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Inflammation Controls B Lymphopoiesis by Regulating Chemokine CXCL12 Expression

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Abstract

Inflammation removes developing and mature lymphocytes from the bone marrow (BM) and induces the appearance of developing B cells in the spleen. BM granulocyte numbers increase after lymphocyte reductions to support a reactive granulocytosis. Here, we demonstrate that inflammation, acting primarily through tumor necrosis factor α (TNFα), mobilizes BM lymphocytes. Mobilization reflects a reduced CXCL12 message and protein in BM and changes to the BM environment that prevents homing by cells from naive donors. The effects of TNFα are potentiated by interleukin 1 β (IL-1β), which acts primarily to expand the BM granulocyte compartment. Our observations indicate that inflammation induces lymphocyte mobilization by suppressing CXCL12 retention signals in BM, which, in turn, increases the ability of IL-1β to expand the BM granulocyte compartment. Consistent with this idea, lymphocyte mobilization and a modest expansion of BM granulocyte numbers follow injections of pertussis toxin. We propose that TNFα and IL-1β transiently specialize the BM to support acute granulocytic responses and consequently promote extramedullary lymphopoiesis.

Key words: bone marrow • innate immunity • TNFα • hematopoiesis • neutrophilia

Introduction

Severe infections in humans deplete BM lymphocytes and induce the appearance of immature lymphocytes in the blood (1, 2). In mice, analogous responses follow infections (3) or administration of adjuvants (3, 4). Within a week of immunization, significant numbers of T cells and both developing and mature B cells are lost from mouse BM, whereas increases in granulocyte numbers and granulocytosis are often observed (3).

The mechanisms whereby adjuvants/inflammation elicit BM lymphopenia are not understood but are independent of adaptive immunity (4). Could innate immune effectors regulate BM lymphopoiesis? In vitro, IL-1 inhibits B lymphopoiesis and promotes myelopoiesis (5, 6). These effects are reversible, and appear to reflect change in nonhematopoietic BM compartments (6). The early stages of B cell development are exceptionally sensitive to apoptosis (7), and the proinflammatory cytokine IFNα/β can suppress B lymphopoiesis by inducing cell death (8–10). However, this suppression only occurs at pharmacologic doses (8), and does not require signal transducer and activator of transcription 1, the physiologic mediator of IFN signaling (11).

Alternatively, as inflammation elicits developing B lymphocytes in the periphery (3, 4, 12–16), BM lymphopenia could reflect mobilization rather than the interruption of a developmental pathway or cell death. For example, cells with the characteristics of pre–B and immature B lymphocytes appear in mouse spleen 2 wk after immunizations with adjuvant (3, 4, 13, 15, 17–19).

Here, we demonstrate that adjuvants suppress chemokine CXCL12 expression in the BM and that these reductions coincide with lymphocyte depletion and mobilization of B cell progenitors to the blood and spleen. Recombinant TNFα alone reduces BM CXCL12, and in TNFα-deficient mice, adjuvant-induced suppression of BM CXCL12 is mitigated, BM lymphopenia is much reduced, and mobilization of developing B cells is absent. Adjuvant effects on BM are largely mimicked by pertussis toxin (PTX), which uncouples most chemokine receptor signaling (20).

Inflammation redirects immunocyte production in BM to favor granulopoiesis. This redirection is an unrecognized

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The online version of this article includes supplemental material.

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Abbreviations used in this paper: CFU-B, pre–B cell CFU; NP-CGG, (4-hydroxy-3-nitrophenyl)acetyl-chicken γ globulin; PTX, pertussis toxin; SA, streptavidin.

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inflammatory response to microbial infection and a novel pathway for the regulation of B lymphopoiesis.

Materials and Methods

Mice. Female C57BL/6 (BL/6, CD45.2), B6.SJL-Ptprca/BoAiTac (B6.SJL, CD45.1), B6.129SF2, TNFα$^{-/-}$ (21), and TNF receptor I– and II–deficient (TNFR$^{-/-}$) mice (22) were obtained from the Jackson Laboratories or Taconic Farms. Mice were housed under 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 old.

Antigens, Adjuvants, and Cytokines. Mice were immunized with single, i.p. injections of 20 or 100 μg (4-hydroxy-3-nitrophenyl)acetyl-chicken γ globulin (NP-CGG) in alum or IFA (Sigma-Aldrich; reference 23). NP-CGG contained 10 or 12 mol NP/mol CGG. NP-CGG was emulsified in IFA or precipitated with alum. Some mice were immunized with SRBCs (Duke University Farm) in PBS or injected with 0.2 ml alum or IFA alone. LPS (*Escherichia coli* O127:B8; Sigma-Aldrich) was resuspended in sterile PBS, and mice were injected i.p. with 75 μg LPS. Mouse rTNFα, rIL-1β, rIL-6, and rIFNβ were purchased from R&D Systems. Pharmacologic doses for each cytokine were confirmed by serial titrations (0.3–3.0 μg/mouse). Single doses of 1 μg rTNFα, rIL-1β, or 1,000 U rIFNβ in 300 μl PBS were given i.v.; these doses did not produce obvious morbidity. PTX and PTX B oligomer were purchased from List Biological Laboratories.

Antibodies. FITC-, PE-, biotin-, or allophycocyanin-conjugated mAb for mouse B220, Gr-1, CD3, IgM, CD4, CD8, and CD11c were purchased from BD Biosciences. PE-Cy5–conjugated mAb for mouse CD4, CD8, TER-119, Gr-1, CD11b, and FITC-conjugated anti-CD45.1 and anti-CD45.2 mAb were purchased from eBioscience. Streptavidin (SA)-allophycocyanin (BD Biosciences) and SA-Texas red (Calbiochem-Novabiochem) identified biotinylated mAb. The 493 mAb (24) binds the fetal stem cell antigen, AA4 (C1qRp/CD93; references 25–27), and was purified from cloned hybridoma cells.

Flow Cytometry. Mice were killed after injection/immunization, and cells were harvested from spleen, femur, tibia, and blood. RBCs were lysed in ammonium chloride buffer (23) before immunolabeling. Typically, ≤10^6 nucleated cells were suspended in 50–100 μl of staining buffer (HBSS with 2% FCS and combinations of labeled mAb) and incubated on ice for 20 min. 7-Aminoactinomycin D (Molecular Probes) was included to identify dead cells. Labeled cells were analyzed/sorted in a FACSCalibur™ flow cytometer (488 nm argon laser; 633 nm helium neon laser) or a FACStarPlus™ flow cytometer (488 nm argon laser; 599 nm dye laser) with the OmniComp option. Cytometry data were analyzed with FlowJo software (Treestar Inc.).

B Cell Colony Forming Unit Assay. B cell progenitors were enumerated as pre–B cell CFU (CFU-B; reference 6). In brief, 10^5 BM cells or 5×10^5 splenocytes were mixed with 1 ml IMDM containing 1% methylcellulose, 30% FCS, 0.1 mM 2-mercaptoethanol, 2 mM glutamine, and 20 ng/ml IL-7. Suspended cells were plated in 35-mm dishes and cultured at 37°C for 7 d. Colonies with B cell morphology were identified and counted by microscope.

