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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,*†,1 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 γ-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 Gα, 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 Ig, 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

KO) (26) were crossed to Cd19-Cre mice (Jackson Laboratory) to generate Cd19-Cre* Fak

KO mice. Cd19-Cre Fak KO mice with the enhanced GFP reporter gene (EGFP* Cd19-Cre Fak KO) were
produced by crossing Cd19-Cre<sup>ERT2</sup> Fak<sup>lo</sup> mice with Rosa26-EGFP<sup>ERT2</sup> 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<sup>ERT2</sup> Fak<sup>lo</sup> mice or littermate CD19-Cre<sup>ERT2</sup> Fak<sup>lo</sup> mice, which we found to be phenotypically equivalent, at 8–12 wk of age were used as wild type (WT) controls. The m<sup>nb–1–Cre</sup> Fak<sup>lo</sup> Rosa26-EGFP<sup>lo</sup> mice were generated by crossing Fak<sup>lo</sup> Rosa26-EGFP<sup>lo</sup> with m<sup>nb–1–Cre</sup> mice, a generous gift from Dr. Michael Reth (Max-Planck Institute for Immunobiology, Freiburg, Germany). The m<sup>nb–1–Cre</sup> Fak<sup>lo</sup> 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<sup>+</sup> Fak<sup>lo</sup> 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 P1: 5'-GACCTCTAACAATCTCTATTTC-3', primer P2: 5'-GAAGTGCTAAGGACACAAATAC-3'; primer P3: 5'-GAGAATGACCTGGGTCGTT-3'. The amplified PCR products consisted of a WT (1.4 kb by P1 and P2 primers, 290 bp by P2 and P3 primers) and a floxed (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'-CACAACAGTTAGTCCGG, reverse 5'-CGTATAGCGCAATTGGCAG-3') as previously described (27). Efgp genotyping was performed using the PCR primers (forward 5'-GACCATGAAAGCCACGCG-3', reverse 5'-CCGATGAGGGTGTTCTGTCG-3') with the conditions: 33 cycles of 94°C for 30 s, 57°C for 1 min, resulting in a 450-bp product (28). In contrast with Cd19-Cre gene expression, m<sup>nb–1–Cre</sup> 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<sup>2+</sup> free, Mg<sup>2+</sup> 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 iliac crest, tibia, and femur with various fluorescent dye-labeled lineage-specific Abs (i.e., CD19, B220, CD43, IgM, and IgD). Flow cytometric analysis was carried out on a FACSVerse (Becton Dickinson), and cell sorting on a FACSARia (Becton Dickinson). The following Abs were purchased from Becton Dickinson, eBioscience, and BioLegend: FITC-, PE-, allophycocyanin-, PE-Cy7-, PerCP-, or allophycocyanin-Cy7–labeled anti-B220 (RA3-6B2), anti-CD93 (AA4.1), anti-CD43 (S7), anti-CD24/HSA (30F1), anti-BP-1 (6C3), anti-CD117/c-Kit (2B8), anti-CD19 (6D5), anti-CD11b/Mac-1(M1/70), anti-GR1 (Gr-1), anti-IgM (II/41), anti-IgD (11–26), anti-B220 (RA3-6B2; eBioscience), followed by Dylight 488–labeled donkey anti-rat IgG (Jackson Immunoresearch). For Rosa26-GFP WT or KO mice, we used chicken anti-GFP Ab (Invitrogen), rat anti-CD20 (BD Biosciences), anti-CD19 (B220), B220<sup>lo</sup> IgM<sup>+</sup> B cells (M3630; Stem Cell Technology), and goat anti-flxed IgG (Jackson Immunoresearch), followed by Dylight 488–labeled streptavidin (Jackson Immunoresearch). For nontargeted sections, were stained with goat anti-osteopontin Ab (R&D Systems) for osteoblasts, rabbit anti-laminin (Chemicon) for vasculature, or goat anti-CD105 (endoglin) Ab (R&D Systems) for sinusoids followed by Dylight 488–labeled donkey antimouse Ab (Jackson Immunoresearch). For Rosa26-GFP<sup>lo</sup> WT or KO mice, we used chicken anti-GFP Ab (Invitrogen), rat anti-CD20 (BD Biosciences), anti-CD19 (B220), B220<sup>lo</sup> IgM<sup>+</sup> B cells (M3630; Stem Cell Technology), and goat anti-flxed IgG (Jackson Immunoresearch), followed by Dylight 488–labeled streptavidin (Jackson Immunoresearch). Pro-B cells were identified by GFP<sup>+</sup> CD43<sup>+</sup> cells. Dilutions of primary and secondary Abs were optimized for Dylight 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 nm), 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-scan excited fluorescein 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 per 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 us to define the thinnest section to allow definition of the edge of the cell (pixel size is 0.25 × 0.25 μm). Isotype Abs were used as negative controls for gating purposes. The total number and morphological distribution of each distinct cellular subpopulation within the entire femoral BM cavity or at specific anatomical locations within the cavity can then be determined using postscan automated image analysis software iCys cytometric analysis software (Compucyte). To assess statistical significance, we analyzed a minimum of three distinct frozen sections from each femur from a minimum of four Fak KO and four WT control animals. LSC analysis in cryopreserved splenic tissue was performed to validate the quantitative imaging cytometry technology because of its well-characterized B cell compartments. The percentage of B220<sup>+</sup> B cells determined via flow cytometry (50%) and LSC (42 ± 6.3%, mean ± SEM) in splenic mononuclear cells was comparable (Supplemental Fig. 3A), if taking under consideration that the laser-scanning cytometer also quantitates nonhemopoietic cells, for example, fibroblasts and endothelial cells. Imaging cytometry further allowed for global characterization of the follicular organization of B220<sup>+</sup> B cells in spleen (Supplemental Fig. 3A). H&E staining of nondecalcified, cryopreserved, 5-μm-thick.
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 Fak<sup>ko<sup> ROsa26-EGFP<sup> KO and mb1-Cre Fak<sup>ko<sup> ROsa26-EGFP<sup> WT mice by FACSia (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 aminomuconar im dye. 5-(and-6)-((4-chloromethyl)benzoyl]amino) tetramethylrhodamine–labeled WT splenic B cells were coinjected as reference cells. A total of 1 x 10<sup>6</sup> 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. After

