

Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow

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The coordinated production of leukocytes in bone marrow is crucial for innate and adaptive immunity. Inflammation alters normal leukocyte production by promoting granulopoiesis over lymphopoiesis, a response that supports the reactive neutrophilia that follows infection. Here we demonstrate that this specialization for granulopoiesis is determined by inflammation-induced reductions of growth and retention factors, most significantly stem cell factor and CXCL12, which act preferentially to inhibit lymphoid development. These hierarchical effects suggest that the normal equilibrium of leukocyte production in bone marrow is determined by lymphopoiesis' higher demand for specific growth factors and/or retention signals. Inflammation regulates this balance by reducing growth factors that have less impact on developing neutrophils than lymphocytes. We demonstrate that granulopoiesis and lymphopoiesis are coupled specifically in the bone marrow by development in a common niche and propose that the leukopoietic equilibrium is specified by limiting amounts of developmental resources.

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Abbreviations used: Ab, antibody; Ag, antigen; APC, allophycocyanin; BAFF, B cell-activating factor; BMp, bone marrow plasma; CFSE, carboxyfluorescein diacetate succinimidyl ester; CLP, common lymphocyte progenitor; CMP, common myeloid progenitor; CMRA, Cell Tracker orange; HPRT, hypoxanthine-guanine phosphoribosyltransferase; PTX, pertussis toxin; SCF, stem cell factor; TXR, Texas red fluorochrome.

Neutrophils develop through proliferative, then nonmitotic stages in BM (1). Mature neutrophils are short-lived and localize rapidly to inflammatory sites where they deliver microbicidal activity (2, 3). This localization produces a brief neutropenia that is corrected by accelerated emigration of BM neutrophils, and subsequently, a reactive neutrophilia that is characterized by abundant, less mature cells (1). Reactive neutrophilia is a crucial component of innate immunity because persistent neutropenia leads to death by infection (4, 5).

The BM regulates the homeostatic release of mature neutrophils and the accelerated production of granulocytes in response to inflammatory signals (6, 7). However, the mechanisms of this regulation is unknown. We (8) and others (9) have shown that infection, adjuvants, or proinflammatory cytokines, especially TNF α and IL-1 β , substantially reduce the numbers of lymphocytes, but not Gr-1⁺ granulocytes, in BM. These depletions correspond with mobilization of BM lymphocytes and concomitant reductions in CXCL12 levels (8). Pertussis toxin (PTX) also mobilizes BM lymphocytes with little effect on the numbers of Gr-1⁺ BM cells (8), which suggests that G-protein-coupled signals control the retention of lymphocytes but not granulocytes in the BM.

We have proposed that inflammation-induced emigration of lymphocytes from BM represents a physiologic response to ensure increased neutrophil production in response to infection, albeit at the expense of central B lymphopoiesis (8). That inflammation might control BM granulopoiesis and lymphopoiesis independently via reductions in CXCL12 expression was a surprising observation; mice deficient for CXCL12 or its receptor, CXCR4, share severe impairments of B lymphocyte and granulocyte production (10–12) that could reflect a common mechanism to localize lymphocyte and granulocyte progenitors in the BM (13).

Here, we show that immature neutrophils in BM are relatively resistant to the inflammatory signals that mobilize lymphocytes and mature neutrophils. Inflammation specifically reduces BM stem cell factor (SCF) and CXCL12 to levels that do not support lymphopoiesis but remain sufficient for continued granulopoiesis. Reactive neutrophilia is the consequence of the expansion of the immature, proliferating neutrophil compartments into BM niches that are vacated by lymphocytes and mature neutrophils. The reciprocal dynamics of the BM lymphocyte and neutrophil populations are consistent with cellular competition within a developmental niche that is defined by a com-

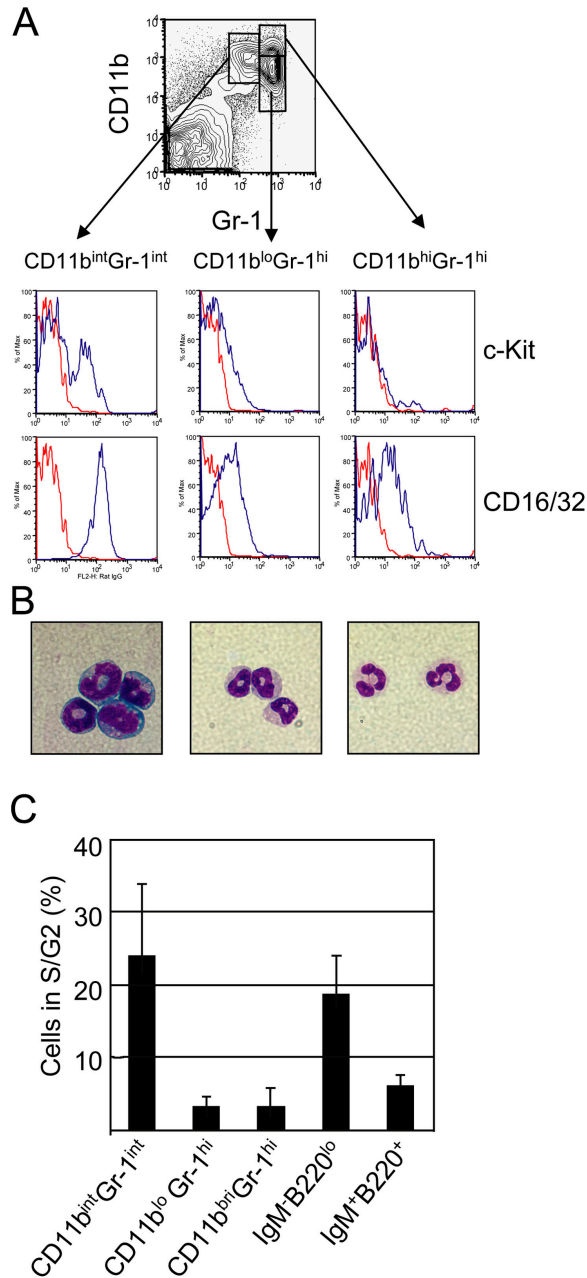


Figure 1. Definition of neutrophil subsets in the BM of naive C57BL/6 mice. BM cells were labeled with mAbs specific for mouse CD11b, CD16/32, c-Kit, or RB6-8C5 Ags. Labeled cells were analyzed/sorted by flow cytometry. Data represent three determinations with two mice/group ($n = 6$). (A) Expression of CD11b and the RB6-8C5 Ag identifies three populations of double-positive cells: CD11b^{int}Gr-1^{int}, CD11b^{lo}Gr-1^{hi}, and CD11b^{hi}Gr-1^{hi}. These populations expressed distinct levels of CD40, c-Kit, F4/80, and CD16/32 (blue) as compared with nonspecific, isotype-matched control Abs (red). (B) Sorted CD11b^{int}Gr-1^{int}, CD11b^{lo}Gr-1^{hi}, and CD11b^{hi}Gr-1^{hi} BM cells after Giemsa staining. CD11b^{int}Gr-1^{int} cells exhibit the morphology of promyelocytes and myelocytes; CD11b^{lo}Gr-1^{hi} and CD11b^{hi}Gr-1^{hi} cells represent increasingly mature cell forms. (C) Fraction of BM neutrophil and B cell populations in the G₂ and S phases of the cell cycle. Among the BM granulocyte compartments, only CD11b^{int}Gr-1^{int} promyelocytes and myelocytes exhibit significant levels of mitosis. The

mon location and shared growth resources. Blocking BM lymphopoiesis results in the specific and reciprocal expansion of the granulocytic compartments of BM, with no effect on other hematopoietic lineages. Thus, inflammation regulates the normal balance of granulopoiesis and lymphopoiesis in BM by suppressing common growth factors that affect lymphopoiesis more strongly than granulopoiesis.