Adoptive Cell Transfer. 3×10^7 BM cells from B6.SJL (CD45.1) mice were injected i.v. into BL/6 (CD45.2) recipients immunized 3 d earlier. 1 d after transfer, femoral BM cells and splenocytes were harvested and stained with FITC-conjugated anti-CD45.1 and biotinylated anti-B220 mAb, followed by SA–Texas red. Labeled, donor-derived cells were enumerated by flow cytometry to determine homing and migration efficiencies. BM cells from TNFR$^{-/-}$ (CD45.2) mice were transferred into naive or immunized B6.SJL (CD45.1) mice. Donor B cells recovered from the BM and spleen of recipients were distinguished from host cells by anti-CD45.2 mAb.

RT-PCR. Total RNA was extracted from BM using RNeasy-kits (QIAGEN); 1 μg RNA was reverse transcribed for 1 h at 42°C (Superscript II reverse transcriptase; Invitrogen). PCR was performed on serial dilutions of cDNA using Taq polymerase (Takara Bio Inc.). PCR primers used were as follows: HPRT, forward, 5′-GCTGGTGAAAAGGACCTCT-3′, reverse, 5′-CACAGGACTAGAACACCTGC-3′; CXCL12, forward, 5′-GTCCTCTTGCTGTCCAGCTC-3′, reverse, 5′-TAATTTCGGGTCAATGCACA-3′; and CXCL12α, reverse, 5′-TGGGCTGTTGTGCTTACTTG-3′; CXCL12β, reverse, 5′-CCTCTCACATCTTGAGCCTCTT-3′. Amplification parameters were as follows: initial denaturation at 94°C for 5 min, 25–32 amplification rounds consisting of denaturation at 94°C for 30 s, annealing at optimal temperatures, and extension for 60 s at 72°C. A final extension round of 72°C for 10 min ended each amplification. Optimal annealing temperatures were as follows: 52°C for HPRT and CXCL12, and 60°C for CXCL12α and -β. PCR products were electrophoresed over 2% agarose gels containing ethidium bromide.

Preparation of BM Plasma and CXCL12 ELISA. BM plasma was prepared by flushing both femurs and tibia with 500 μl of cold PBS into Eppendorf-type centrifuge tubes. Cells/debris were removed by centrifugation at 3,000 *g* for 10 min at 4°C; BM plasma was stored at −20°C.

CXCL12 protein concentrations were determined by ELISA. In brief, 96-well plates (BD Falcon™; BD Biosciences) were coated overnight with anti-CXCL12 mAb 79018 (R&D Systems) (2 μg/ml in 0.1 M carbonate buffer) at 4°C. Serially diluted BM plasma samples were loaded, incubated overnight at 4°C, and washed with PBS containing 0.1% Tween 20. Bound CXCL12 was detected by biotinylated anti-CXCL12α mAb (BAF310; R&D Systems) and horseradish peroxidase–SA (Southern Biotechnology Associates, Inc.). Horseradish peroxidase activity was visualized using a tetramethylbenzidine peroxidase substrate kit (Bio-Rad Laboratories). CXCL12 concentrations were determined from purified CXCL12 standards (PeproTech).

Online Supplemental Material. Table S1 summarizes the effects of several inflammatory agents on thymocytes. Fig. S1 illustrates reductions of CXCL12 message in BM by adjuvant and TNFα. Fig. S2 shows that the PTX B oligomer has no effect on BM. Fig. S3 compares the ability of antigens/adjuvants to induce BM lymphopenia. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20031104/DC1.

Results

Adjuvants Deplete BM Lymphocytes and Induce the Appearance of Developing B Cells in the Periphery. Immunization with NP-CGG/IFA reduces the numbers of developing (CD93$^+$B220^{lo}) and mature (CD93$^-$B220^{hi}; reference 24) BM B cells (Fig. 1 A); losses are evident 3 d after immunization, with maximal reductions coming on days 4–6 (CD93$^+$B220^{lo} B cells, four- to fivefold reductions; CD93$^-$B220^{hi} B cells, sevenfold) (Fig. 1 B). Thereafter, developing and mature B cell numbers in the BM return to normal levels (Fig. 1 B). Both B cell populations decline at

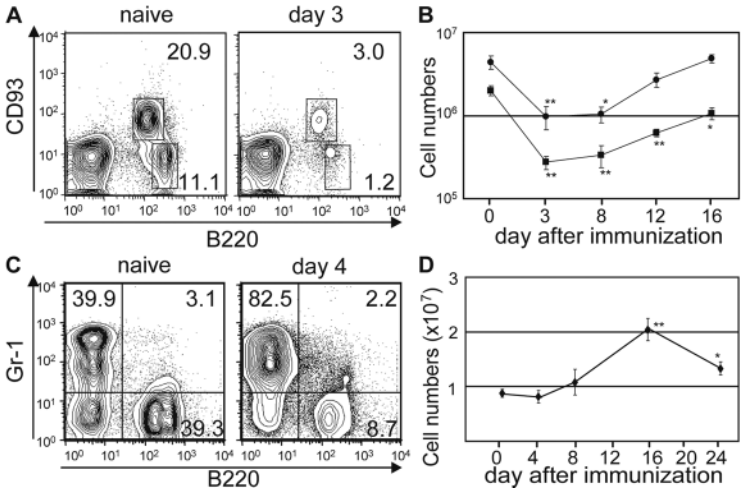

Figure 1. Immunization depletes BM B cells but increases BM granulocyte numbers. BL/6 mice were immunized with NP-CGG/IFA. Lymphocytes and granulocytes from femur and tibia were analyzed by flow cytometry; representative FACS® profiles of BM cells (A, B220 and CD93; C, B220 and Gr-1) 3 or 4 d after immunization. Percentages of gated $CD93^+B220^{lo}$, $CD93^-B220^{hi}$, and $Gr\text{-}1^+B220^-$ cells are given. 3 d after immunization, $CD93^+B220^{lo}$ and $CD93^-B220^{hi}$ cell numbers fall in the BM (A), whereas $Gr\text{-}1^+B220^-$ cell numbers change little (C). Dynamics of BM B cell (B) or granulocyte (D) populations indicate that B lymphopenia persists for ≥12 d (B), whereas granulocyte numbers increase (D). Points represent mean ± SEM of $CD93^+B220^{lo}$ (●), $CD93^-B220^{hi}$ (■), and $Gr\text{-}1^+B220^-$ cells (◆). Asterisks indicate significant differences from controls: *, $P < 0.05$; **, $P < 0.01$.

similar rates, but losses of $CD93^-B220^{hi}$ cells are significantly ($P < 0.05$) greater and more sustained than that of $CD93^+B220^{lo}$ cells. Similar kinetics of loss and recovery are also observed for BM T cells (Table I and unpublished data), indicating that all BM lymphocyte populations are sensitive to adjuvant-induced depletion.

In contrast, BM granulocyte numbers, especially less mature $Gr\text{-}1^{int}$ cells (28), increase after immunization (Fig. 1, C and D). $Gr\text{-}1^+$ cell numbers significantly increase 1 wk after immunization, reach maximal levels on day 16 (naive, $8.8 \pm 1.8 \times 10^6$; and day 16, $20.4 \pm 3.5 \times 10^6$, $P < 0.01$), and then gradually fall to normal levels (Fig. 1 D).

