

Mobilization of Hematopoietic Progenitor Cells by Yeast-Derived β -Glucan Requires Activation of Matrix Metalloproteinase-9

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ABSTRACT

Poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-glucopyranose (PGG) β -glucan is a soluble yeast-derived polysaccharide that has previously been shown to induce hematopoietic progenitor cell (HPC) mobilization. However, the mobilizing mechanism of action remains unknown. Here, we confirmed that PGG β -glucan alone or in combination with granulocyte colony-stimulating factor (G-CSF) mobilizes HPC into the periphery. Optimal mobilizing effects were seen 24–48 hours after PGG β -glucan doses of 4.8–9.6 mg/kg. Animals treated with G-CSF and PGG β -glucan showed a collaborative effect in HPC mobilization compared with G-CSF treatment alone. Additional studies demonstrated that neither complement 3 nor complement receptor 3 played a role in this effect and that PGG β -glucan treatment did

not induce proinflammatory cytokine secretion. However, bone marrow cells from PGG β -glucan-treated mice secreted abundant matrix metalloproteinase-9 (MMP-9), and PGG β -glucan-induced HPC mobilization was abrogated in MMP-9 knockout mice. Moreover, we demonstrated that both hematopoietic and nonhematopoietic cells contributed to MMP-9 secretion upon PGG β -glucan treatment. In addition, HPCs mobilized by PGG β -glucan had similar levels of engraftment in host and lineage differentiation capability compared with those mobilized by G-CSF. Thus, PGG β -glucan is an agent that enhances HPC mobilization and may improve the outcome of clinical stem cell transplantation. *STEM CELLS* 2008;26:1231–1240

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Hematopoietic progenitor and stem cell (HPSC) transplantation has been widely used to treat a variety of neoplastic diseases [1]. Both hematopoietic cytokines and myelosuppressive chemotherapy can be used to mobilize HPSCs from the bone marrow (BM) niche to the peripheral blood (PB). Granulocyte colony-stimulating factor (G-CSF) is the most commonly used mobilizing agent because of its superior effectiveness and low side effect profile [2]. However, studies have shown that heavily pretreated patients who have received previous chemotherapy and/or radiation fail to mobilize sufficient stem cells for autotransplantation [3, 4]. Of note, even in normal, previously untreated donors, mobilization with G-CSF alone results in variable CD34⁺ cell numbers. Up to 15% of these healthy donors mobilize suboptimal numbers (fewer than 2.5×10^6 CD34⁺ cells per kilogram) [5, 6]. Therefore, novel strategies to manage patients who mobilize poorly or do not mobilize at all are drastically needed.

Yeast-derived β -glucans are long polymers of β (1,3)-glucose, with 3%–6% of the backbone glucose units possessing a β (1,6) branch [7]. Many yeast-derived β -glucans have been observed to demonstrate significant bioactivity in vivo, including antitumor efficacy and stimulation of hematopoiesis [8–10]. For example, soluble poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-

glucopyranose (PGG) β -glucan was shown in both in vivo and ex vivo models to enhance murine and primate myelopoiesis [11]. In addition, stem-cell-mobilizing effects of PGG β -glucan and the combination of PGG β -glucan with G-CSF have been studied [12]. A single dose of PGG β -glucan was found to mobilize hematopoietic progenitor cells (HPCs) to the PB. Furthermore, an additive effect was seen when PGG β -glucan was used in conjunction with G-CSF. However, the mechanism of action of PGG β -glucan for mobilizing HPSC has not been elucidated. Our previous studies have shown that low molecular weight soluble β -glucan is capable of binding to the lectin site domain of complement receptor 3 (CR3; CD11b/CD18, Mac-1, $\alpha_m\beta_2$ integrin) [8, 13]. In addition, yeast-derived particulate β -glucan has been demonstrated to enhance complement-mediated hematopoietic recovery after BM injury, presumably via stimulation of CR3⁺ HPC proliferation [14]. A previous study also showed that the mobilizing activity of PGG β -glucan was independent of cytokine or chemokine induction [12]. Thus, it is proposed that the mobilizing effect of PGG β -glucan occurs via a novel but previously uncharacterized mechanism.