RESULTS

Three neutrophil compartments in BM

Granulocytes in mouse BM can be categorized into increasingly mature subsets on the basis of nuclear and granular morphology, or by the expression of CD11b and the RB6-8C5 antigen (Ag; 14, 15). mAbs that are specific for CD11b and the RB6-8C5 Ag (Gr-1⁺) identify three distinct double-positive populations in naive BL/6 mice: CD11b^{int}Gr-1^{int}, CD11b^{lo}Gr-1^{hi}, and CD11b^{hi}Gr-1^{hi} (Fig. 1 A). The CD11b^{int}Gr-1^{int} cell population expressed significantly higher levels of c-Kit and CD16/32, both markers of developmental immaturity (Fig. 1 A). The neutrophil-specific mAb NIMP-R14 (16) efficiently labeled the CD11b^{lo}Gr-1^{hi} and CD11b^{hi}Gr-1^{hi} cells and bound a subset (60%) of the CD11b^{int}Gr-1^{int} compartment (unpublished data).

After sorting to high purity, CD11b^{int}Gr-1^{int} cells were found to be primitive, with little nuclear condensation and azureophilic cytoplasm (Fig. 1 B). The morphology of CD11b^{int}Gr-1^{int} cells is consistent with that of promyelocytes and myelocytes (with occasional metamyelocytes; reference 17), the mitotic progenitors of all granulocytes. CD11b^{hi}Gr-1^{hi} cells were identical to mature peripheral neutrophils, whereas CD11b^{lo}Gr-1^{hi} cells represented an intermediate stage of neutrophil maturation (metamyelocytes and band forms) with faintly stained cytoplasm and incompletely condensed, ring-shaped nuclei (Fig. 1 B; reference 17).

Proliferating cells in each neutrophil population were identified by determining the DNA content in fixed and permeabilized cells (Fig. 1 C; reference 18). Consistent with its expression of c-Kit and morphology, the CD11b^{int}Gr-1^{int} population contained a high frequency (24 vs. 3%) of proliferating cells (Fig. 1 C). As controls, 19% of IgM⁻B220^{lo} pro-/pre-B cells were in cycle compared with 6% in the mature, IgM⁺B220^{hi} population (Fig. 1 C). Thus, CD11b and Gr-1 expression reveal three increasingly mature granulocyte populations. CD11b^{int}Gr-1^{int} cells are mitotically active promyelocytes and myelocytes; CD11b^{lo}Gr-1^{hi} cells are nondividing, immature neutrophils; and CD11b^{hi}Gr-1^{hi} cells are mature neutrophils.

Reciprocal dynamics of BM lymphocyte and granulocyte populations

Inflammation mobilizes lymphocytes from the BM without reducing Gr-1⁺ cell numbers (8). The subsequent granulocyte

DNA content of single cells in each phenotypic compartment was determined by intracellular propidium iodide staining. Bars represent the average fraction (\pm SD) of cells in the G₂ and S phases of the cell cycle.

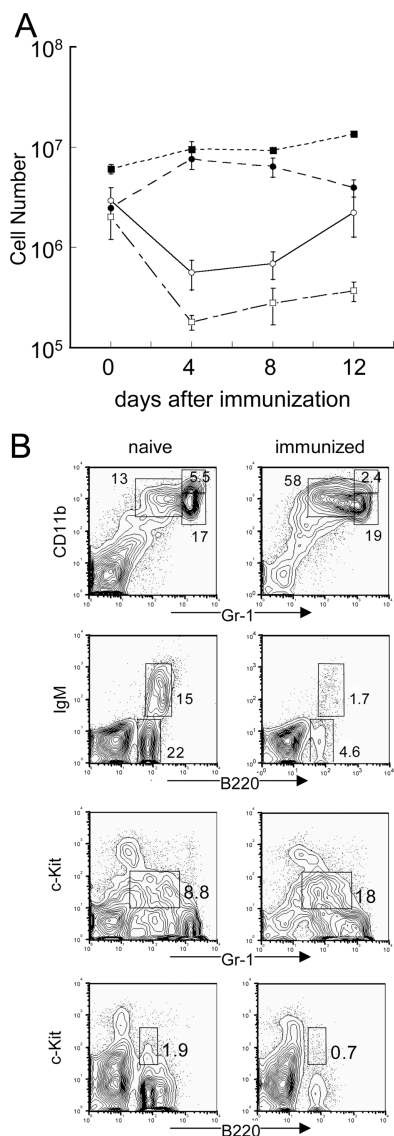


Figure 2. Immunization with adjuvant depletes mature, but not immature, neutrophils from BM. (A) Population dynamics of BM neutrophil and B cell subsets after immunization. 4 to 16 d after immunization, BM cells from C57BL/6 mice ($n = 4-6$) were recovered and labeled with mAb specific for CD11b, the RB6-8C5 Ag, or B220. CD11b^{int}Gr-1^{int}, CD11b^{lo}Gr-1^{int}, and CD11b^{hi}Gr-1^{hi} neutrophils and B220^{lo} and B220^{hi} lymphocytes were enumerated by flow cytometry to determine the effects of immunization on each population. CD11b^{int}Gr-1^{int} and CD11b^{lo}Gr-1^{int} cell numbers expand and contract reciprocally to B220^{lo} B cells. Points represent average (\pm SEM) numbers of Gr-1^{int} (●), Gr-1^{hi} (■), B220^{lo} (○), or B220^{hi} (□) cells from a tibia and femur at the times indicated; naive controls are shown (day 0). (B) FACS profiles of CD11b⁺Gr-1⁺ and B220⁺ BM cell populations before (left), and 4 d after (right) immunization represent three independent experiments with two mice/group ($n = 6$). Immunization reduces the CD11b^{hi}Gr-1^{hi} neutrophil subset and both B220^{lo} and B220^{hi} lymphocytes, but expands CD11b^{int}Gr-1^{int} and CD11b^{lo}Gr-1^{int} cell sets.

cytosis that was elicited by inflammatory stimuli could result from a specific expansion of granulocytic progenitors or a general enhancement of the ability of BM to support he-

matopoiesis (8). To investigate these possibilities, we followed the dynamics of developing and mature neutrophils and lymphocytes in the BM after immunization (Fig. 2).

4 d after immunization, the proliferating CD11b^{int}Gr-1^{int} neutrophil compartment grew three- to fourfold, whereas the mature CD11b^{hi}Gr-1^{hi} populations changed little (Fig. 2 A); coincidentally, B220^{lo} and B220^{hi} cell numbers decreased approximately threefold (Fig. 2 A; reference 8). The reciprocal behavior between CD11b^{int}Gr-1^{int} neutrophils and B220⁺ lymphocytes was repeated subsequently; as B220⁺ BM cell numbers returned to normal, the expanded, immature neutrophil compartments diminished (days 8 and 12; Fig. 2 A).