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**FIGURE 1.** Selective decrease of progenitor B cell number in B cell–specific FAK KO mice. (A) BM cells from Cd19-Cre Fak<sup>ko</sup> 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 (B220<sub>lo</sub> CD19+ IgM<sub>2</sub> CD43+), pre-B (B220<sub>hi</sub> CD19<sup>+</sup> IgM<sup>-</sup> CD43<sup>-</sup> ), immature B (imm B; B220<sup>+</sup> CD19<sup>+</sup> IgM<sup>-</sup> CD43<sup>-</sup> ), and mature B cells (Mat B; B220<sup>+</sup> CD19<sup>+</sup> IgM<sup>+</sup> AA4.1<sup>+</sup>); spleen cell gates: total B (CD19<sup>+</sup>), imm B (CD19<sup>+</sup> CD23<sup>-</sup> CD21<sup>-</sup>/35<sup>-</sup> ), follicular B (FB: CD19<sup>+</sup> CD23<sup>+</sup> CD21<sup>-</sup>/35<sup>-</sup> ), and marginal zone B cells (MZB; CD19<sup>+</sup> CD23<sup>-</sup>/35<sup>-</sup> CD21<sup>-35</sup> ). Data are pooled from four independent experiments.

**FIGURE 2.** Fak deletion affects pro-B cell growth. (A and B) B220<sup>+</sup> IgM<sup>+</sup> CD43<sup>+</sup> EGFP<sup>+</sup> sorted BM pro-B cells (2 x 10<sup>3</sup> per plate) were sorted from Cd19-Cre<sup>+</sup> Fak<sup>ko</sup> ROsa26-EGFP<sup>-</sup> KO mice 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<sup>-</sup> cells and IgM<sup>-</sup> c-Kit<sup>+</sup> cells is marked under each gate as mean ± SEM. Data are pooled from 10 experiments. (C) B220<sup>+</sup> IgM<sup>+</sup> CD43<sup>+</sup> EGFP<sup>+</sup> BM cells (2 x 10<sup>3</sup> 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 x 10<sup>3</sup> 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<sup>+</sup> 7-AAD<sup>-</sup>) and dead (Annexin V<sup>+</sup> 7-AAD<sup>+</sup>) cells were assessed among the CD19<sup>+</sup> IgM<sup>-</sup> cell population. Percentage Annexin V<sup>+</sup> (apoptotic and dead) cells were plotted (mean ± SEM). Data are pooled from four experiments. Student t test, *p < 0.05, **p < 0.01.
CD43 or IgD, and DAPI (blue) followed by iCys imaging. longitudinal femur sections from C57BL/6 WT mice were stained for B220 cells in the endosteal region of the metaphyses and the diaphysis. Longitudinal surface (white dotted line). Arrowheads indicate B220+ CD43+ and B220+ IgD+ cells to the bone.

**Statistical analysis**

Statistical analysis was performed on all numerical data as indicated in each figure using GraphPad Prism software (GraphPad, San Diego, CA).

