B-lymphopoiesis is stopped by mobilizing doses of G-CSF and is rescued by overexpression of the anti-apoptotic protein Bcl2

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ABSTRACT

Osteoblasts are necessary to B lymphopoiesis and mobilizing doses of G-CSF or cyclophosphamide inhibit osteoblasts, whereas AMD3100/Plerixafor does not. However, the effect of these mobilizing agents on B lymphopoiesis has not been reported. Mice (wild-type, knocked-out for *TNF-α* and *TRAIL*, or over-expressing *Bcl-2*) were mobilized with G-CSF, cyclophosphamide, or AMD3100. Bone marrow, blood, spleen and lymph node content in B cells was measured. G-CSF stopped medullar B lymphopoiesis with concomitant loss of B-cell colony-forming units, pre-pro-B, pro-B, pre-B and mature B cells and increased B-cell apoptosis by an indirect mechanism. Overexpression of the anti-apoptotic protein Bcl2 in transgenic mice rescued B-cell colony forming units and pre-pro-B cells in the marrow, and prevented loss of all B cells in marrow, blood and spleen. Blockade of endogenous soluble TNF-α with Etanercept, or combined deletion of the *TNF-α* and *TRAIL* genes did not prevent B lymphopoiesis arrest in response to G-CSF. Unlike G-CSF, treatments with cyclophosphamide or AMD3100 did not suppress B lymphopoiesis but caused instead robust B-cell mobilization. G-CSF, cyclophosphamide and AMD3100 have distinct effects on B lymphopoiesis and B-cell mobilization with: 1) G-CSF inhibiting medullar B lymphopoiesis without mobilizing B cells in a mechanism distinct from the TNF-α-mediated loss of B lymphopoiesis observed during inflammation or viral infections; 2) CYP mobilizing B cells but blocking their maturation; and 3) AMD3100 mobilizing B cells without affecting B lymphopoiesis. These results suggest that blood mobilized with these three agents may have distinct immune properties.

Introduction

The interface between the compact bone and the bone marrow (BM), the endosteum, is a privileged site where bone formation and turnover take place. In the past decade, it has emerged that this endosteal region of the BM, particularly the metaphyseal spongiosa rich in trabecular bone, harbors the most primitive hematopoietic stem cells (HSC) able to reconstitute long-term multi-lineage hematopoiesis upon serial transplantation into lethally irradiated mice.[1-5] Hence, it was concluded that HSC niches are not distributed randomly in the BM tissue but preferentially locate within 2-3 cell diameters from endosteal bone surfaces.[3-4] These conclusions were further supported by the observation that HSC express calcium receptors sensing the calcium gradient formed by osteoclast-mediated bone degradation and helping HSC to lodge in these endosteal niches.[6] This drew the attention to the potential role of osteoblasts, osteoprogenitors and their mesenchymal precursors in regulating most primitive HSC. Conditional gene deletion in, and specific ablation of osteoblasts,[7] osteoprogenitors[8] or mesenchymal stem cells[9] have shown that osteoblast-lineage and mesenchymal progenitor cells are critical to maintain normal HSC within the BM. It has also recently emerged that in addition to regulating HSC, osteoblasts and their progenitors critically regulate medullar B lymphopoiesis. Indeed, ablation of osteoblasts or conditional deletion of the *Gsα* gene specifically in osteoblasts impairs primitive B lymphopoiesis in the BM.[10-11] Therefore, osteoblastic lineage cells at the endosteum control the maintenance of two different arms of hematopoiesis: 1) primitive hematopoiesis via HSC; and 2) B-lymphopoiesis.

We and others have previously reported that specific populations of BM macrophages are critical to maintain HSC within their BM niches. Indeed, ablation of these macrophages[12] and/or their stimulation by granulocyte colony-stimulating factor (G-CSF)[13] results in inhibition of bone formation, disappearance of endosteal osteoblasts, and impairment of HSC niche function as measured by expression of HSC-supportive factors such as CXCL12, Kit ligand, angiopoietin-1, and VCAM-1, leading to robust mobilization of HSC into the peripheral blood.[12-14] We identified two macrophage subsets that potentially exert this regulatory role: 1) osteomacs, a specific population of BM macrophages that form a canopy over active osteoblasts at the endosteum and are necessary to maintain osteoblast function; and 2) $CD11b^{+}F4/80^{+}Ly6\text{-}G^{+}$ macrophages.[15] It is still unclear as to whether osteomacs are a subset of the $CD11b^{+}F4/80^{+}Ly6\text{-}G^{+}$

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macrophages that specifically support osteoblasts or whether these are separate populations. Nevertheless, we and others have found that continuous treatment with the cytokine G-CSF causes HSC mobilization by depleting these niche-supportive macrophages, causing depletion of endosteal osteoblasts, and reducing HSC niche function leading to HSC mobilization into the peripheral blood.[13,15] We have also found that the alkylating agent cyclophosphamide (CYP) also depletes osteomacs and osteoblasts from endosteal surfaces leading to impairment of HSC niches and HSC mobilization.[16] In contrast, the CXCR4 antagonist AMD3100 (Plerixafor), which mobilizes HSC by binding directly to CXCR4 and blocking the chemotactic signaling elicited by the binding of the chemokine CXCL12,[17] has no effect on osteoblasts or niche-supportive macrophages.[16]

Considering that both G-CSF and CYP inhibit osteoblasts and HSC niches, whereas AMD3100 does not,[16] and that endosteal osteoblasts are critical to maintain medullar B lymphopoiesis,[10-11] we have evaluated the effect of these three mobilizing agents on B lymphopoiesis in the mouse.

Design and Methods

All experiments were approved by the Animal Experimentation Ethics Committees of the University of Queensland and University of Sydney, Australia.