In contrast with the rapid changes in B220⁺ lymphocyte and CD11b^{int}Gr-1^{int} neutrophil numbers, the mature neutrophil compartment of BM is more stable. In naive mice, a tibia and femur contain $\sim 7 \times 10^6$ CD11b^{hi}Gr-1^{hi} neutrophils; 4 d after immunization that number increases $\sim 40\%$, and by day 12 doubles to $\sim 1.4 \times 10^7$ (Fig. 2 A).

Adjuvant-induced expansion of the BM's primitive granulocytic compartment includes the c-Kit⁺CD11b^{int}Gr-1^{int} cell subset and occurs even as c-Kit⁺ pro-B cells are lost from BM (Fig. 2 B; reference 8). This reciprocity suggests a regulatory mechanism that acts by reducing lymphopoietic resources while sparing factors that are important for granulocyte development.

Inflammation differentially modulates growth factors in BM

The inflammation-induced emigration and subsequent recovery of BM lymphocytes correlates with changes in CXCL12 mRNA and protein expression (8). Mindful that CXCL12 is crucial for granulocytic and lymphoid progenitors to colonize the BM (11–13), we used quantitative RT-PCR to identify differentially regulated factors that might expand granulopoiesis in BM that is transiently deficient in CXCL12.

mRNA specific for B cell-activating factor (BAFF), CXCL2, CXCL12, GAPDH, GM-CSF, hypoxanthine-guanine phosphoribosyltransferase (HPRT), IL-1 β , IL-3, IL-7, and SCF were quantified relative to GAPDH message at various times after immunization (Table I and Fig. 3 A). As expected, CXCL12 message decreased ~ 10 -fold at days 4 and 8 after immunization (8); recovers to 50% of naive levels were achieved by day 12; and 16 d after immunization, CXCL12 mRNA levels were ≥ 10 -fold higher than naive controls (Fig. 3 A). SCF mRNA followed a similar kinetics, and decreased 10-fold by day 4 and recovered to supranormal levels by day 16. Immunization decreased BAFF and IL-7 message levels by not $>50\%$, a decrease that also was observed in the HPRT control (Fig. 3 A). However, although HPRT message returned to naive levels, mRNA that was specific for IL-7 and BAFF became elevated (170% and 400% of naive controls, respectively) 16 d after immunization. Overexpression of CXCL12, SCF, BAFF, and IL-7 in BM coincides with recovery of central B lymphopoiesis and a return to normal levels of granulocyte production (Fig. 2 A; reference 8).

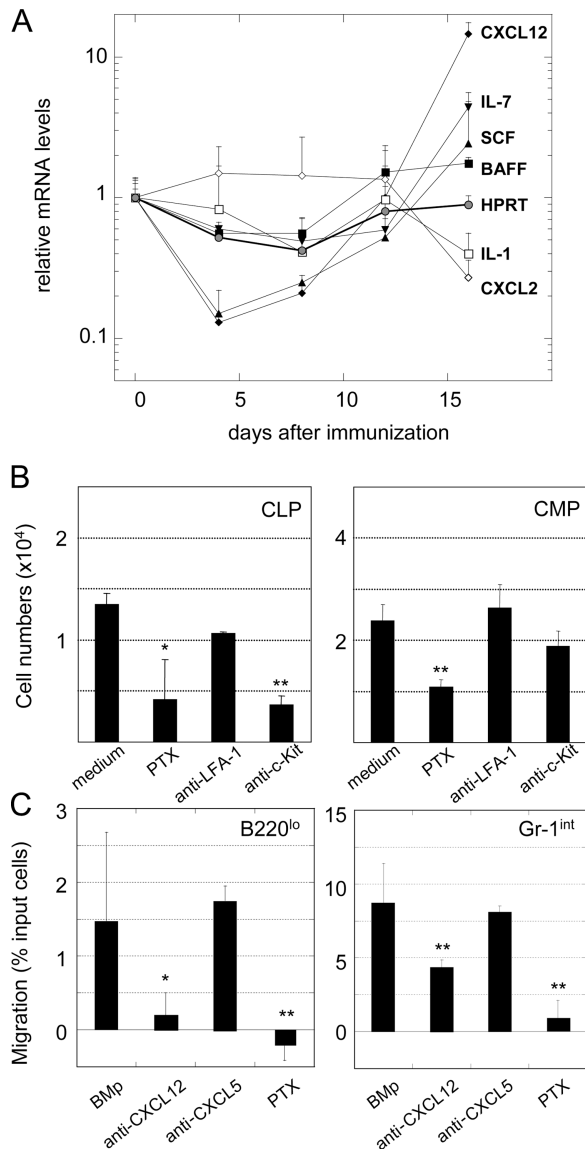


Figure 3. Inflammation modulates the expression of lymphoid, but not myeloid, growth factors in the BM. (A) Immunization strongly (>10-fold) reduced steady-state levels of mRNA specific for CXCL12 and SCF; relative to HPRT, BAFF and IL-7 message levels were not suppressed significantly. By 16 d after immunization, mRNA levels for lymphocyte growth factors were supranormal. mRNA specific for various growth factors was quantitated in reference to GAPDH message present in the same sample. Each point ($n = 2-5$) represents the average (\pm SD) amount of specific mRNA relative to mean levels present in naive controls. Levels of mRNA for GM-CSF and IL-3 were below the limits of detection. (B) Reduced c-Kit signaling preferentially inhibits proliferation/survival and differentiation of CLP over CMP. 100 CLP or CMP were cultured on adherent BM cell layers in media supplemented with hematopoietic growth factors ($n = 2-3$). PTX, or mAb specific for LFA-1 or c-Kit were added to cultures to determine the effect of reduced c-Kit signaling on lymphopoiesis and myelopoiesis. PTX inhibits lymphopoiesis and myelopoiesis equally but antagonistic anti-c-Kit mAb preferentially suppressed only the CLP cultures. (C) Preferential inhibition of chemotaxis of B220^{lo} and Gr-1^{int} BM cells by anti-CXCL12 mAb. In vitro, B220^{lo} and Gr-1^{int} BM cells migrate to BMP. This migration is abrogated completely by PTX and unaffected by mAb to

In contrast, mRNAs that are specific for IL-1 β and CXCL2, a potent activator of granulopoiesis (19) and neutrophil chemokines (20), respectively, were not changed significantly by immunization until day 12; both then decreased to 30–40% of naive levels. mRNA levels in BM for two important myelopoietic growth factors, IL-3 and GM-CSF, were below the detection limit ($\leq 10^{-5}$ GAPDH levels; Table I) of our PCR assay.

Inflammatory reductions of CXCL12 message in BM correlate with lower levels of CXCL12 protein (8). To determine if reduced SCF mRNA also decreased BM SCF protein, SCF in the BM plasma (Bmp) of naive and immunized mice (day 4; $n = 4$) was quantified by ELISA. SCF levels in naive mice averaged 163 pg/ml Bmp; Bmp from immunized mice contained only 35 pg/ml SCF, $\sim 21\%$ of control levels (unpublished data).