To determine if adjuvant-induced depletion of BM lymphocytes includes cell mobilization, we enumerated $CD93^+B220^{lo}$ blood cells after immunization. $CD93^+B220^{lo}$ blood cell numbers increased soon after immunization (Fig. 2, A and B), with a peak at day 3 (naive, $5.3 \pm 1.8 \times 10^3$ cells/ml; and day 3, $14.7 \pm 3.7 \times 10^3$ cells/ml, $P < 0.05$). Developing B cell numbers in blood returned to normal levels (Fig. 2 B, day 8, $3.4 \pm 0.7 \times 10^3$ cells/ml, $P = 0.42$; day 12, $7.4 \pm 1.2 \times 10^3$ cells/ml, $P = 0.08$; and day 16, $8.9 \pm 2.3 \times 10^3$ cells/ml, $P = 0.10$).

Immunization also increased the fraction of IgM^- cells among $CD93^+B220^{lo}$ blood cells. In naive animals, 80–90% of $CD93^+B220^{lo}$ blood cells are IgM^{hi}, transitional B cells (Fig. 2 C; reference 29). 3 d after immunization, the IgM^- fraction of $CD93^+B220^{lo}$ blood cells increased to ~45% (Fig. 2 C); adjuvants increase the numbers of $CD93^+B220^{lo}$ blood cells by expanding the IgM^- compartment. As reported previously (3, 13, 17, 18), $CD93^+B220^{lo}$ IgM^- cell numbers in the spleen also increased after immunization (Fig. 2 A). Transient, nonsignificant ($P = 0.06$) increases immediately followed the peak of $CD93^+B220^{lo}IgM^-$ cells in blood, but larger and sustained increases began at day 8 (naive, $1.6 \pm 0.1 \times 10^5$; day 8, $3.6 \pm 0.6 \times 10^5$, $P < 0.05$) and continued until day 16 (Fig. 2 B, $11.0 \pm 0.6 \times 10^5$, $P < 0.01$). In both blood and spleen, $CD93^+B220^{lo}IgM^-$ cells did not express CD11b, CD11c, CD8, or TER-119 (unpublished data).

Table I. *Effects of Various Inflammatory Agents on BM Cell Populations*

Bone marrow	n[a]	Total	$B220^{lo}$	$B220^{hi}$	Gr-1	CD3
PBS	19	216 ± 9.7[b]	43.0 ± 4.2	31.7 ± 3.2	88.1 ± 4.0	8.1 ± 1.7
NP-CGG/IFA	5	264 ± 11.5[c]	13.7 ± 0.7[c]	7.9 ± 0.2[c]	184 ± 9.6[c]	1.2 ± 0.2[c]
TNFα	15	178.4 ± 16.3[d]	23.2 ± 2.4[c]	13.1 ± 1.7[c]	108.2 ± 10.6	1.3 ± 0.1[c]
IL-1β	7	218 ± 7.5	33.7 ± 3.6	24.6 ± 1.5	116.6 ± 5.7[c]	4.5 ± 0.7
IL-6	5	199 ± 17.2	45.3 ± 1.8	22.2 ± 1.8	76.1 ± 2.0	3.9 ± 0.3[d]
IFNβ	6	229 ± 14.8	44.0 ± 7.7	35.4 ± 1.7	75.6 ± 7.7	11.9 ± 2.4
TNFα + IL-1β	4	281 ± 17.3[c]	13.2 ± 3.2[c]	8.5 ± 2.2[c]	218.3 ± 17.4[c]	1.3 ± 0.3[c]
PTX	4	148 ± 9.3[c]	6.9 ± 0.5[c]	2.2 ± 0.1[c]	99.6 ± 7.5	1.3 ± 0.1[c]

[a]Total number of mice from multiple (two to eight) experiments (TNFα and IL-1β is a single experiment).
[b]Mean ± SEM (×10^6) number of cells recovered from the femurs of individual mice 6 d after treatment.
[c]$P < 0.01$.
[d]$P \leq 0.05$.

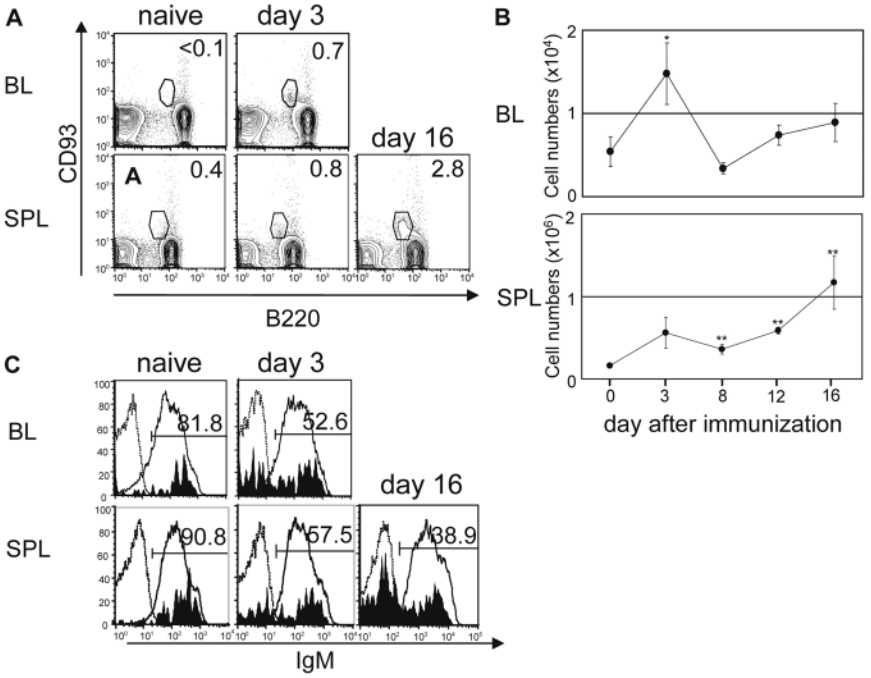

Figure 2. Appearance of $CD93^+B220^{lo}$ cells in the periphery after immunization. $CD93^+B220^{lo}$ B cells in the blood (BL) and spleen (SPL) of immunized BL/6 mice at days 3–16 were characterized and enumerated by flow cytometry. (A) Representative FACS® profiles of $CD93^+B220^{lo}$ and $CD93^-B220^{hi}$ cells in BL and SPL. Percentages of gated $CD93^+B220^{lo}$ cells are indicated. (B) Kinetics of $CD93^+B220^{lo}$ cell numbers in BL and SPL after immunization. Asterisks indicate significant differences from controls: *, $P < 0.05$; **, $P < 0.01$. (C) Surface IgM expression by $CD93^+B220^{lo}$ cells in BL and SPL. Shaded areas, solid lines, and dashed lines represent surface IgM expression by $CD93^+B220^{lo}$, $CD93^-B220^{hi}$, and $B220^-$ cells, respectively. Percentages of IgM^+ cells in $CD93^+B220^{lo}$ cell gates are shown.

To determine whether these $CD93^+B220^{lo}$ IgM^- cells were B lineage progenitors, we enumerated CFU-B in BM and spleen after immunization. CFU-B are abundant in the BM of naive mice ($12.9 \pm 1.0 \times 10^3$ cells/femur), but rare in the spleen (Fig. 3, A and B, $0.2 \pm 0.1 \times 10^3$ cells/spleen). In 4 d, adjuvants decrease the numbers of BM CFU-B to ~25% of controls ($P < 0.01$); these reductions are sustained until day 8 (Fig. 3 A). Significant increases in splenic CFU-B occur 8 d after immunization ($0.5 \pm 0.1 \times 10^3$ cells/spleen, $P < 0.05$), with CFU-B numbers peaking at day 12 ($4.3 \pm 1.3 \times 10^3$ cells/spleen, $P < 0.01$) and declining thereafter (Fig. 3 B).

