+ CD23- CD21/35- immature B cells, FO – CD19+ CD23+ CD21/35mid follicular B cells, MZ – CD19+ CD23-fo CD21/35hi marginal zone B cells. (B) Splenic transitional B cells were analyzed by flow cytometry. IgM+ CD23- transitional 1 (T1), IgM+ CD23+ transitional 2 (T2), and IgM- CD23+ transitional 3 (T3) B cells were gated as shown. % of gated cell population is marked next to each gate. Representative data from four independent experiments are shown. (C) Total numbers of each transitional B subset cells per spleen were calculated using flow cytometry. Student’s t-tests were performed as shown *, P<0.05 and **, P<0.01 (Unpaired, two-tailed).

**Figure S3. Laser scanning cytometry analysis of spleen sections and morphology of the femoral BM cavity.**

(A) Validation of quantitative imaging cytometry on 5μm histologic sections of cryopreserved splenic tissue. B220+ B cells (green) and nucleated cells (blue, DAPI) in C57BL/6 mouse spleen. Image: laser scan mosaic image of the entire tissue section. Scale bar: 500μm. Frozen sections were stained with rat anti-B220 antibody (RA3-6B2, BD Biosciences) or isotype control antibody (rat IgG2a, BD Biosciences) followed by Alexa 488- labeled secondary antibody and DAPI nuclear staining dye. Individual cellular events are identified by nuclear staining (DAPI). % of B220+ cells were gated from dot plot. (B) Hematoxylin and Eosin (H&E) staining of the femoral bone from C57BL/6 wild type
mouse. The magnified diaphyseal cavity shows cortical bone surrounding dense BM and a portion of central sinus (pink cavity). The distal metaphysis shows the characteristic trabecular bone structure with BM within the trabeculae. Blue arrows: bone surface, Black dotted region: endosteal region, Black arrows: vascular system, Green dotted region: central medullary region. Representative data from four independent experiments are shown.

**Figure S4. Localization of progenitor B cells in femoral BM microenvironments by solid-phase laser scanning cytometry.** Longitudinal femur sections from C57BL/6 wild type (WT) mice were stained with B cell markers. Sinusoidal niche was stained by goat anti-CD105 (endoglin) antibody / Dylight 649-labeled donkey anti-goat IgG antibody (white), followed by nuclear DAPI staining (blue). Stained slides were analyzed by iCys imaging cytometry. (A) A square area of a femur is magnified to show a representative region image. (B) Representative field images of CD43, B220, and endoglin /DAPI channels are shown. Arrowheads indicate B220⁺ CD43⁺ pro-B cell. (C) A field image of DAPI channel is shown with threshold contours which are expanded by 2 pixels for integration contours. (D) Fluorescence intensity in different channels is quantified for each individual event as shown in a dot plot with specific gates based on isotype control staining. Numbers within the plot indicate the percentage of cells in each population. Representative data from four independent experiments are shown.
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A

CD19+ WT

MZ 5% FO 64%

Imm 14%

CD23

KO

CD19+ MZ 6% FO 57%

Imm 10%

CD23

B

WT

CD19+AA4.1+

T3 18% 27%

21% 24%

KO

CD19+AA4.1+

T3 23% 29%

21% 23%

IgM

C

Number of transitional B cells in spleen

<table>
<thead>
<tr>
<th>Subset</th>
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<th>KO</th>
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<td>T1</td>
<td>2.39 ± 0.15</td>
<td>1.65 ± 0.17**</td>
</tr>
<tr>
<td>T2</td>
<td>1.82 ± 0.13</td>
<td>2.31 ± 0.14*</td>
</tr>
<tr>
<td>T3</td>
<td>1.17 ± 0.08</td>
<td>1.38 ± 0.10</td>
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(mean ± SEM, millions, n=8)