To determine whether reductions in SCF and CXCL12 expression might depress lymphopoiesis while sparing granulopoiesis, we determined the effects of reduced c-Kit signaling on the proliferation and differentiation of common lymphocyte progenitors (CLP) and common myeloid progenitors (CMP) in vitro (21, 22). In brief, ~ 100 CLP or CMP were introduced into established cultures of adherent BM cells that were supplemented with lymphoid or myeloid growth factors. PTX or antagonistic mAb specific for LFA-1 or c-Kit were added to replicate cultures. 4 d later, the effects of these treatments on CLP and CMP differentiation were determined by enumerating B220⁺ and Gr-1⁺ cells (Fig. 3 B).

PTX efficiently inhibits proliferation of CLP and CMP, and reduces the production of differentiated B220⁺ and Gr-1⁺ cells to $\sim 50\%$ of control cultures (Fig. 3 B). Although mAb to LFA-1 had little effect ($P > 0.05$) on CLP or CMP cultures, addition of c-Kit mAb significantly inhibited ($P < 0.01$) B220⁺ cell production from CLP (27% of controls), but had little activity in CMP cultures (80% of controls; Fig. 3 B).

Similarly, whereas B220^{lo} and Gr-1^{int} cells migrated to Bmp in vitro, lymphocyte migration was inhibited by mAb to CXCL12 far more effectively (13% of controls; $P = 0.04$) than granulocyte migration ($\leq 50\%$ of controls; $P = 0.05$), despite the greater sensitivity of Gr-1^{int} cells to the dilute source of chemoattractant (Fig. 3 C; reference 8). mAb to CXCL5 had no effect on B220^{lo} or Gr-1^{int} cell migration. In these in vitro assays, SCF and CXCL12, two BM growth factors specifically diminished by inflammation (Fig. 2), preferentially affect lymphocyte progenitors.

Homing of immature neutrophils to BM is PTX resistant

The different effects of anti-CXCL12 on the ability of developing B cells and neutrophils to migrate toward Bmp (Fig. 3 C) suggest distinct sensitivities to $G_{\alpha i}$ signals. Al-

CXCL5 ($n = 2$). However, mAb specific for CXCL12 suppresses the migration of B220^{lo} cells (13% of Bmp control) more than Gr-1^{int} cells (50% of Bmp control; $n = 5-7$). * $P \leq 0.05$; ** $P \leq 0.01$.

Table I. RT-PCR primers and relative message levels for lymphoid and myeloid growth factors in the BM of naive mice

Gene	Primers	Relative levels mRNA in BM of naive mice ^a
HPRT	forward 5'-GCTGGTGAAGGACCTCT-3' reverse 5'-CACAGGACTAGAACACCTGC-3'	1.76 ± 0.45 × 10 ⁰ C _T = 21.3 ± 0.2 ^b
GAPDH	forward 5'-AACTTGGCATTGTGGAAGG-3' reverse 5'-ACACATTGGGGGTAGGAACA-3'	1.00 × 10 ⁰ C _T = 22.4 ± 2.3
BAFF	forward 5'-TTCCATGGCTTCTCAGCTTT-3' reverse 5'-GGAATTGTTGGCAGTGTTT-3'	3.07 ± 1.00 × 10 ⁻¹ C _T = 23.9 ± 0.4
SCF	forward 5'-CGGGAATCCTGTGACTGATAA-3' reverse 5'-GGCCTCTCGGAGATTCTTT-3'	6.16 ± 2.03 × 10 ⁻³ C _T = 29.5 ± 0.4
CXCL12	forward 5'-GTCCTTGTCTGCCAGTC-3' reverse 5'-TAATTCGGGCAATGCACA-3'	3.95 ± 1.53 × 10 ⁻³ C _T = 30.3 ± 4.0
IL-1β	forward 5'-GACCTCCAGGATGAGGACA-3' reverse 5'-AGGCCACAGGTATTTTGTGCG-3'	8.74 ± 0.28 × 10 ⁻⁴ C _T = 32.6 ± 0.2
IL-7	forward 5'-TGGAAATCCTCCACTGATCC-3' reverse 5'-ACCAGTGTTTGTGCCTTG-3'	4.93 ± 1.75 × 10 ⁻⁴ C _T = 33.1 ± 0.3
CXCL2	forward 5'-AGTGAAGCTGCTGTCAATG-3' reverse 5'-AGGCACATCAGGTACGATCC-3'	1.19 ± 0.17 × 10 ⁻⁴ C _T = 36.1 ± 0.7
IL-3	forward 5'-TAGGGAAGCTCCCAGAACCT-3' reverse 5'-TTAGGAGAGACGGAGCCAGA-3'	<1.00 × 10 ⁻⁵ NA
GM-CSF	forward 5'-CATCAAAGAAGCCCTGAACC-3' reverse 5'-TGCATTCAAAGGGGATATCAG-3'	<1.00 × 10 ⁻⁵ NA

^amRNA levels are standardized to GAPDH message; levels of a second housekeeping gene, HPRT, control for nonspecific changes in message levels.

^bC_T values represent average threshold cycle number (±SD) for amplifications of cDNA reverse transcribed from 0.025 μg total RNA.

NA, not applicable.

though PTX depletes the BM of lymphocytes, it increases the numbers of Gr-1⁺ BM cells (8).

To determine the relative importance of G_{αi} signaling in the homing of BM lymphocytes and granulocytes, we incubated BM cells (1.5 × 10⁷) from B6.SJL mice (CD45.1) in media which contained 0, 25, or 100 ng/ml of PTX and then injected them into BL/6 (CD45.2) recipients. The next day, we enumerated CD45.1⁺ neutrophils and B cells in BM to determine homing efficiencies.

CD45.1⁺Gr-1⁺ and CD45.1⁺IgM⁻B220^{lo} cells were detected easily in recipient BM 14 h after transfer of untreated cells; treatment with PTX reduced the homing efficiency of mature CD45.1⁺CD11b^{hi}Gr-1^{hi} neutrophils and IgM⁻B220^{lo} pro-/pre-B cells more than fivefold (Fig. 4). In contrast, pretreatment with PTX resulted in nonsignificant, twofold reductions in the numbers of CD45.1⁺CD11b^{int}Gr-1^{int} pro-/myelocytes that returned to the BM (Fig. 4). Homing by immature, CD45.1⁺CD11b^{lo}Gr-1^{hi} neutrophils also was resistant to PTX. Doses of PTX that abrogated BM reentry by mature, CD11b^{hi}Gr-1^{hi} neutrophils and B220^{lo} lymphocytes reduced homing by immature neutrophils <20% (Fig. 4). Thus, CD11b^{int}Gr-1^{int} and CD11b^{lo}Gr-1^{hi} granulocytes localize in BM by a mechanisms that is rela-

tively insensitive to disruption of G_{αi}-coupled signaling; homing by B220^{lo} lymphocytes and mature, CD11b^{hi}Gr-1^{hi} neutrophils is PTX-sensitive.