TNFα Mimics Inflammation's Effects on BM. To determine if a single proinflammatory cytokine could reproduce adjuvant's effects on BM, we administered rTNFα, rIL-1β, rIL-6, or rIFNβ to BL/6 mice and followed changes in lymphocyte and granulocyte numbers.

Of these cytokines, only TNFα recapitulated the cell mobilizations induced by adjuvant (Table I). 6 d after injection, TNFα reduced BM $CD93^+B220^{lo}$, $CD93^-B220^{hi}$, and $CD3^+$ cell numbers to 54, 41, and 16% of controls, respectively. In addition, TNFα modestly increased BM granulocyte numbers (123% of controls, $P = 0.07$). These effects are similar to those of adjuvants, albeit less profound and persistent. For example, adjuvant reduced $CD93^+B220^{lo}$, $CD93^-B220^{hi}$ B cells in the BM by five- and sevenfold, respectively, whereas TNFα reduced $CD93^+B220^{lo}$ and $CD93^-B220^{hi}$ BM cells two- and threefold (Fig. 1 and Table I). In contrast to their strong effects on BM, both adjuvants and TNFα induced only modest and transient changes in the thymus (Table S1, available at http://www.jem.org/cgi/content/full/jem.20031104/DC1).

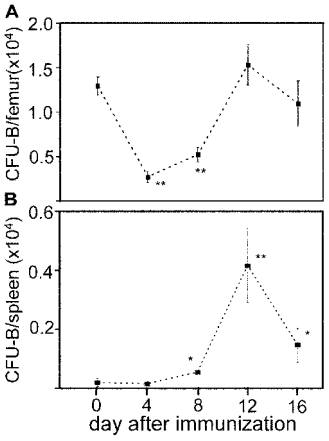

Figure 3. Immunization increases splenic CFU-B numbers. BL/6 mice were immunized with 5×10^8 SRBCs in 200 μl IFA. 4–16 d later, spleen and BM cells were harvested, and CFU-B were enumerated after culture for 7 d. (A) Femoral BM CFU-B. (B) Splenic CFU-B numbers after immunization. Asterisks indicate significant differences from controls: *, $P < 0.05$; **, $P < 0.01$.

Neither IL-6 nor IFNβ significantly altered $CD93^+B220^{lo}$ or $CD93^-B220^{hi}$ BM cell numbers, nor did they change granulocyte numbers (Table I). IL-1β lowered $B220^+$ BM cell numbers nonsignificantly (~80% of controls, $P = 0.11$) but significantly expanded granulocytes (Table I, 132% of controls, $P < 0.01$).

Noting that TNFα primarily reduced BM lymphocyte numbers, whereas IL-1β expanded the BM granulocyte compartment, we tested whether these cytokines synergize by injecting 0.5 μg TNFα and 0.5 μg IL-1β singly or in combination. Synergy was obvious; TNFα and IL-1β together reduced $B220^+$ (~30% of controls, $P < 0.01$) and $CD3^+$ (16% of controls, $P < 0.01$) BM cell numbers more effectively than higher doses either cytokine alone (Table I). Potentiation was also apparent in significantly larger increases in the BM $Gr\text{-}1^+$ populations (Table I, 250% of controls, $P < 0.01$). In combination, TNFα and IL-1β fully recapitulate adjuvant-induced change in BM lymphocyte and granulocyte populations.

TNFα Mobilizes B Cell Progenitors from the BM. To determine if TNFα mobilized BM lymphocytes, we enumer-

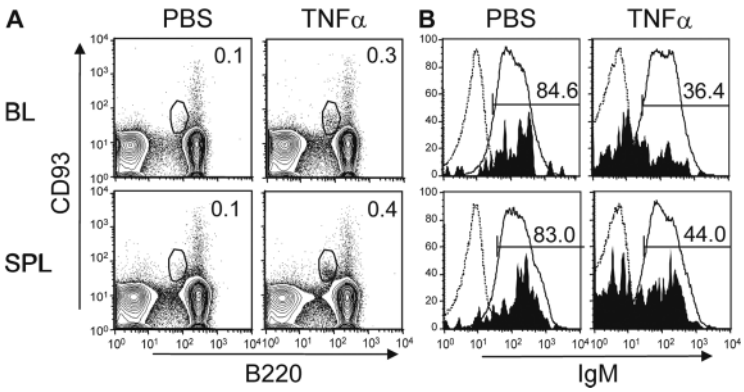

Figure 4. TNFα mobilizes CD93$^+$B220^{lo}IgM$^-$ cells. (A) Representative FACS® profiles of blood (BL) and spleen (SPL) cells from PBS- and TNFα-injected mice. Percentages indicate CD93$^+$B220^{lo} cells in the lymphocyte gate. (B) IgM expression by CD93$^+$B220^{lo} B cells from BL and SPL. Shaded areas, solid lines, and dashed lines represent surface IgM expression by CD93$^+$B220^{lo}, CD93$^-$B220^{hi}, and B220$^-$ cells, respectively. Numbers indicate percentages of IgM$^+$ cells in CD93$^+$B220^{lo} cell gates.

ated CD93$^+$B220^{lo} cells in the periphery after injecting rTNFα. 3 d after injection, CD93$^+$B220^{lo} cells increased two- to threefold in the blood ($P < 0.05$) and spleen ($P < 0.05$) (Fig. 4 A). In control mice, ~15% of CD93$^+$B220^{lo} cells in blood and spleen were IgM$^-$; after TNFα treatment, IgM$^-$ cells comprised 55–65% of both CD93$^+$ B220^{lo} populations (Fig. 4 B). Thus, rTNFα mobilizes CD93$^+$B220^{lo}IgM$^-$ cells to peripheral tissues.

If TNFα plays a principal role in adjuvant-induced BM lymphopenia, lymphocyte mobilization should be reduced or absent in TNF knockout animals. We immunized TNFα$^{-/-}$ mice and congenic controls and followed changes in CD93$^+$B220^{lo} and CD93$^-$B220^{hi} cell numbers in BM, blood, and spleen. 4 d after immunization, substantial losses of CD93$^+$B220^{lo} (27% of controls) and CD93$^-$B220^{hi} (7% of controls) BM cells were evident in B6.129SF2 mice. In contrast, immunization of TNFα$^{-/-}$ mice resulted in approximately twofold reductions of CD93$^+$B220^{lo} (65% of controls) and CD93$^-$B220^{hi} (42% of controls) cells (Fig. 5 A).