Mouse mobilization and tissue harvesting

All experiments were performed on 8-12 week-old male C57BL/6 mice. *vavBcl2* transgenic mice[18] were produced by breeding transgenic males with wild-type C57BL/6 females. B6.*TNFa*$^{-/-}$ mice[19] were crossed with B6.*TRAIL*$^{-/-}$ mice[20] to generate mice heterozygous for both alleles. These were interbred to yield B6.*TNFa*$^{-/-}$.*TRAIL*$^{-/-}$ (B6.TT) mice defective in both alleles. Pups were identified by PCR on ear punch biopsy using primers specific to the Bcl2 transgenene as described.[18] Polymerase chain reaction (PCR) detection of *TNF-α* and *TRAIL* wild-type and targeted alleles was performed essentially as described previously[20] but using *TRAIL* gene specific neomycin-target allele primers.

Recombinant human G-CSF (Filgrastim, Amgen, Thousand Oaks, CA, USA) was injected twice daily subcutaneously at 125 μg/kg per injection for up to six consecutive days. Control mice were injected with an equivalent volume of saline. In some experiments, 20 mg/kg/day etanercept was injected once daily intraperitoneally for four days to block endogenous soluble tumor-necrosis factor (TNF)-α[21] and G-CSF administered for the last three days of the experiment, as described above.

Cyclophosphamide (CYP)-treated mice were injected intraperitoneally with a single dose of 200 mg/kg CYP diluted in saline.

AMD3100 octohydrochloride (Tocris Bioscience, Bristol, UK) was injected intraperitoneally as a single 16 mg/kg dose corresponding to 10 mg/kg of AMD3100 base. Tissues were harvested 1 h after AMD3100 administration.

At specified time points, mice were anesthetized with isofluorane and approximately 1 mL of blood collected into heparinized tubes by cardiac puncture before cervical dislocation. Femoral BM was flushed and spleens dissociated in phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) for further analyses. For flow cytometry analyses, red cells were lysed from blood samples as previously described.[22] Spleens were harvested, weighed, and dissociated in PBS with 2% FCS. Cells and RNA from the central region of the BM, and RNA from the endosteum were isolated from femurs as previously described.[23] Inguinal and popliteal lymph nodes draining hind legs[24] were harvested and dissociated in PBS with 2% FCS similar to spleens.

Cell counts and colony assays

Leukocytes were counted on a Sysmex KX-21 automated cell counter. For colony-forming unit B cell (CFU-B) assays, 10^5 cells were plated in 1 mL of Methocult CFU-B medium containing 10 ng/mL human interleukin (IL)-7 following manufacturer's instructions (Stem Cell Technologies, Vancouver, Canada). Colonies of small lymphocytes were counted after seven days culture.

CFU-C assays to test the mobilization of myeloid progenitors were performed in methylcellulose medium containing recombinant mouse granulocyte-monocyte colony-stimulating factor (GM-CSF), Kit ligand and IL-6, as previously described.[12]

Quantitative real-time RT-PCR (RT-qPCR)

RNA was precipitated, DNase was treated and reverse transcribed using random hexamers. qRT-PCR with CXCL12 and IL-7 were performed using Taqman probes (labeled 5'with 6-carboxyfluorescein (FAM) and 3' with blackhole quencher-1 (BHQ-1)). Oligonucleotide sequences are shown in *Online Supplementary Table S1* except for mouse G-CSF receptor (*Csf3r*) primers. Primers and probe set were purchased from Applied Biosciences (set Mm00432735_m1). All primers crossed intron-exon boundaries and did not amplify genomic DNA in the absence of reverse transcription. RNA levels were standardized by parallel RT-qPCR using primers to the housekeeping gene β2-microglobulin.

TNF-α measurement in bone marrow fluids

At sacrifice, femurs were flushed with 1 mL ice-cold PBS, BM cells dissociated by serial pipetting and then pelleted at 400 x g for 5 min at 4°C. BM fluids in supernatants were collected, aliquoted and stored at -70°C until use. TNF-α protein concentration was measured using the BD Cytometric Bead Array for mouse inflammatory cytokines following the manufacturer's instructions (BD Biosciences, Sydney, Australia) and analyzed on an LSRII flow cytometer (BD Biosciences).

Flow cytometry

Bone marrow, blood and spleen leukocytes were pelleted at 370 x g for 5 min at 4°C and resuspended in 2.4G2 anti-CD16/CD32 hybridoma culture supernatant to block IgGFc receptors. Measurement of B-progenitor cells were performed by staining cell suspensions with CD11b-PECy7, NK1.1-PE, biotinylated donkey F(ab')2 anti-mouse-IgM with streptavidin-Pacific Blue, B220-APCCy7, CD19-PercPCy5.5 or CD19-APC and CD43-FITC (BD Biosciences) monoclonal antibodies. For sorting, cells were labeled in an identical manner and sorted in medium containing 50% FCS on an Aria cell sorter (BD Biosciences).

Mobilization of HSPC was measured by flow cytometry using a cocktail of anti-lineage (Lin) antibodies (CD3ε, CD5, B220, CD11b, Gr-1, CD41, Ter119), anti-Sca-1, anti-Kit, CD48 and CD150 antibodies, as previously described.[25]

For apoptosis assays, BM cells were labeled as above, pelleted, resuspended in 100 μL of annexin V binding buffer (BD Biosciences) and incubated with APC-conjugated annexin V at room temperature for 15 min. Cells were then analyzed using an LSRII flow cytometer following addition of 2 μg/mL 7-actinomycin D (7-AAD).