A common niche for B220⁺ and Gr-1⁺ cell development

The reciprocal expansion of the Gr-1^{int} BM compartment following adjuvant-induced emigration of B220⁺ lymphocytes (Fig. 2; reference 8) suggested to us that granulocytes and lymphocytes might occupy a common developmental niche. If so, Gr-1^{int} and B220^{lo} cells must co-locate within the BM. Histologic sections of femurs from naive mice (Fig. 5 A) demonstrate that Gr-1⁺ and B220⁺ cell clusters are interspersed closely. 4 d after immunization, expansion by Gr-1⁺ cell clusters is obvious, whereas B220⁺ lymphocyte clusters become rare (Fig. 5 B). The proximity of granulocytes and B lymphocytes in the BM is consistent with a shared developmental niche but does not exclude the possibility of a tight mosaic of sites that is specific for myelo- or lymphopoiesis.

To determine whether granulocytes and B lymphocytes can develop within a common microenvironment, we prepared cultures of BM stromal cells free of hematopoietic progenitors (23) and seeded them with autologous c-Kit⁺ B220^{lo} and c-Kit⁺Gr-1^{int} cells labeled by carboxyfluorescein

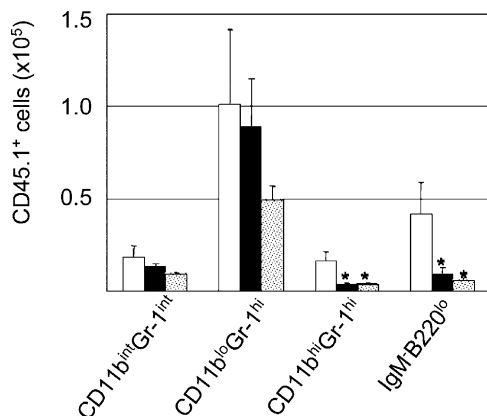


Figure 4. The ability of immature, CD11b^{int}Gr-1^{int} and CD11b^{lo}Gr-1^{hi} granulocytes to home to the BM is resistant to PTX. BM cells from B6.SJL (CD45.1) mice were exposed to PTX, 0 ng/ml ($n = 3$; open bars); 25 ng/ml ($n = 4$; filled bars); and 100 ng/ml ($n = 2$; stippled bars) and injected into C57BL/6 (CD45.2) recipients. 14 h later, CD45.1⁺ cells in host BM were enumerated by flow cytometry. Bars represent mean (\pm SD) numbers of CD45.1⁺ cells; significant ($P < 0.05$) changes from controls are indicated (*). Whereas exposure to 25 ng/ml PTX significantly reduced the numbers of transferred CD11b^{hi}Gr-1^{hi} neutrophils and B220^{lo} lymphocytes, homing by CD11b^{int}Gr-1^{int} and CD11b^{lo}Gr-1^{hi} granulocytes was inhibited insignificantly ($P > 0.05$), even after exposure to 100 ng/ml PTX.

diacetate, succinimidyl ester (CFSE; green) or Cell Tracker orange (CMRA; red). 1 d later, the numbers and distributions of B220⁺ (CFSE/green) and Gr-1⁺ (CMRA/red) cells in cultures with and without stromal cell layers were identified by fluorescence microscopy.

In the absence of stromal cells, brightly fluorescent, non-adherent B220⁺ and Gr-1⁺ cells were distributed uniformly and relatively infrequent (Fig. 5 C). In the presence of stromal cell layers, B220⁺ and Gr-1⁺ cell numbers were increased substantially (not depicted) and many B220⁺ and Gr-1⁺ cells had migrated beneath stromal cells to form “cobblestone clusters” (Fig. 5 D). The majority of cobblestone clusters contained B220⁺ and Gr-1⁺ cells that were dimly fluorescent and suggestive of cell division; mixed cell populations often were beneath a single stromal cell and demonstrated that c-Kit⁺B220^{lo} and c-Kit⁺Gr-1^{int} cells can develop in a common microenvironment (Fig. 5 D).

If the B220⁺ and Gr-1⁺ BM compartments also share a developmental niche in vivo, reductions in one lineage will result in a specific and compensatory expansion of the other (Fig. 2; reference 8). To test this prediction without disturbing the cytokine milieu of BM (Fig. 4), we enumerated myeloid (Gr-1⁺), lymphoid (B220⁺), and erythroid (TER119⁺) cells in the femurs of normal and RAG1-deficient mice. In brief, BM cells from C57BL/6 ($n = 5$) and congenic *Rag-1*^{-/-} mice ($n = 5$) were labeled with fluorochrome-coupled mAbs specific for the B220, TER119, or RB6-8C5 Ags; labeled and unlabeled cells from individual femurs were enumerated by flow cytometry.

On average, femurs from control mice contained $155.9 \pm 15.9 \times 10^5$ nucleated cells comprising (all $\times 10^{-5}$) 76.1 (± 8.5) B220⁺ cells; 42.9 (± 3.0) Gr-1⁺ cells; 18.3 (± 4.6) TER119⁺ cells; and 18.5 (± 2.6) unlabeled cells (Fig. 5 E). Femoral cell numbers in *Rag-1*^{-/-} mice ($130.6 \pm 13.4 \times 10^5$) were significantly ($P = 0.002$) lower than that of BL/6 controls, with all cell losses confined to B220⁺ compartments (Fig. 5 E). In RAG1-deficient BM, cells with a pre-/pro-B phenotype were increased (from 2×10^5 to 10×10^5), which was consistent with blockade of D-to-J_H joining (unpublished data; reference 24). Despite this expansion, femurs of RAG1-deficient mice contained only one third as many B220⁺ cells ($27.5 \pm 3.3 \times 10^5$ vs. 76.1×10^5) as controls. Without compensating increases in other BM cell compartments, femurs from RAG1-deficient mice should have contained only $\sim 108 \times 10^5$ nucleated cells.

Instead, losses in B220⁺ BM cell numbers were offset by increased numbers of Gr-1⁺ cells. The mean number of Gr-1⁺ femoral cells in RAG1-deficient mice was significantly higher ($P < 0.001$) than in controls; they increased from $42.9 \pm 3.0 \times 10^5$ cells to $62.9 \pm 9.5 \times 10^5$ cells. This increase represented expansions in all neutrophil compartments, including the proliferating CD11b^{int}Gr-1^{int} cell subset ($P = 0.01$). Significantly, femurs from *Rag-1*^{-/-} and control mice contained virtually identical numbers of TER119⁺ ($20.0 \pm 2.6 \times 10^5$ vs. $18.3 \pm 4.6 \times 10^5$, respectively; $P = 0.36$) and unlabeled cells ($20.2 \pm 2.8 \times 10^5$ vs. $18.5 \pm 2.6 \times 10^5$; $P = 0.21$). Removal of BM lymphocytes by a specific developmental blockade results in the compensatory expansion by Gr-1⁺ cells only. The specific and reciprocal coupling between Gr-1⁺ and B220⁺ cell numbers and their co-localization defines a common developmental niche for BM granulocytes and lymphocytes.