Although BM lymphopenia was reduced, mobilization of CD93$^+$B220^{lo} cells was not detectable in TNFα knockouts. 4 d after immunization, CD93$^+$B220^{lo} cells increased two- to threefold in the blood (naive, $5.8 \pm 3.9 \times 10^3$ cells/ml; day 4, $18.7 \pm 0.5 \times 10^3$ cells/ml; $P < 0.01$) and spleen (naive, $1.1 \pm 0.6 \times 10^5$ cells/spleen; day 4, $2.5 \pm 0.7 \times 10^5$ cells/spleen; $P < 0.05$) of TNFα sufficient controls (Fig. 5 B). In contrast, CD93$^+$B220^{lo} cell numbers did not increase in the blood (naive, $4.7 \pm 2.6 \times 10^3$ cells/ml; day 4, $3.2 \pm 3.1 \times 10^3$ cells/ml; $P = 0.44$) or spleen (naive, $0.9 \pm 0.5 \times 10^5$ cells/spleen; day 4, $0.5 \pm 0.2 \times 10^5$ cells/spleen; $P = 0.15$) of immunized TNF$^{-/-}$ mice (Fig. 5 B). Thus, TNFα is a principal component of adjuvant-induced BM lymphopenia and mobilization.

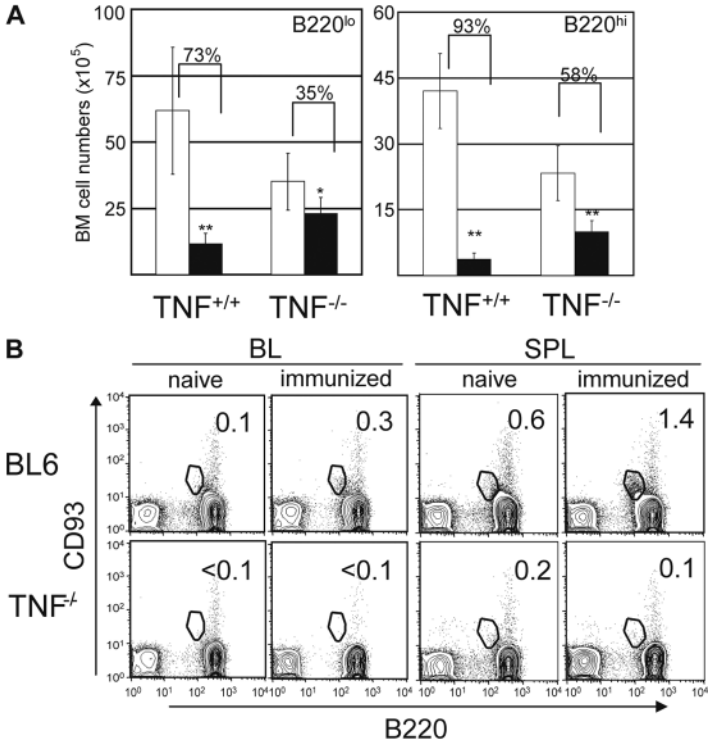

Figure 5. Adjuvant-induced BM lymphopenia and B cell mobilization in TNFα$^{-/-}$ mice. TNFα$^{-/-}$ mice and congenic controls were immunized with NP-CGG/IFA. 3 d after immunization, B cells in BM and spleen (SPL) were analyzed by flow cytometry. (A) Adjuvant-induced BM lymphopenia is mitigated in TNFα$^{-/-}$ mice. Bars represent mean ± SD of B220^{lo} and B220^{hi} cell numbers from two femurs of naive (unshaded) or immunized (shaded) mice. Asterisks indicate significant differences from controls: *, $P < 0.05$; **, $P < 0.01$. (B) Mobilization of CD93$^+$B220^{lo} cells is absent in TNF$^{-/-}$ mice. Representative FACS® profiles for CD93$^+$B220^{lo} and CD93$^-$B220^{hi} cells in the blood (BL) and SPL of naive and immunized mice are shown. Percentages of BL and SPL CD93$^+$B220^{lo} lymphocytes (CD11c-, Gr-1–, Mac-1–, CD8-, and TER-119–negative) are shown.

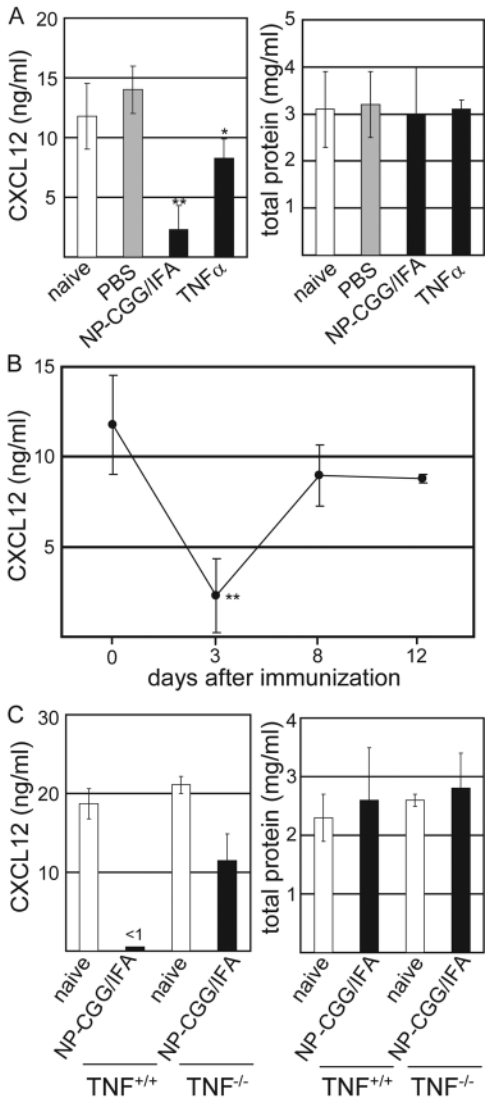

Figure 6. Adjuvants and TNFα suppress BM CXCL12. (A) Adjuvants and TNFα reduce BM CXCL12 protein, but do not alter total protein levels. Bars represent mean ± SD protein concentrations. (B) CXCL12 protein levels in BM plasma of BL/6 mice after immunization. Points represent mean ± SD CXCL12 concentrations. Asterisks indicate significant differences from controls: *, $P < 0.05$; **, $P < 0.01$. (C) Adjuvant-induced suppression of BM CXCL12 is mitigated in TNFα$^{-/-}$ mice. 3 d after immunization, CXCL12 protein concentrations in BM plasma of TNFα$^{-/-}$ and control mice were determined. Experimental groups are as follows: naive TNF$^{+/+}$, untreated B6.129SF2; NP-CGG/IFA TNF$^{+/+}$, immunized B6.129SF2; naive TNF$^{-/-}$, untreated TNFα$^{-/-}$; and NP-CGG/IFA TNF$^{-/-}$, immunized TNF$^{-/-}$.

Immunization and TNFα Reduce CXCL12 in the BM. CXCL12 and its receptor, CXCR4, are crucial for the homing of hematopoietic progenitor cells (30–33), and interruption of CXCL12/CXCR4 interaction mobilizes BM stem cells (34, 35). Could inflammation/TNFα mobilize BM B cells by reducing BM CXCL12/CXCR4 expression and/or signaling? We measured CXCL12 message in BM by semi-quantitative RT-PCR. In naive mice, CXCL12 mRNA was detected in unsorted BM cells, but absent or much reduced in the B cell, T cell, and granulocyte compartments (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20031104/DC1), consistent with the production of CXCL12 by nonhematopoietic stromal cells (36). 3 d after immunization or i.v. TNFα, levels of CXCL12α and -β mRNA (36) fell twofold in BM, whereas HPRT mRNA levels remained constant (Fig. S1).