Statistical analysis

Calculations for whole body content in B-cell progenitors were based on the assumption that one femur represents 5.6% of total mouse BM and total volume of blood is 0.08 mL per g of body

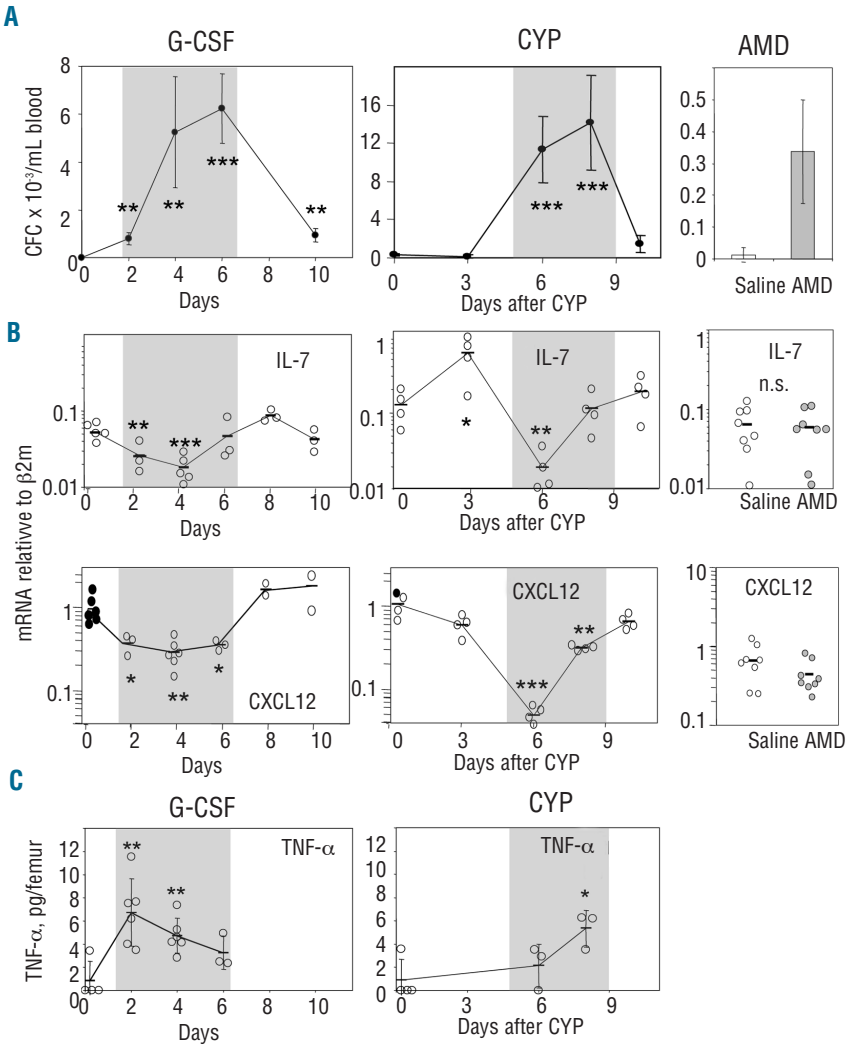

Figure 1. Effect of G-CSF, CYP and AMD3100 on CFU-C mobilization and expression of IL-7, CXCL12 and TNF-α. (A) Mobilization of CFU-C in peripheral blood was measured at indicated time points. Data are means±SD, 4 mice per time point. (B) IL-7 and CXCL12 mRNA were measured in the endosteal region of the BM by RT-qPCR. Data are relative to β2-microglobulin mRNA. Each dot is the result from an individual mouse. Bars are the average of each group. (C) Concentration of TNF-α protein in BM fluids. Each dot is the result from an individual mouse. Bars are the average of each group. ***P<0.001, **P<0.01, and *P<0.05. Gray boxes on kinetics show time period during which HSPC were mobilized into the blood.

weight.[26] Significance levels were calculated using the Student's t-test for colony assays, flow cytometry analyses, and RT-qPCR.

Results

G-CSF inhibits medullar B-lymphopoiesis

Adult mice were mobilized by bi-daily injections of G-CSF for up to six days (Figure 1A) and expression of IL-7 and CXCL12 was measured in the endosteal region of the BM by RT-qPCR (Figure 1B). TNF-α protein concentration was directly measured in BM fluids (Figure 1C). IL-7 and CXCL12 mRNA expression were both significantly inhibited by G-CSF treatment whereas TNF-α protein concentration in BM fluids increased during the first four days of G-CSF treatment. As CXCL12 and IL-7 are B-lymphopoietic cytokines[27-29] and TNF-α has been reported to inhibit B-lymphopoiesis,[30] these results suggested that G-CSF may impair B lymphopoiesis in the BM.

We next measured the number of $B220^+CD11b^-NK1.1^-$ B cells in BM, blood and spleen by flow cytometry. The frequency of these B cells in the BM declined 28-fold at Day 6 of G-CSF treatment and progressively returned to normal levels eight days following cessation of G-CSF administration (Figure 2A). This decrease in medullar B cells was not accompanied by any significant increase in the number of B cells in blood or spleen during G-CSF treatment (Days 0-6) (Figure 2A) although a significant increase in blood B cells was observed at Day 10 when the number of B cells in the BM began to recover, returning to normal values by Day 14. Assuming that a femur represents 5.6% of total BM, and that total blood volume is proportional to mouse body weight (0.08 mL/g),[26] we found that the total number of B cells in whole BM, blood and spleen per mouse declined progressively by 60% at Day 6 of G-CSF administration and returned to baseline values by Day 14. To further clarify whether this decline in BM B cells was due to a halt in medullar B-lymphopoiesis or involved relocation of some B progenitors into the spleen, B progenitors and B cells were enumerated by flow cytometry. B-lineage cells were gated within the $side\text{-}scatter^{low}CD11b^-NK1.1^-$ population as surface IgM $(sIgM)^-B220^+CD19\text{-}CD43^+$ pre-pro-B cells, $sIgM^-B220^{low}CD19^+CD43^-$ pro-B cells, $sIgM\text{-}B220^+CD19^+$ pre-B cells, and $sIgM^+B220^+$ mature B cells, as previously reported by Zhu *et al.*,[10] and Hardy *et al.* for the specific expression of CD43 in pre-pro-B cells[31] (Figure 3A). These analyses revealed a rapid and pronounced decline of all B-