DISCUSSION

Leukocyte production in the BM is controlled by inflammatory cytokines. In concert, TNF α and IL-1 β induce the loss of BM lymphocytes by emigration while significantly expanding granulocyte production (8). This expansion is realized by increasing CD11b^{int}Gr-1^{int} pro-/myelocyte numbers (Fig. 1) in concordance with lymphocyte losses (Fig. 2). We conclude that the expansion of this generative, CD11b^{int}Gr-1^{int} compartment fuels the reactive CD11b^{lo}Gr-1^{hi}, CD11b^{hi}Gr-1^{hi} neutrophilia of inflammation and infection (Figs. 1 and 2; references 3–5, 25).

Inflammation’s reciprocal effects on BM lymphopoiesis and granulopoiesis could be coupled or independent; however, coupled regulation is the more parsimonious explanation to link these leukocyte compartments. To identify an inflammation-dependent mechanism that regulates granulopoiesis and lymphopoiesis we assayed mRNA specific for a variety of growth and localization factors (CXCL 12, CXCL 2, IL-1 β , IL-3, IL-7, GM-CSF, SCF, BAFF) that influence lymphocytes and neutrophils (Table I; Fig. 3). We observed

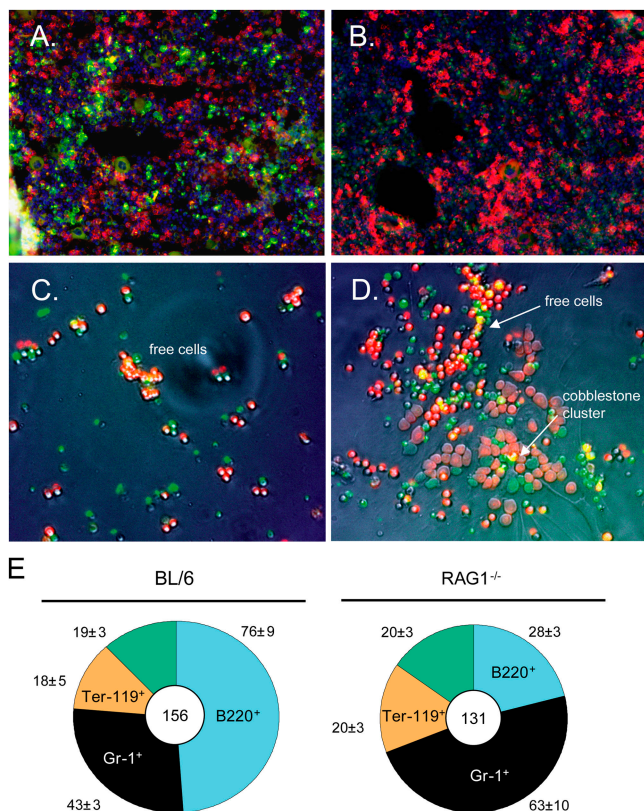


Figure 5. BM granulocytes and B lymphocytes share a common developmental niche. (A) The femoral BM of naive mice contains many closely associated Gr-1⁺ (red) and B220⁺ (green) cell clusters. (B) 4 d after immunization with adjuvants, the size of Gr-1⁺ BM cell clusters is much increased; this expansion includes areas that formerly were occupied by B220⁺ lymphocytes (see Fig. 1). (C) Culture of c-Kit⁺Gr-1⁺ (red) and c-Kit⁺B220⁺ P (green) in the absence of BM stromal cell layers results in little or no proliferation and very limited development. (D) In the presence of stromal cell layers, c-Kit⁺Gr-1⁺ and c-Kit⁺B220⁺ cells migrate beneath stromal cell bodies to form cobblestone clusters that contain lymphocyte- and granulocyte precursors. (E) Hematopoietic compartments in the BM of *Rag-1*^{-/-} and congenic C57BL/6 mice. Femoral BM cells from C57BL/6 ($n = 5$) and RAG1-deficient mice ($n = 5$) were labeled with the Gr-1 mAb and mAbs specific for B220 and TER119; labeled and unlabeled BM cells from individual femurs were enumerated by flow cytometry. Control femurs contained an average of 156×10^5 nucleated cells (center), comprising $\sim 76 \times 10^5$ B220⁺ cells, 43×10^5 Gr-1⁺ cells, 18×10^5 TER119⁺ cells, and 19×10^5 unlabeled cells (outer segments). Femoral BM cell numbers in *Rag-1*^{-/-} mice (center; 31×10^5) were significantly ($P = 0.002$) lower than controls with losses confined to B220⁺ compartments. Femurs of RAG1-deficient mice contained an average of only 28×10^5 B220⁺ cells but a compensating increase of Gr-1⁺ cell numbers to 63×10^5 cells/femur. In contrast, RAG1-deficient and control mice contained virtually identical numbers of TER119⁺ and unlabeled cells.

that inflammation's effects on the BM are specific; immunization with adjuvant significantly reduced only CXCL12 and SCF, and these reductions correlated exactly with the depletion of BM lymphocytes and the expansion of CD11b^{int}Gr-1^{int} cell numbers (Fig. 3; reference 8). Although

our search for regulated factors was not exhaustive, it is significant that in vitro and in vivo, reductions in CXCL12 and SCF preferentially disturb CLP and B220^{lo} lymphocyte development and localization with no or lesser effects on CMP and CD11b^{int}Gr-1^{int} granulocytes (Figs. 3 and 4). Adult viable c-Kit-deficient mice exhibit normal myelopoiesis with little or no B-lymphopoiesis (26); administration of AMD-3100, a specific antagonist of the CXCL12 receptor, CXCR4, mobilizes CD34⁺ human stem cells but not myelocytes or metamyelocytes (27). Given this biased activity, how could reductions of CXCL12 and SCF effect granulopoietic expansions?

An attractive possibility is that the reciprocal control of granulopoiesis and lymphopoiesis reflects competition between the generative CD11b^{int}Gr-1^{int} and B220^{lo} compartments in the BM. Thus, when inflammation reduces the capacity of the BM to retain lymphocytes and promote lymphopoiesis (Figs. 3 and 4; reference 8), granulocyte precursors acquire increased resources for proliferation and/or survival and expand into areas that are vacated by migrant lymphocytes. A prediction of this hypothesis is that lymphocyte and granulocyte progenitors occupy and compete in a common BM niche.

Histologic examination of femoral BM reveals B220⁺ and Gr-1⁺ cells which are organized in small, adjacent clusters (Fig. 5 A). Immunization with adjuvant alters this distribution by dramatically expanding Gr-1⁺ cell areas and reducing B220⁺ cell zones (Fig. 5 B); these histologic changes match the dynamics of the Gr-1⁺ and B220⁺ cell numbers after immunization (Fig. 2). To determine whether B lymphocyte and granulocyte development can occur in a common microenvironment, we cultured c-Kit⁺B220^{lo} and c-Kit⁺Gr-1^{int} cells with autologous BM stromal cells that were depleted of hematopoietic activity (23). These cultures supported large numbers of developing leukocytes that had migrated beneath the stromal cell layer to form cobblestone clusters. These clusters contained Gr-1⁺ and B220⁺ cells, often beneath a single stromal cell body (Fig. 5 D). The close association of B220⁺ and Gr-1⁺ cell clusters in the BM (Fig. 5 A) and their adjacent development in vitro (Fig. 5 D) provide strong support for our hypothesis that these cell compartments share a developmental niche in the BM.