CXCL12 protein in BM plasma also declined after immunization or TNFα injection. In control mice, the average concentration of CXCL12 in BM plasma was 14.0 ± 2.0 ng/ml. 3 d after immunization, CXCL12 protein levels dropped sixfold to 2.3 ± 2.0 ng/ml ($P < 0.01$; Fig. 6 A). These losses were specific, as total protein levels in the BM plasma of control and immunized mice were identical (Fig. 6 A). After immunization, CXCL12 returned to near normal levels by day 8 (Fig. 6 B). This pattern of decreased CXCL12 expression follows the course of BM lymphocyte loss and recovery after immunization (Fig. 1 B).

TNFα also inhibited BM CXCL12 protein production, but less profoundly than adjuvant. 3 d after TNFα, BM CXCL12 levels fell to 8.3 ± 1.6 ng/ml ($P < 0.05$; Fig. 6 A). Adjuvant-induced reductions of CXCL12 protein are greater than reductions in mRNA, whereas TNFα induces comparable reductions in both (Fig. 6 and Fig. S1). We interpret this difference to reflect distinct regulatory mechanisms (35, 37–39).

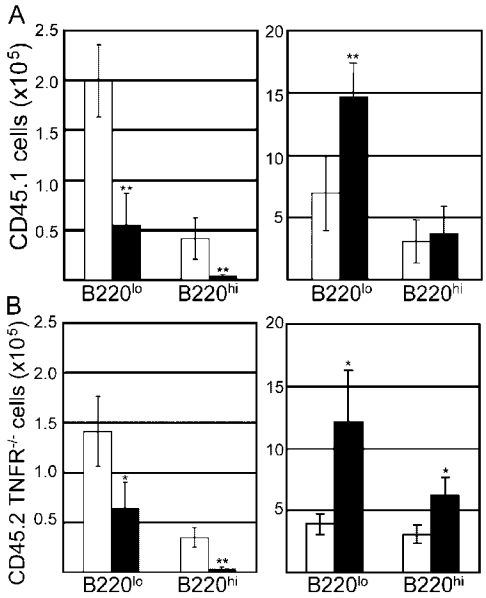

Figure 7. Transferred BM B cells do not home efficiently in immunized recipients. (A) BM cells from naive B6.SJL (CD45.1) animals were transferred into naive or immunized, congenic (CD45.2) recipients. 1 d after transfer, donor B220^{lo} and B220^{hi} cells in the BM and SPL of recipients were enumerated by flow cytometry. (B) Homing of TNFR$^{-/-}$ BM B cells in immunized recipients is impaired. BM cells from naive, TNFR$^{-/-}$ (CD45.2) mice were transferred into naive and immunized (CD45.1) recipients. 1 d after transfer, donor B cells in the BM and SPL of recipients were enumerated. Bars indicate mean ± SD numbers of CD45.2 B cells recovered from naive (unshaded) and immunized (shaded) recipients. Asterisks indicate significant differences from controls: *, $P < 0.05$; **, $P < 0.01$.

CXCL12 Reduction in TNFα$^{-/-}$ Mice. In naive B6.129SF2 mice, the average concentration of CXCL12 protein in BM plasma (Fig. 6 C, 19 ± 1.9 ng/ml) is similar to BL/6 mice (Fig. 6 A); 4 d after immunization, BM CXCL12 concentrations fell below detectable levels (Fig. 6 C, <1 ng/ml). In contrast, immunization of TNFα$^{-/-}$ mice reduces BM CXCL12 levels by ≤50% (Fig. 6 C, naive, 21 ± 1.1 ng/ml; immunized, 11 ± 3.4 ng/ml). These reductions were specific, as total BM plasma protein remained constant in all groups (Fig. 6 C).

Homing to BM Is Reduced in Immunized Mice. If adjuvants mobilize BM lymphocytes by reducing CXCL12, normal cells should be unable to colonize the BM of immunized mice. To test this prediction, BM cells from naive, CD45.1 donors were transferred into naive or immunized CD45.2 recipients; 24 h later, CD45.1 B cells in the BM and spleen were enumerated by flow cytometry.

CD45.1 donor B cells readily entered the BM of naive recipients. We typically recovered 2.0 ± 0.4 × 10^5 CD45.1$^+$B220^{lo} cells and 0.4 ± 0.2 × 10^5 CD45.1$^+$B220^{hi} cells from each femur and tibia from naive hosts (Fig. 7 A). In immunized recipients, we only recovered 0.6 ± 0.3 × 10^5 CD45.1$^+$B220^{lo} cells (30% of controls, $P < 0.01$) and 0.03 ± 0.02 × 10^5 CD45.1$^+$B220^{hi} cells (7% of controls, $P < 0.01$). In contrast, equivalent numbers of CD45.1$^+$ B220^{hi} cells were present in the spleens of both naive (3.1 ± 1.7 × 10^5) and immunized hosts (3.7 ± 2.2 × 10^5), and more CD45.1$^+$B220^{lo} cells were consistently recovered from the spleens of immunized than from naive recipients (Fig. 7 A, 14.7 ± 2.7 × 10^5 vs. 7.0 ± 2.9 × 10^5; $P < 0.01$).

To exclude the possibility that defective BM homing in immunized recipients was due to TNFα-mediated change in the transferred cells, we transferred 1.5 × 10^7 BM cells from TNFR$^{-/-}$ mice (CD45.2) into naive or immunized B6.SJL (CD45.1) hosts (Fig. 7 B). Average recoveries from the BM of naive recipients were 1.4 ± 0.3 × 10^5 CD45.2$^+$B220^{lo} and 0.4 ± 0.1 × 10^5 CD45.2$^+$B220^{hi} TNFR-deficient cells. However, only 0.6 ± 0.3 × 10^5 CD45.2$^+$B220^{lo} cells (43% of controls, $P < 0.05$) and 0.03 ± 0.02 × 10^5 CD45.2$^+$B220^{hi} TNFR-deficient cells (10% of controls, $P < 0.01$) were recovered from the BM of immunized recipients.

PTX Depletes BM Lymphocytes and Mobilizes Developing B Cells. In association with reductions in CXCL12, adjuvant mobilizes BM lymphocytes and increases granulocyte numbers (Figs. 1 and 6), with little effect on thymocytes (Table S1). If CXCL12 reductions cause these changes, a blockade of CXCL12 signals must produce similar results. We injected BL/6 mice with PTX, an inhibitor of many chemokine receptors, including the CXCL12 receptor, CXCR4 (20), and followed its effects on BM and thymus.

3 d after injecting PTX, CD93$^+$B220^{lo} and CD93$^-$B220^{hi} BM cell numbers fell significantly and remained suppressed until day 12. B cell numbers began to recover 12–18 d after PTX treatment, reaching normal levels by day 24 (Fig. 8 A). PTX also lowered CD3$^+$ BM cell numbers (Table I) with similar kinetics (unpublished data). These effects were dependent on the ribosyltransferase activity of PTX, as the enzymatically inactive PTX B oligomer had no effect on BM (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20031104/DC1). PTX had little effect on thymocyte populations (Table S1).

PTX did not elicit losses of BM granulocytes. Instead, granulocyte numbers in the BM began to increase as soon as 6 d after PTX treatment with peak numbers at day 18.