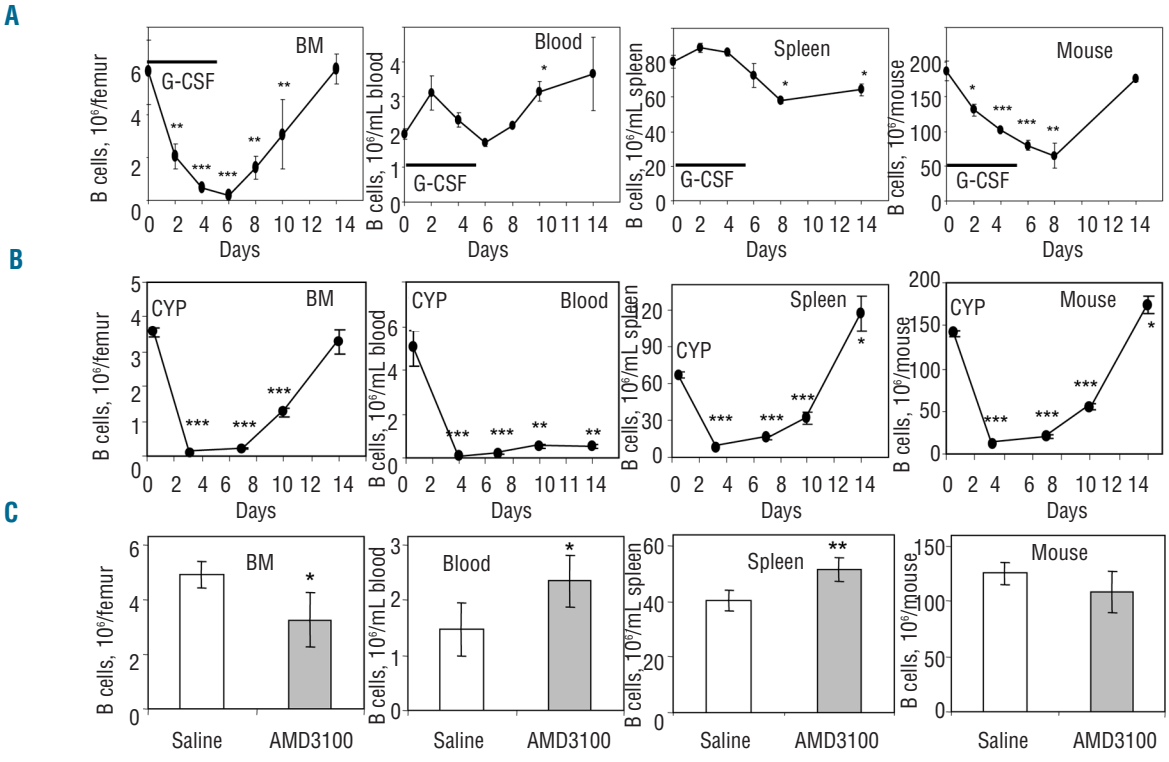

Figure 2. Comparative effect of G-CSF, CYP and AMD3100 on B cells in BM, blood and spleen. At indicated time points, SSC^{low} CD11b^{-} B220^{+} B cells were measured by flow cytometry in BM, blood and spleen. The charts on the right column show the number of B cells in total BM, blood, spleen per mouse calculated after summation of content in BM, blood and spleen. Data are average±SD of 4 mice per time point per treatment group. ***P<0.001, **P 0.01, and *P<0.05.

cell populations in the BM, with the exception of pro-B cells whose decline was delayed, occurring only at Day 4 of G-CSF administration (Figure 3B). Importantly, although some pre-pro/pro/pre-B cells relocated to blood and spleen, this did not compensate for the loss of BM B cells and their progenitors, resulting in a net decrease in pre-pro-B (3.5-fold), pro-B (1.7-fold), pre-B (4.6-fold), and mature B (1.9-fold) cells on a per mouse basis for the sum of the BM, blood and splenic compartments (Figure 3B). These results were confirmed at the level of more primitive CFU-B in functional B-cell colony assays, with a rapid and severe decline in medullar CFU-B with only marginal mobilization into blood and spleen (Figure 3B). Importantly, while mature sIgM^{+}B220^{+} B cells were abundant in inguinal and popliteal lymph nodes, all sIgM^{-}B220^{+} B-progenitor subsets were very rare (less than 1/100 of mature B cells). Furthermore, their number did not increase after four days of G-CSF (*Online Supplementary Figure S1*), excluding the possibility that B-cell progenitors home to lymph nodes in response to G-CSF.

To determine whether this loss in medullar B cells was mediated by apoptosis, BM cells were stained with 7-AAD and annexin-V (Figure 3C). Apoptosis of immature sIgM^{-} and mature sIgM^{+} B cells was in part responsible for this collapse in medullar B-lymphopoiesis with a significant increase in the proportion of 7-AAD^{+} and/or annexin-V^{+} apoptotic cells in these two B-cell populations after four days of G-CSF. Conversely, the proportion of apoptotic BM myeloid cells was significantly decreased by G-CSF treatment.

Blockage of TNF-α does not prevent HSPC mobilization or arrest in B-lymphopoiesis

To determine whether the effect of G-CSF on B cells was direct, sIgM^{-}B220^{+}CD19^{-}CD43^{+} pre-pro-B cells, sIgM-B220^{+}CD19^{+}CD43^{-} pre-B and pro-B cells, and sIgM^{+}B220^{+} mature B cells were sorted from the BM, RNA extracted and G-CSF receptor mRNA quantified by RT-qPCR. G-CSF receptor mRNA were undetectable in all B-cell populations but abundantly expressed in BM CD11b^{+} myeloid cells and Lin^{-}Sca1-Kit^{+} myeloid progenitors (*Online Supplementary Figure S2*). Therefore, in the absence of receptor, the effect of G-CSF on B cells must be indirect.