If developing granulocytes and lymphocytes compete for a BM niche, reductions in one compartment must result in expansions by the other. We tested this prediction by comparing the numbers of cells belonging to three hematopoietic lineages in the BM of *Rag-1*^{-/-} mice and their normal, congenic controls. Losses of B220⁺ cells in the BM of RAG1-deficient mice were compensated only by increased numbers of Gr-1⁺ cells; the numbers of TER119⁺ erythroid lineage and unlabeled cells in RAG1-deficient and control mice were virtually identical (Fig. 5 E).

Our lymphocyte ablation experiment had three possible outcomes (Fig. 6). If B220⁺, Gr-1⁺, and TER119⁺ cells oc-

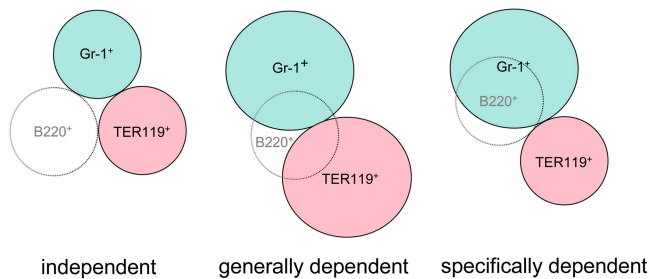


Figure 6. Models for interaction between the B220⁺, Gr-1⁺, and TER119⁺ hematopoietic compartments of BM. In normal femoral BM, B220⁺ lymphocytes, Gr-1⁺ granulocytes, and TER119⁺ erythroid cells are present in stable ratios (4:2:1; Fig. 5 E) that define one of three possible relationships between these hematopoietic compartments: independence, general dependence, or specific dependence. In the case of independence (left), growth resources for each compartment are distinct and the compartments do not compete. Under these conditions, removal of the large B220⁺ compartment by a lymphocyte-specific, developmental blockade would not alter the size/state of the other cell compartments. If all hematopoietic cell compartments share growth resources and are generally dependent (center), reductions in B220⁺ cell numbers would result in compensatory increases in the Gr-1 and TER119 compartments. Specific dependence (right) between hematopoietic compartments exists when some, but not all, compartments compete for a growth resources. If the B220⁺ and Gr-1⁺ BM compartments share a developmental niche that excludes the erythroid lineages, depletion of the B220⁺ cells would result in compensatory increases in Gr-1 cell numbers without changes in the TER119 compartment. This outcome is observed in the BM of RAG1-deficient mice (Fig. 5 E).

copy separate BM niches, reduction in any one cellular compartment will not affect the others. Conversely, if all hematopoietic lineages compete generally in BM, losses in any one compartment would be compensated by expansions of all of the others. Neither of these outcomes was observed in RAG1-deficient mice. Instead, reduced B220⁺ cell numbers expanded only the Gr-1⁺ cell compartment. This specific and reciprocal coupling between B220⁺ and Gr-1⁺ BM cell numbers defines a common developmental niche (Fig. 6) that may be visualized *in vitro* and *in vivo* (Fig. 5). Granulopoiesis and lymphopoiesis compete in the BM, whereas under these experimental conditions, erythropoiesis is independent of both.

The relative abundance of mRNAs for lymphoid growth factors (Table I) suggests that lymphopoiesis and granulopoiesis are balanced normally by a surfeit of growth resources that are lymphoid-specific or -preferent (Figs. 3 and 4; Table I). If so, reductions in CXCL12 and SCF would reduce BM lymphocyte numbers directly but promote granulopoiesis indirectly, by removing lymphocyte competitors. Reductions in SCF (mimicked by inhibiting *c-Kit* signaling) and CXCL12 primarily inhibit the activities of lymphocyte progenitors (Figs. 3 and 4). These biased effects provide a mechanism to shift the leukopoietic equilibrium in the BM toward granulopoiesis and the support of neutrophilic responses to infection (4, 5).

The new leukopoietic equilibrium that is established by inflammation may require additional signals to normalize.

We note that reestablishment of normal lymphopoietic levels coincides with supranormal expression of lymphoid growth factor genes and decreased IL-1 β and CXCR2 message (Fig. 3). Excess lymphoid support may be necessary to restore lymphoid progenitors to BM niches that are colonized by progenitor and immature granulocytes, and thereby, reverse increased granulopoiesis.

The reductions of BM CXCL12 and SCF elicited by the proinflammatory cytokines, TNF α and IL-1 β (8), preferentially affect lymphopoiesis and lymphocytes over the other hematopoietic compartments (Figs. 3 and 4). These differential effects and competition between the BM's B220⁺ and Gr-1⁺ compartments (Fig. 6) offer a simple, but robust, mechanism for innate immunity to regulate granulopoiesis and lymphopoiesis. During infection, acute granulocytic responses are crucial for host protection; in contrast to lymphocytes, mature granulocytes are very short-lived and are incapable of proliferation, and effective granulocytic responses require expanded leukopoiesis in the BM.

Why does the specialization of the BM lead to peripheral lymphopoiesis? We note that neutrophils, especially those elicited by myelopoietic growth factors and/or proinflammatory cytokines, release large amounts of BAFF (28, 29). Perhaps the reactive neutrophilia that is elicited by TNF α and IL-1 β amplifies extramedullary lymphopoiesis by increasing local levels of BAFF to support cell survival and/or differentiation to plasmacytes.

MATERIALS AND METHODS

Mice and immunizations. C57BL/6 (BL/6, CD45.2), congenic B6.SJL-Ptprca/BoAiTac (B6.SJL, CD45.1), B6.129S7-Rag1^{tm1Mom}/J were purchased (Jackson ImmunoResearch Laboratories or Taconic Farms). Mice were housed in specific pathogen-free conditions at the Duke University Animal Care Facility and given sterile bedding, water, and food. Mice used in these experiments were 6–18 wk of age; these studies were reviewed and approved by the Duke University Institutional Animal Care and Use Committee.

Mice were immunized by i.p. injections of (4-hydroxy-3-nitrophenyl)acetyl-chicken γ globulin (50 μ g) emulsified in IFA (Sigma-Aldrich; reference 23); (4-hydroxy-3-nitrophenyl)acetyl-chicken γ globulin contained 10 or 12 mol nitrophenyl/mol chicken γ globulin.

Flow cytometry. FITC, PE, biotin, or allophycocyanin (APC), PE-Cy5-conjugated mAb specific for mouse B220, IgM, CD11b, CD4, CD8, CD40, TER119, Thy1.2, CD117, CD45.1, F4/80, or RB6-8C5 were purchased from BD Biosciences or eBioscience. The Gr-1 mAb binds RB6-8C5 Ag, a marker of granulocyte development (15). CD93-specific 493 mAb (30) was purified from culture supernatants in our laboratory. Texas red (TXR) conjugates of antibody (Ab) for mouse IgM were purchased from Southern Biotechnology Associates, Inc. We used the neutrophil-specific mAb, NIMP-R14 (16), to confirm histologic identification of granulocyte subsets.