**Results**

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

Fak was conditionally deleted in B cells by breeding Fak−/− mice with CD19-Cre−/− mice to generate CD19-Cre−/− Fak−/− 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+ B220lo CD43+ IgM−, CD19+ B220lo CD43− IgM−, and CD19+ B220lo 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−/lo 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−/− Fak−/− KO 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−/− Fak−/− 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\(^6\) IgM\(^-\) CD43\(^+\) EGFP\(^+\) pro-B cells cultured in IL-7 ± CXCL12 were 40–50% lower compared with the number of colonies and cells from WT B220\(^6\) IgM\(^-\) CD43\(^+\) EGFP\(^+\) pro-B cells (Fig. 2A). Of interest, B220\(^6\) IgM\(^-\) CD43\(^+\) 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\(^-\) progenitor B cells, of which 20 and 14% are c-Kit\(^+\) pro-B cells, respectively (Fig. 2B). The colony-replating 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 cultured in IL-7 ± CXCL12.}

**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\(^6\) CD43\(^+\) 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 (C) Mb1-Cre mice, and in the endosteal area (ER) versus central medullary region (CMR) of diaphysis as shown in (A). Student t test: \(*p < 0.05, \**p < 0.01, \***p < 0.001, n = 4.

The distances of CD19-Cre Fak KO and WT control B220\(^6\) CD43\(^+\) 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 < 0.01, \**p < 0.001, \***p < 0.001. Accumulated percentage of B220\(^6\) CD43\(^+\) 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 line indicates 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\(^6\) CD43\(^+\) pro-B cell (white arrowhead) on the osteopontin\(^+\) niche. Cellular events in osteopontin\(^+\) integration contour (within 5 μm outside of osteopontin\(^+\) signals; Cyan) are identified as cells contacting Opm\(^+\) cells. Percentages (mean ± SEM) of total cells, B220\(^+\) B cells, and B220\(^+\) CD43\(^+\) 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 t test, \(*p < 0.05, \**p < 0.01, \***p < 0.001, n = 4. Data are pooled from four independent experiments. (D) The frequency of B220\(^6\) CD43\(^+\) pro-B cells within 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) as shown in (A). Student t test: \(*p < 0.05, \**p < 0.01, n = 3.

Nonrandom, gradient distribution of pro-B cells in longitudinal sections of femoral BM

Although highly desirable, a comprehensive analysis of the global spatial distribution of phenotypically defined B cell populations in the context of entire BM cavities has not been carried out. This is mostly due to the technical difficulties associated with imaging of long bones and the need for cell-surface marker combinations used to track B cell populations at a single cell level. To this end, we used LSC, which provided a detailed quantitative map of the spatial distribution of B cell subsets in whole-longitudinal femoral BM sections (Supplemental Fig. 4A–D). The fluorescence levels associated with each cellular event are measured and plotted while retaining the localization of the cellular event within the entire longitudinal BM section (Supplemental Fig. 3A, 4D). This approach allows for the generation of tissue maps (Fig. 3B) and the objective quantification of discrete B cell subsets within different regions, for example, microenvironments. Distribution data are shown for pro-B cell and mature B cell stage populations, for example, B220\(^6\) CD43\(^+\) and B220\(^6\) IgD\(^+\) B cells, respectively (Fig. 3). Although B cells of all stages were found throughout the BM cavity, the distribution of pro-B cells differed from that of mature B cells. B220\(^6\) CD43\(^+\) pro-B cells were localized preferentially in

**FIGURE 3.** Longitudinal femur sections of Mb1-Cre Fak KO and WT control mice were stained with Abs against Fak (red), CD43 (green), and osteopontin (white), and DAPI (blue). (A) Longitudinal femur sections were stained with Abs against Fak (red), CD43 (green), and osteopontin (white), and DAPI (blue). Representative field images show B220\(^6\) CD43\(^+\) pro-B cell (white arrowhead) on the osteopontin\(^+\) niche. Cellular events in osteopontin\(^+\) integration contour (within 5 μm outside of osteopontin\(^+\) signals; Cyan) are identified as cells contacting Opm\(^+\) cells. Percentages (mean ± SEM) of total cells, B220\(^+\) B cells, and B220\(^+\) CD43\(^+\) 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 t test, \(*p < 0.05, \**p < 0.01, \***p < 0.001, n = 4. Data are pooled from four independent experiments. (B) The frequency of B220\(^6\) CD43\(^+\) pro-B cells within 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) as shown in (A). Student 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+ FakKO 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.
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> 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>CD93<sup>+</sup> progenitor B cells, of which 42% were 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>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>+</sup> CD19<sup>+</sup> IgM<sup>-</sup>CD93<sup>-</sup> progenitor B cells residing intravascularly 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).

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/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 decrease 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 pre-dilection 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 immunobiology, Freiburg, Germany) and Klaus Rajewsky (Harvard Medical School, Boston, MA) for critical discussion.

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References
11. Dar, O., P. Goulich, V. Verheij, A. Kalinkovich, O. Kollet, N. Netzer, R. Margalit, M. Zsak, A. Nagler, I. Hardan, et al. 2005. Chemokine receptor deletion also leads to the egress of progenitor 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 BCR/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.

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