As TNF-α inhibits B-lymphopoiesis[30] and is increased in the BM during G-CSF treatment (Figure 1), we treated mice for four days with etanercept, a human TNFR2-IgG1 Fc chimera that cross-reacts with mouse TNF-α and inhibits endogenous soluble mouse TNF-α.[21] Mice were then mobilized with G-CSF for the last three days. Etanercept treatment did not alter mobilization of CFU-C, Lin^{-}Sca1^{+}Kit^{+} HSPC or Lin^{-}Sca1^{+}Kit^{+}CD48^{-}CD150^{+} HSC into the blood or spleen (*Online Supplementary Figure S3*). Etanercept did not prevent the loss of pre-pro-B cells or more mature B-cell precursors in the BM following G-CSF treatment, although it did increase the number of mature B cells in steady-state (*Online Supplementary Figure S3*). These results were confirmed in mice knocked-out for both *TNF-α* and *TRAIL* genes. A 4-day treatment with G-CSF also strongly reduced medullar B lymphopoiesis in these mice (*Online Supplementary Figure S4*). Therefore,

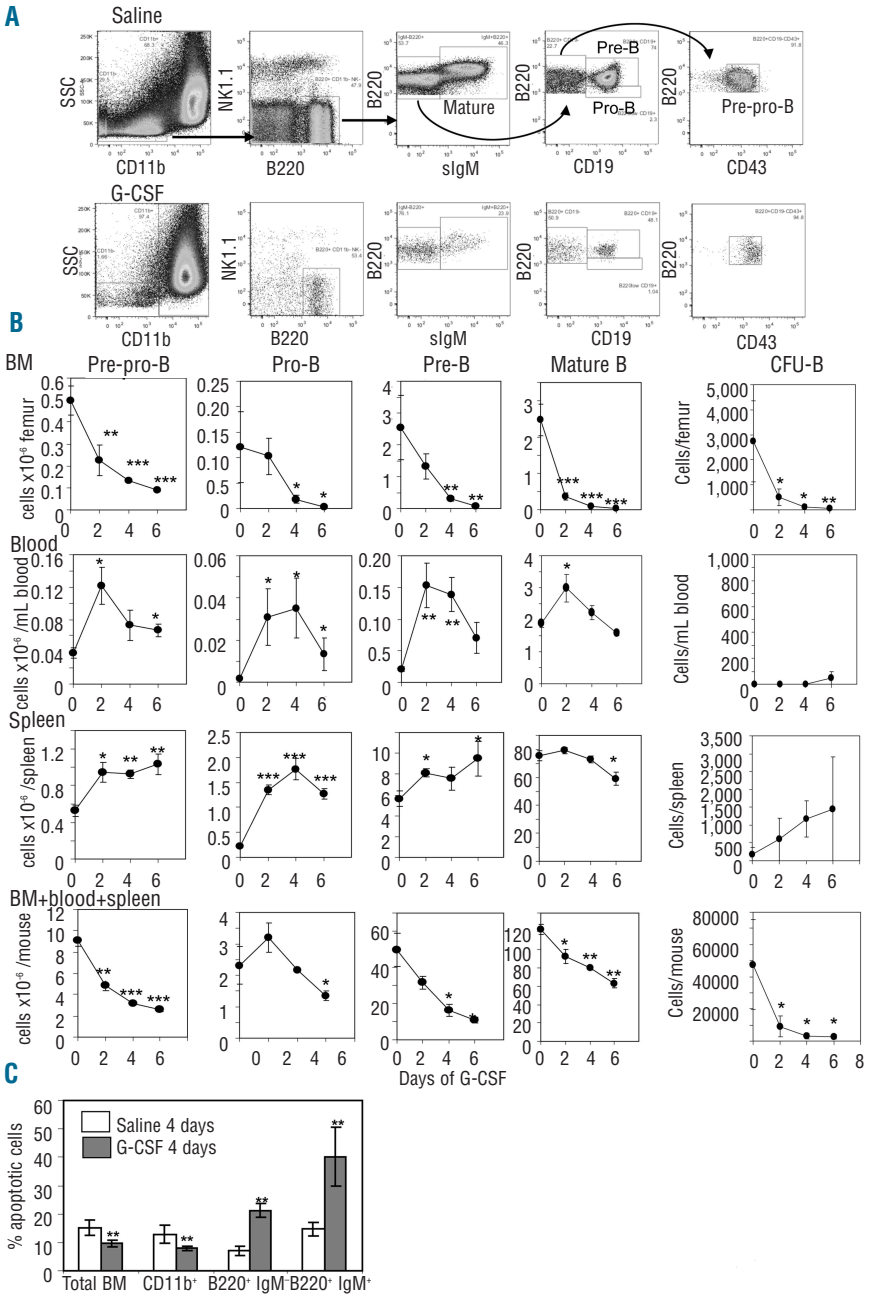

Figure 3. G-CSF induces loss of all B-cell subsets in the BM. (A) Gating strategy to identify B-cell subsets during mobilization. B-cell subsets were defined within the SSC^{low} CD11b^{-} NK1.1- B220^{+} population as shown in the top two dot-plots. Mature B cells were defined as sIgM^{+} whereas immature B cells were sIgM^{-}. Immature B cells were then subdivided in B220^{+} CD19^{-} CD43^{+} pre-pro-B cells, B220^{low} CD19^{+} pro-B cells and B220^{+} CD19^{+} pre-B cells. Typical B-cell profiles are shown for BM from control saline treated mice (top row) and G-CSF-treated mice (bottom row). Note the strong reduction of all B-cell subsets in G-CSF-mobilized BM. (B) The number of cells in each B-cell subset (pre-pro-B, Pro-B, pre-B and mature B cells) was measured by flow cytometry in BM, blood and spleen at indicated time points of G-CSF treatment. Results per mouse were calculated after summation of content in BM, blood and spleen. The number of CFU-B was measured in B-cell colony assays. (C) Apoptosis in BM cells was measured by flow cytometry. Cells were considered apoptotic when they were positive for annexin-V and/or 7-AAD. Data are average±SD of 4 mice per time-point per treatment group. ***P<0.001, **P<0.01, and *P<0.05.