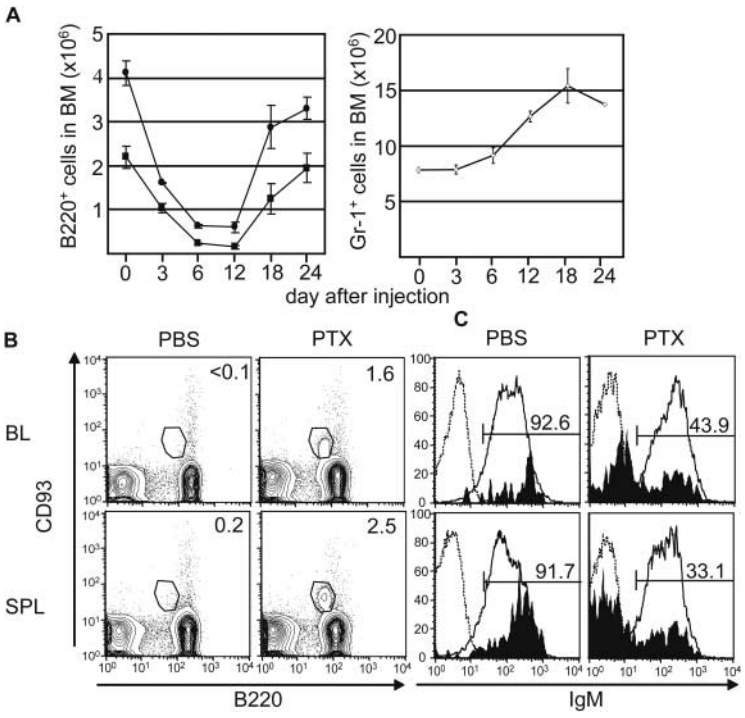

Figure 8. PTX elicits BM lymphopenia and mobilizes CD93$^+$B220^{lo}IgM$^-$ cells. BM, blood (BL), and spleen (SPL) cells were harvested from BL/6 mice 0–24 d after injection of 300–500 ng PTX and analyzed by flow cytometry. (A) Dynamics of BM B cell subsets (left) and granulocytes (right). Points represent mean ± SEM numbers of B220^{lo} (●), B220^{hi} (■), and Gr-1$^+$ (◇) cells from two femurs. (B) Appearance of CD93$^+$B220^{lo}IgM$^-$ cells in the periphery after PTX treatment. FACS® profiles of CD93$^+$B220^{lo} and CD93$^-$B220^{lo} cells from BL and SPL in PBS- and PTX-treated mice are shown. Percentages of cells in the CD93$^+$B220^{lo} gate are indicated. (C) PTX elicits populations of CD93$^+$B220^{lo}IgM$^-$ cells in BL and SPL. Representative FACS® profiles of surface IgM expression by CD93$^+$B220^{lo} cells. Shaded areas, solid lines, and dashed lines represent surface IgM expression by CD93$^+$B220^{lo}, CD93$^-$B220^{hi}, and B220$^-$ cells, respectively. Percentages of IgM$^+$ cells in CD93$^+$B220^{lo} cell gates are shown.

Later, BM granulocyte numbers began a return to basal levels (Fig. 8 A).

PTX also mobilized $CD93^+B220^{lo}$ BM cells; 3 d after injecting PTX, $CD93^+B220^{lo}$ cell numbers were significantly higher in blood ($P < 0.01$) and spleen (Fig. 8 B, $P < 0.01$). The majority of these peripheral $CD93^+B220^{lo}$ cells (blood, 56%; spleen, 66%) were surface IgM^- (Fig. 8 C).

Discussion

Infections and adjuvants can produce BM lymphopenia (1–3, 9). BM B ($CD93^+B220^{lo}$ and $CD93^-B220^{hi}$) and T lymphocyte numbers fall significantly in the first week after immunization with adjuvant, but both compartments begin recoveries in the second week and approach normal levels in the third (3). Recovery in the BM is coincident with the appearance of splenic $CD93^+B220^{lo}$ cells that express RAG1/2 and λ5 (3). In contrast, BM granulocyte numbers do not fall but increase after immunization (3). These observations suggest that inflammation affects BM hematopoiesis to favor granulocyte production. However, the mechanisms responsible for inflammation-induced changes in the BM are unknown.

Here, we show that adjuvants suppress BM CXCL12 and mobilize functional B cell progenitors ($B220^{lo}CD93^+IgM^-$ cells and CFU-B) into the periphery. Both phenomena can be mediated by rTNFα, and both are reduced or absent in $TNF\alpha^{-/-}$ mice. A blockade of $G_{\alpha i}$-dependent signaling by PTX recapitulates these effects in the absence of an overt inflammatory response. We conclude that inflammation acts via TNFα and CXCL12 to reduce the BM lymphocyte compartments in preparation for expanded granulocyte production. This model outlines a novel inflammatory response and predicts that innate immune responses are physiologic regulators of central hematopoiesis.

Regulation of B Lymphopoiesis during Inflammation. Adjuvants, LPS, and gram-negative bacteria, but not noninflammatory antigens such as SRBCs (references 3, 4; Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20031104/DC1) deplete all BM lymphocyte compartments equally (Fig. 1 and Table I; references 3, 4). The mechanism of depletion has been unclear, but cytokine-driven apoptosis was a favored candidate (7–10). Developing B cells are sensitive to apoptotic signals (7) and severe viral infection or high doses of IFNα/β suppress B lymphopoiesis by apoptosis (8–10). However, this apoptosis likely represents a pathologic or pharmacologic response (8). In our hands, adjuvant-induced depletions of lymphocytes are not biased for developmentally immature compartments (Fig. 1), are restricted to the BM (Table S1), and unassociated with obvious morbidity or pathology.

Although we cannot rule out an apoptotic component, adjuvant-induced BM lymphopenia is coincident with a massive mobilization of BM lymphocytes that results in the appearance of $CD93^+B220^{lo}IgM^-$ cells in the blood and spleen (Fig. 2). These $CD93^+B220^{lo}IgM^-$ cell populations include functional CFU-B (Fig. 3), providing an explanation the findings that the RAG^+ splenocytes elicited by adjuvant are not mature lymphocytes and require functional BM (4, 19).

TNFα Mobilizes BM Lymphocytes. Adjuvant's effects on BM could be fully reproduced by two proinflammatory cytokines, TNFα and IL-1β (Table I). TNFα significantly decreases BM lymphocyte numbers (Table I), mobilizes $B220^{lo}CD93^+IgM^-$ cells (Fig. 4), and modestly expands the BM granulocyte compartment (Table I). A central role for TNFα in adjuvant-induced loss of BM lymphocytes was confirmed in $TNF\alpha^{-/-}$ mice (Fig. 5) that exhibited much reduced BM lymphopenia and no mobilization of $CD93^+B220^{lo}$ cells.

However, residual losses of BM B cells in immunized $TNF\alpha^{-/-}$ mice indicate that inflammation does not act via TNFα only. IL-1β elicited a nonsignificant BM lymphopenia but greatly expanded granulocyte numbers (Table I). The effects of IL-1 in vivo are similar to those observed in vitro by Dorshkind (6) and complement TNFα. Suboptimal doses of TNFα and IL-1β synergize to act on BM as profoundly as complex inflamogens (Table I and Fig. S3). Thus, the primary effect of TNFα appears to be the mobilization of BM lymphocytes, whereas IL-1β promotes granulocytic expansion. A similar potentiation has been observed in rats (40).