Mice were killed at various times after injection/immunization and single cell suspensions were prepared from femur and tibia. Erythrocytes were lysed in ammonium chloride buffer. For analysis, cells were suspended in 50–100 μ l of ice-cold HBSS containing 2% FCS and various combinations of labeled antibodies for 20 min. 7-Aminoactinomycin D (Molecular Probes) or propidium iodide (Sigma-Aldrich) was included to identify dead cells. Labeled cells were analyzed in FACScan (488 nm argon laser) or FACS Vantage SE (FACSDiVa digital option; 488 nm argon laser and 633 nm helium/neon laser) flow cytometers. For sorting, BM cell suspensions

were labeled with fluorochrome-antibody conjugates; flow cytometric data were analyzed with FlowJo software (Treestar Inc.).

Definition and isolation of BM cell populations. BM cells were labeled with FITC-conjugated Gr-1 mAb, PE- or TXR-conjugated anti-IgM, and APC-conjugated anti-B220. CD11b^{hi}Gr-1^{hi}IgM⁻B220⁻, CD11b^{lo}Gr-1^{hi}IgM⁻B220⁻, and CD11b^{int}Gr-1^{int}IgM⁻B220⁻ cells were doubly sorted to >98% purity in a FACS Vantage SE flow cytometer.

CMP and CLP were recovered from BM as described previously (21, 22). In brief, BM cells were labeled with PE-CY5-conjugated mAbs specific for CD4, CD8, TER119, Gr-1, CD11b, and B220. Labeled, Lin⁺ cells were depleted by incubation with anti-PE magnetic beads (Miltenyi Biotec; GmbH). To isolate CMP, Lin⁻ cell populations were stained with APC-conjugated anti-c-Kit mAb, TXR-conjugated anti-Sca-1, FITC-conjugated anti-CD34, and PE-conjugated anti-CD16/32; CLP were identified by labeling with APC-conjugated anti-c-Kit, TXR-conjugated anti-Sca-1, FITC-conjugated anti-Thy1.2, and PE-conjugated anti-IL-7R. CMP and CLP were recovered by sorting with a FACS Vantage SE flow cytometer.

Determination of cell cycle status. BM cells that were labeled by various combinations of fluorochrome-mAb conjugates were fixed in 1% paraformaldehyde at 4°C for 30 min and subsequently incubated with 1 µg/ml propidium iodide in 2% saponin/PBS at 4°C for 30 min. The DNA content in various cell populations was determined by quantifying levels of bound propidium iodide by flow cytometry (18). Cells containing 2N (G_{0/1}) or >2N levels (G₂ and S) of DNA were enumerated. Doublet cells were excluded by forward and side-scatter gating (18).

Quantification of mRNA levels. mRNA from BM cells was precipitated in Trizol reagent (Invitrogen) and reverse transcribed (Superscript II; Invitrogen). Quantitative PCR amplifications of cDNA were performed in an iCycler thermal cycler (Bio-Rad Laboratories) with SYBR Green PCR core reagents (Applied Biosystems) and primers specific for BAFF, CXCL2, CXCL12, GAPDH, GM-CSF, HPR1, IL-1β, IL-3, IL-7, or SCF transcripts (Table I). Standard amplification parameters for the quantitative PCR were as follows: initial denaturation at 94°C for 10 min; amplification cycle; denaturation at 94°C for 45 s; and anneal/extension at 64°C for 45 s. The relative expression levels for growth factor genes were calculated by the comparative C_T (threshold cycle) method recommended by the manufacturer (Applied Biosystems) normalized to GAPDH message in the same sample. In brief, ΔC_T values were determined by subtracting C_T(target) from C_T(GAPDH). Expression levels relative to GAPDH were defined as: 2^{-ΔC_T}. For the individual genes studied, expression data were standardized to averaged, homologous message in naive mice (n = 3–5); mRNA levels in the BM of naive mice for GAPDH, HPR1, BAFF, CXCL2, CXCL12, SCF, IL-1β, IL-7, IL-3, and GM-CSF vary over a ≥10⁵-fold range (Table I).

Adoptive cell transfer. The ability of cells to localize in the BM was determined by cell transfer. In brief, 2 × 10⁷ BM cells from B6.SJL (CD45.1) mice were incubated at 37°C in media containing PTX (0, 25, 100 ng/ml) for 1 h, washed, and injected i.v. into naive, congenic C57BL/6 recipients (CD45.2). Femoral and tibial BM cells were harvested 14 h later and labeled with mAb specific for CD45.1, the RB6-8C5 Ag, CD11b, B220, or IgM. Labeled CD45.1⁺ cells were analyzed and enumerated by flow cytometry to determine migration efficiencies.

Preparation of BM plasma. BMP was prepared by flushing femurs and tibias from four mice with 500 µl RPMI 1640 containing 0.5% BSA and 10 mM Hepes (migration media). Suspended cells were transferred into Eppendorf-type centrifuge tubes; cells and debris were removed by repeated (2×) centrifugation at 5,000 g for 10 min.

Chemotaxis assays. BM cell chemotaxis was quantified in 24-well Transwell plates (Corning Costar) containing cells at 5 × 10⁶/ml in migration media. Mouse CXCL5 (PeproTech) or CXCL12α (PeproTech) or BMP

was added to migration media to specific concentrations, and 500-µl aliquots were placed in the lower chambers of plates. In some experiments, neutralizing Ab to mouse CXCL12 (100 µg/ml; R&D Systems) or CXCL5 (50 µg/ml; R&D Systems) also was added. The 5-µm pore inserts were placed on the wells and preconditioned at 37°C for 1 h; BM cells (5 × 10⁵) were loaded (100 µl) into the upper chambers. Cells were allowed to migrate into the lower chamber for 4 h at 37°C. Subsequently, cells in the upper and lower chambers were incubated with antibodies specific for B220 and the RB6-8C5 Ag; labeled cells in duplicate cultures were enumerated by microscopy. In some experiments, the BM cells were pretreated with 100 ng/ml PTX for 1 h at 37°C before their introduction into the migration plate.

Cell culture. c-Kit⁺IgM⁻B220⁺ and c-Kit⁺CD11b⁺Gr-1⁺ BM cells were sorted and labeled (10⁴) with CFSE IMS (Molecular Probes, Inc.) or CMRA (Molecular Probes, Inc.). Labeled cells were cultured in established, long-term BM cultures containing ~2.5 × 10³ stromal cells (23); 1 d after co-culture, CFSE- and CMRA-labeled cells were analyzed by fluorescence microscopy.

CMP or CLP (10²) were cultured with the OP9 stromal cell line (31) in the presence of specific cytokines to promote proliferation and differentiation. For CMP cultures, 10 ng/ml IL-3 (R&D Systems) and GM-CSF (PeproTech) were added; CLP cultures contained 10 ng/ml IL-7 (R&D Systems) and Flt3-ligand (R&D Systems). After 5 d of culture, total myeloid or lymphoid cell numbers were determined by microscopy.

ELISA for growth factors. SCF levels in BMP were determined in naive, control, and adjuvant immunized mice (day 4) by the ELISA methods described by Ueda and colleagues (8). In brief, 96-well plates were coated with goat anti-SCF Ab (AF-455-NA); bound SCF was detected with biotinylated with a second anti-SCF goat Ab (BAF455; both R&D Systems). rMouse SCF (R&D Systems) was used to calibrate the assay and determine its detection limit (1 pg/ml).

Statistics. Statistical significance (P ≤ 0.05) of data was determined by Student's *t* test.

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