TNF-α, and TNF-α related TRAIL are dispensable to the B-lymphopoiesis inhibition in response to G-CSF.

Overexpression of Bcl-2 rescues CFU-B and pre-pro-B cells in response to G-CSF

To further investigate the contribution of apoptosis in the inhibition of B-lymphopoiesis in response to G-CSF, we next mobilized vavBcl2 transgenic mice which overexpress the *Bcl2* gene under the vav gene promoter in all hematopoietic cells. Bcl2 is an anti-apoptotic protein necessary to early B-cell development[32] and mutations in *Bcl2* gene are frequent in B-cell neoplasms.[33-34] Overexpression of Bcl2 in hematopoietic cells causes a pan-leukophilia, B-cell accumulation in the spleen, evolving to follicular lymphoma after 40-week latency.[18,35] We conducted experiments in 8-week old mice that did not shown any sign of malignancy despite a pronounced leukophilia in steady-state (*data not shown*). Colony assays in the presence of GM-CSF, Kit ligand and IL-6 showed that the number of myeloid progenitors CFU-C in blood and spleen was more than a degree of magnitude higher in vavBcl2 mice compared to wild-type, both in steady-state and after a 4-day course of G-CSF (Figure 4A). There was no significant difference in CFU-C content in the BM between the two strains in steady-state or during mobilization.

B-cell subset analysis revealed that, unlike the wild-type, there was no significant loss of pre-pro-B cells and CFU-B in mobilized BM from *vavBcl2* transgenic mice (Figure 4B). Mobilization of pre-pro-B cells and CFU-B into the blood was also more pronounced (Figure 4B). Therefore, overexpression of Bcl2 rescued the loss of most primitive B-cell progenitors in G-CSF mobilized BM. CFU-

B were also dramatically increased in the spleen of mobilized vavBcl2 mice (Figure 4B).

Analysis of more mature B cells revealed that, although the number of pro-B, pre-B and mature B cells was dramatically decreased in the BM of mobilized vavBcl2 mice, this loss in the BM was fully compensated by a robust mobilization of these B-cell subsets into the blood. Consequently, the total number of CFU-B, pre-pro-B, pro-B, pre-B and mature B cells did not decrease on a per mouse basis that included the summation of BM, blood and spleen compartments (Figure 4B). This is in sharp contrast to wild-type mice in which the loss of B cells in the BM was not compensated by their number in the blood, spleen or lymph nodes, resulting in an overall decrease of all B-cell types on a per mouse basis (Figure 3B). Taken together, these data show that Bcl2 overexpression rescues the arrest in medullar B lymphopoiesis during G-CSF administration with maintenance of pre-pro-B cell and CFU-B pools in the BM, and redistribution of all B-cell stages from the BM into the blood and spleen without loss in B cells.

Cyclophosphamide (CYP) and AMD3100 induce B-cell mobilization without impairing B-lymphopoiesis

We next examined the effect of two other mobilizing agents on medullar B-lymphopoiesis. A single dose of the alkylating agent CYP causes rapid myelosuppression in BM during the acute cytotoxic phase in the first three days of administration in mice. This is followed by a robust rebound of hematopoiesis between Days 6-8 that coincides with HSPC mobilization into the blood.[16,36] Total $B220^+$ B cells were profoundly reduced in BM, blood and spleen at Day 3 and slowly recovered in BM and spleen between Days 6 and 14 (Figure 2). To better understand the effect of CYP on lymphopoiesis, B-cell precursors were further analyzed in these three tissues following the same gating strategy as that used in G-CSF-mobilized mice. At Day 3, during the acute cytotoxic phase, pro-B, pre-B and mature B cells were almost undetectable in the BM (*Online Supplementary Figure S5*). Although significantly reduced, detectable numbers of pre-pro-B cells remained in the BM. At Days 6 and 8, when HSPC are