Adjuvants and TNFα Suppress BM CXCL12. CXCL12 attracts many hematopoietic cells (34, 41, 42), including progenitor B cells, and is important for their survival, differentiation, and localization (33). Both adjuvants and TNFα reduce CXCL12 in the BM, and these reductions mirror lymphocyte mobilization (Fig. 6, A and B). Adjuvant-induced reductions of CXCL12 in $TNF\alpha^{-/-}$ mice were substantially less than in controls (Fig. 6 C) and consistent with reduced BM lymphopenia and lack of $CD93^+B220^{lo}$ cell mobilization (Fig. 5). Inflammation mobilizes BM lymphocytes by suppressing CXCL12 expression. Although adjuvants lower CXCL12 mRNA approximately threefold, CXCL12 protein falls to <20% of controls; rTNFα reduces BM CXCL12 mRNA and protein to ~50% of control levels (Fig. 6 and Fig. S1). The contrasting ranges of CXCL12 message and protein levels in the BM suggest that inflammation regulates chemokine expression transcriptionally and posttranslationally.

Consistent with this idea, Fedyk et al. (37) showed that TNFα modestly suppressed CXCL12 transcription in dermal fibroblasts, whereas Petit et al. (35) found that granulocytes substantially lowered CXCL12 levels by elastase-driven proteolysis. Other enzymes secreted by granulocytes (e.g., matrix metalloproteinases and cathepsin G) also inactivate CXCL12 (38, 39).

Inflammation Alters the BM to Prevent Cell Homing. $B220^{lo}$ and $B220^{hi}$ BM cells from naive donors inefficiently home in immunized recipients (Fig. 7). Reduced homing efficiency is not due to TNF-mediated change in the transferred cells, as $TNFR^{-/-}$ cells do not enter the BM of immunized recipients (Fig. 7 B). We conclude that inflammation modifies the BM by reducing CXCL12 sufficiently to

no longer attract and/or retain lymphocytes. The observation that PTX, a $G_{\alpha i}$ poison that inhibits most chemokine signaling (20), mimics inflammation's effects on BM and mobilizes $CD93^+B220^{lo}IgM^-$ cells (Fig. 8 and Table I) is consistent with this model, but is not a proof.

Retention of BM Granulocytes. In vitro, granulocytes display strong, $G_{\alpha i}$-dependent chemotaxis to CXCL12 (reference 43 and unpublished data). CXCL12 and CXCR4 are crucial for both myelopoiesis and B lymphopoiesis (30, 31), and mice reconstituted with CXCR4-deficient fetal liver cells have increased numbers of developing granulocytes and B cells in their blood (33). How is it that reductions of CXCL12 or inhibition of chemokine signaling by PTX depletes BM lymphocytes but not granulocytes?

One possibility is that the chemotactic sensitivities of B cells and granulocytes to CXCL12 differ. If granulocytes respond to significantly lower concentrations of CXCL12 ($\sim$1 ng/ml) than lymphocytes, reductions of CXCL12 would favor the retention of granulocytes in the BM. In the absence of CXCL12/CXCR4, such selectivity would be lost. Increased sensitivity to CXCL12 signals would also make granulocytes relatively resistant to nonsaturating doses of PTX.

Although early myeloid progenitors depend on CXCL12 to enter the BM, $Gr\text{-}1^{int}$ and $Gr\text{-}1^{hi}$ granulocytes (28) might be retained there by other chemokines. For example, LPS induces BM stromal cells to express CCL21, a chemokine for myeloid progenitors (44). Other myeloid chemoattractants, CCL3 and CCL8, are expressed in BM as well (45). In this model, granulocytes would normally depend on CXCL12 homing/retention signals, but are held in the BM by other chemokines during infection.

A third possibility is that $G_{\alpha i}$-independent mechanisms retain granulocytes in the BM. Although the initial localization of myeloid progenitors in the BM is CXCL12 and $G_{\alpha i}$ dependent (30, 31, 33), retention of developing and mature granulocytes in the BM could be $G_{\alpha i}$-independent (46–49). These PTX-insensitive pathways could be constitutive or induced by inflammation.

None of these models are mutually exclusive, and we are in the process of testing each. However, our data show that BM lymphocytes and granulocytes respond differently to environmental cues that control their retention in the BM (Figs. 1 and 8; Table I).

Lymphopoiesis and Granulopoiesis during Inflammation. Although severe inflammation may induce apoptosis in BM lymphocytes (9, 40), milder inflammation mobilizes BM lymphocytes to the blood and spleen (Fig. 2) and establishes extramedullary B lymphopoiesis (Fig. 3). Lymphocyte mobilization is associated with increased numbers of $Gr\text{-}1^{int}$ cells and expansion of the BM granulocyte compartment (Fig. 1, C and D). The coordination of these changes suggests a regulated, physiologic response. We propose that inflammatory agents elicit TNFα (and other potentiating cytokines) at sites of infection, and perhaps in the BM (50), sufficiently to suppress BM CXCL12. Initially, this suppression occurs by transcriptional inhibition (reference 37 and Fig. S1), but later it occurs by proteolysis from an expanded granulocyte compartment (35, 38, 39). IL-1β promotes granulocytic expansion, especially in the presence of TNFα (Table I). Reduced CXCL12 levels mobilize BM lymphocytes, and initiate extramedullary lymphopoiesis. In the spleen, displaced CFU-B proliferate and differentiate into pre–B and immature B cells that express RAG1/2 and λ5 (Figs. 2 and 3; references 3, 4, 13, 16, 19).

BM granulocytes appear to expand into generative niches abandoned by mobilized lymphocytes. Although IL-1β promotes myelopoiesis (Table I; reference 6), its effects are strongly potentiated by TNFα-induced mobilizations. This synergy of TNFα and IL-1β suggests that lymphopoiesis and myelopoiesis compete in the BM. Competition for space or resources is also implicit in pharmacologic modulations of hematopoiesis (i.e., factors that promote granulocyte development mobilize lymphocyte progenitors [reference 51] and vice versa [references 52, 53]).

Alternatively, it is possible that the recovery of BM lymphocyte compartments and increased granulocyte numbers represents a general increase in the ability of the BM to support hematopoiesis. This increased generative capacity might result from the accumulation of growth resources over the lymphopenic period.

The utility of increasing granulopoiesis in response to inflammation is obvious. Mature granulocytes are unable to divide and once activated, survive only hours to days. The ability to increase granulocyte production to replenish cells lost in inflammatory responses would be considerably advantageous as persistent neutropenia leads to death from infection (54, 55)

The advantage of extramedullary B lymphopoiesis is less obvious. Nonetheless, it is clear that inflammation promotes transient, extramedullary B lymphopoiesis. We think it unlikely that this splenic lymphopoiesis has no physiologic role. The appearance of CFU-B in the spleen is well regulated (unpublished data) and the number of pre–B and immature B cells that arise there can comprise 10–20% of splenic $B220^+$ cells (references 3, 4, 18; unpublished data). Perhaps some fraction of these cells are recruited into local humoral responses (15–17, 56)?

In conclusion, adjuvant-induced BM lymphopenia reflects the mobilization of lymphocytes. This mobilization is mediated by a TNFα/CXCL12 axis that intimately links the innate and adaptive immune systems. Proinflammatory cytokines not only act as immune effectors and organizers of lymphoid tissue but also direct BM hematopoiesis.

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