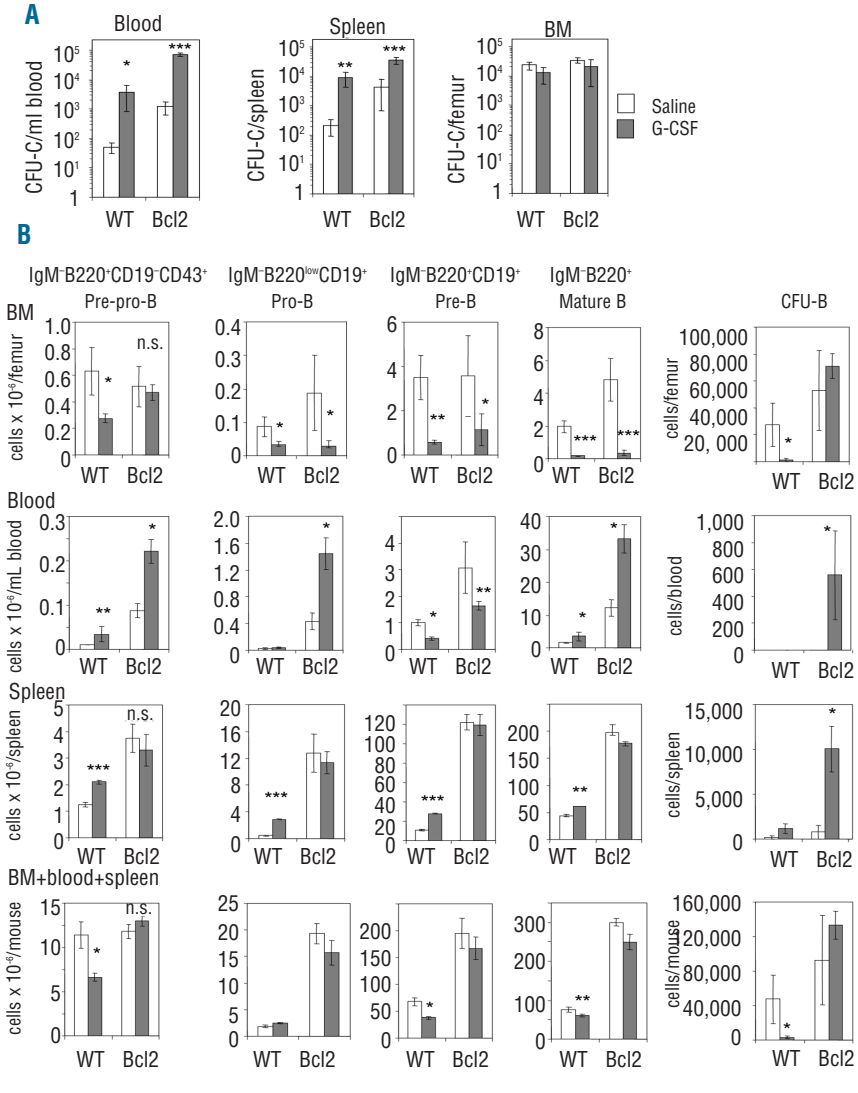

Figure 4. Overexpression of Bcl-2 in vavBcl2 mice rescues medullar B lymphopoiesis during G-CSF treatment. (A) CFU-C in blood, BM and spleen were measured in colony assays. (B) The number of cells in each B-cell subset (pre-pro-B, Pro-B, pre-B and mature B cells) was measured by flow cytometry in BM, blood and spleen at Day 6 of G-CSF or saline treatments. Results per mouse were calculated after summation of content in BM, blood and spleen. The number of CFU-B was measured in B-cell colony assays. Data are average±SD of 4 mice per time-point per treatment group. ***P<0.001, **P<0.01, and *P<0.05.

mobilized into the blood (Figure 1A),[36] medullar B-lymphopoiesis was re-initiated with increased pre-pro-B, pro-B and pre-B cells in the BM. These B cell precursors were also robustly mobilized into the blood and spleen (*Online Supplementary Figure S5*). As a result, all these B-cell precursor populations rebounded from the Day 3 nadir on a per mouse basis based on the summation of the BM, blood and spleen compartments (*Online Supplementary Figure S5*). Therefore, unlike G-CSF, HSPC mobilization in response to CYP is not concomitant with an arrest in medullar B-lymphopoiesis. Importantly, we noted that B-cell maturation to sIgM$^+$ was delayed with the numbers of mature B cells remaining low in BM, spleen and BM even ten days after CYP injection (*Online Supplementary Figure S5*). As mature B cells represent a large proportion of B cells in the blood, this low number of mature B cells explains the persistent low number of total B cells in the blood even 14 days after CYP treatment (Figure 2B).

Finally, to determine the effects of CXCR4, mice were treated with AMD3100, a CXCR4 antagonist that blocks the chemotactic effect of the chemokine CXCL12,[37] and tissues harvested 1 h later at the peak of HSPC mobilization[17] (Figure 1A). AMD3100 caused a slight decrease in medullar B cells and significant B-cell mobilization into blood and spleen (Figure 2C). A more detailed analysis of B-cell subsets showed very robust mobilization of all B-cell types into the blood (*Online Supplementary Figure S6*). A significant proportion of pro-B, pre-B and mature B cells had already homed to the spleen after 1 h of AMD3100 injection, demonstrating that these B cells can very rapidly home to the spleen. There was no significant reduction in pre-pro-B cells and pro-B cells in the BM following AMD3100 treatment. However, the number of more differentiated pre-B and mature sIgM$^+$ B cells was significantly reduced in the BM (*Online Supplementary Figure S6*) explaining the overall reduction of B cells in the BM (Figure 2C). Finally, AMD3100 treatment did not alter the numbers of pre-pro-B, pro-B and mature B cells on a per mouse basis (in BM, blood and spleen) (*Online Supplementary Figure S6*). Together, these results suggest that AMD3100 does not compromise medullar B lymphopoiesis and B-cell survival despite a robust mobilizing effect on all differentiation stages of the B-cell lineage.

Discussion

G-CSF causes a rapid loss of osteoblasts on endosteal surfaces[12,16] with reduction in the transcription of both CXCL12 and IL-7 mRNA in the endosteal region, and increased TNF-α protein in BM fluids. This is accompanied by a very pronounced reduction in the numbers of pre-pro-B, pro-B, pre-B and sIgM$^+$ mature B cells in the BM. The loss of B-cell precursors in the BM is not compensated by relocation to the blood, spleen or lymph nodes. Furthermore, the proportion of apoptotic B cells increased in the BM in response to G-CSF, a loss that was rescued by the overexpression of the anti-apoptotic protein Bcl2. This suggests that G-CSF blocks medullar B-lymphopoiesis by increasing B-progenitor cell apoptosis via the mitochondrial-triggered pathway. This is consistent with the observation that Bcl2 overexpression also rescues B-cell numbers in the BM and spleen of mice deficient in BOB.1/OBF.1, a B-cell specific transcriptional co-activator.[32]

This effect of G-CSF was indirect as B cells and their progenitors do not express G-CSF receptor. A possible candidate for mediating this indirect effect was TNF-α as: 1) TNF-α administration directly inhibits B lymphopoiesis; and 2) inhibition of B-lymphopoiesis by LPS- or adjuvant-induced acute inflammation are in part TNF-α dependant.[30] However, we have found that neither blockade of endogenous TNF-α with etanercept nor targeted deletion of the *TNF-α* or *TRAIL* genes prevents the arrest in B-lymphopoiesis caused by G-CSF. Therefore, although G-CSF and acute inflammation[38-39] both enhance myelopoiesis and suppress B-lymphopoiesis, the mechanisms are somewhat distinct in respect to the role of TNF-α. Clearly, cytokines other than TNF-α and TRAIL play a role in inhibiting B-lymphopoiesis in response to G-CSF. One possibility is lymphotoxin-α, since this cytokine is known to alter B-cell maturation *in vivo*[19,40] but this remains to be investigated in relation to G-CSF. A possible mechanism leading to increased B-cell apoptosis in the BM in response to G-CSF could be the downregulation of both CXCL12 and IL-7 mRNA in the endosteal region. Indeed, both CXCL12[27-28] and IL-7[29] are indispensable to B-lymphopoiesis. CXCL12 and IL-7 have been reported to be produced by separate BM stromal cells and from distinct niches for pre-pro-B cells and pro-B cells respectively.[41] An important role of osteoblasts in regulating B-lymphopoiesis is suggested by expression of both IL-7 and CXCL1211 in osteoblasts. Furthermore, deletion of the protein *Gsα* gene specifically in primitive osteoprogenitors and osteoblasts using Cre recombinase under the control of the *osterix* gene promoter (Gsα^{osxKO} mice) also reduces the number of osteoblasts on endosteal surfaces, and down-regulates IL-7 expression leading to impaired medullar B lymphopoiesis with reduced numbers of pro-B- and pre-B cells, whereas pre-pro-B and sIgM$^+$ B cells are unaffected in the BM of these mice.[11] This phenotype is somewhat similar to our observation in wild-type mice mobilized with G-CSF except that G-CSF strongly reduced pre-pro-B cells and sIgM$^+$ B cells in addition to pro-B and pre-B cells (Figure 3). In these Gsα^{osxKO} mice, administration of exogenous IL-7 was also able to partially rescue medullar B-lymphopoiesis.[11] Therefore, it is exciting to speculate that: 1) the attenuation of IL-7 expression in pro-B and pre-B cell niches observed in Gsα^{osxKO} mice mice or wild-type mice treated with G-CSF, induces apoptosis of IL-7-dependent pro-B and pre-B cells; and 2) that the subsequent B-cell apoptosis can be rescued either completely by overexpression of the Bcl2 anti-apoptotic protein, or partially by administering IL-7. The main difference between Gsα^{osxKO} mice mice and G-CSF-treated wild-type mice is that CXCL12 was down-regulated in the latter, not in the former. This may explain the pronounced decrease in pre-pro-B cells and mature sIgM$^+$ B cells that was not observed in Gsα^{osxKO} mice mice.

The lesser effect of CYP on medullar B-lymphopoiesis was unexpected as CYP also ablates osteoblasts, although this occurs during the rebound phase between Days 6-10 following CYP administration when HSPC are mobilized into the blood.[16] The persistent absence of endosteal osteoblasts at Days 6-10 of CYP[16] did not prevent the re-expression of IL-7 at Day 8, or the rebound in medullar B-lymphopoiesis with the number of pro-B and pre-B cells increasing in the BM from Day 6 and more primitive pre-pro-B cells from Day 8 after CYP administration. This was highly unexpected as osteoblasts have been reported to be necessary to maintain medullar B-lymphopoiesis *in*

vivo.[10] Of note, less primitive pro-B and pre-B cells rebounded in the BM two days prior to pre-pro-B cells which rebounded in the BM at Day 8 only. As pre-pro-B cells rebounded in the spleen earlier than in the BM (Day 6 instead of Day 8), this suggests that B-lymphopoiesis re-started from the spleen sometime between Day 3 and Day 6 post-CYP and seeded the BM despite persistent absence of osteoblasts. Clearly, expression of IL-7 and CXCL12 resumed in other BM stromal cells in the absence of osteoblasts. This is consistent with previous observations showing that IL-7 and CXCL12 are also expressed by distinct stromal cells away from the endosteum.[41-42] However, it is important to note that despite the re-expression of both IL-7 and CXCL12 and re-initiation of medullar B-lymphopoiesis, the maturation of pre-B cells into sIgM$^+$ cells was still blocked at Day 10 after CYP when osteoblasts are still absent,[16] with very few sIgM$^+$ B cells in BM, blood and spleen.

Finally, we found that all B-cell subsets were robustly mobilized 1 h after AMD3100 administration without inhibition of CXCL12 or IL-7 expression or medullar B-lymphopoiesis. This is consistent with our previous observations that AMD3100 has no effect on osteoblast numbers and expression of the osteoblast marker osteocalcin.[16] Therefore, AMD3100 mobilizes B cells by directly antagonizing CXCR4 which is expressed by B cells,[43-44] without interfering with B-cell niches or medullar lymphopoiesis.

In conclusion, the three HSC mobilizing agents G-CSF, CYP and AMD3100 have very distinct effects on B-lymphopoiesis and B-cell mobilization with: 1) G-CSF inhibiting medullar B-lymphopoiesis without mobilizing B cells in a mechanism distinct from the loss of B-lymphopoiesis observed during inflammation or viral infections;[30,38] 2) CYP mobilizing B cells but blocking their maturation into sIgM$^+$ B cells; and 3) AMD3100 mobilizing B cells without affecting B lymphopoiesis. These results suggest that blood mobilized with these three agents may have distinct immune properties.

Finally, as G-CSF is a potent inhibitor of B-lymphopoiesis, it could be a useful agent to treat B-cell neoplasms. However, as Bcl2 overexpression is common in and drives B-cell neoplasms, combinations of G-CSF with Bcl2 inhibitors such as ABT-737[45-47] or obatoclax[48] could efficiently target B-cell malignancies or lymphoproliferative disorders associated with Bcl2 overexpression.

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