In this study, we examined the ability of pharmaceutical-grade PGG β -glucan to mobilize HPCs alone or in conjunction with G-CSF. We found that PGG β -glucan, as a single agent, causes HPC mobilization, with a peak time at 24–48 hours. In addition, we confirmed that PGG β -glucan enhances the mobilizing effect of G-CSF. These effects were demonstrated to be

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independent of proinflammatory cytokine secretion or complement 3 (C3) activation but were dependent on matrix metalloproteinase-9 (MMP-9) release. Our additional studies demonstrated that both hematopoietic BM cells, predominantly Ter-119⁺ erythroid cells, and nonhematopoietic BM endothelial cells are responsible for the PGG β -glucan-stimulated MMP-9 secretion. Thus, these studies demonstrate a novel mechanism for PGG β -glucan-induced HPC mobilization and suggest a potential clinical application in combination with G-CSF in HPC transplantation.

MATERIALS AND METHODS

Experimental Animals

All experimental protocols using animals or biological animal specimens were conducted in accordance with all relevant federal guidelines and had been approved by the University of Louisville Institutional Animal Care and Use Committee. Wild-type (WT) C57Bl/6 and FVB mice were obtained from the National Cancer Institute (Frederick, MD). CR3 knockout (KO) and C3 KO mice on C57Bl/6 background and MMP-9 KO mice on FVB background were purchased from Jackson Laboratory (Bar Harbor, ME, <http://www.jax.org>). All mice were given access to food and water ad libitum and were maintained in the university's specific pathogen-free animal facility.

HPC Mobilization by PGG β -Glucan

C57Bl/6 WT, FVB WT, CR3 KO, C3 KO, and MMP-9 KO mice (six mice per group), 4–6 weeks old, were given a single i.v. injection via tail vein of pharmaceutical-grade PGG β -glucan (Imprime PGG; Biothera, Eagan, MN, <http://www.biotherapharma.com>) at the indicated doses. PB was collected by cardiac puncture on sedated mice after PGG β -glucan injection at the indicated time. HPC mobilization was assayed using in vitro methylcellulose culture. All mice had a baseline complete blood count with automated differential analysis performed on a Hemavet 840 (Drew Scientific, Oxford, CT, <http://www.drew-scientific.com>) prior to treatment and at the time of peripheral blood lymphocyte (PBL) collection after treatment.

Combination of G-CSF and PGG β -Glucan for HPC Mobilization

Six- to 8-week-old C57Bl/6 female mice were treated with PGG β -glucan with or without recombinant human G-CSF (Amgen, Thousand Oaks, CA, <http://www.amgen.com>) or phosphate-buffered saline (PBS). PGG β -glucan was administered i.v. at a single dose of 9.6 mg/kg. G-CSF was injected subcutaneously (s.c.) on four consecutive days at a dose of 125 μ g/day. In PGG β -glucan/G-CSF combination-treated groups, mice received G-CSF (125 μ g/day) s.c. from day 1 to day 4, with PGG β -glucan (9.6 mg/kg) i.v. injected on day 2, 48 hours prior to the last dose of G-CSF. PB was collected on day 4, 4 hours after the last dose of G-CSF injection.

MethoCult Colony-Forming Unit Assay

Mouse cells were assayed for total colony-forming units (CFUs) and differential CFUs, such as granulocyte-macrophage colony-forming units (CFU-GM), CFU-G, CFU-M, and erythroid burst-forming unit, using cytokine combinations as described previously [15]. In brief, PB was collected by cardiac puncture, diluted with 5 ml of PBS, and underlaid with 1.5–2 ml of GE Ficoll-Hypaque (GE Healthcare, Little Chalfont, U.K., <http://www.gehealthcare.com>). After centrifugation, the leukocyte layer was carefully removed, gently washed, and then resuspended in 1–2 ml of M-199. Purified leukocyte cell suspension (4×10^5) was mixed with 4 ml of MethoCult medium (StemCell Technologies, Vancouver, BC, Canada, <http://www.stemcell.com>) containing 5 ng/ml recombinant mouse (rm) granulocyte-macrophage colony-stimulating factor (GM-CSF) and 5 ng/ml rm interleukin (IL)-3 (Cell Sciences, Can-

ton, MA, <http://www.cellsciences.com>). Cells were plated at 2×10^5 cells per well in duplicate six-well tissue culture plates and incubated in a humidified atmosphere with 5% CO₂ at 37°C. Colonies were enumerated after approximately 6–7 days of incubation.

Complement Activation Assay

Mice ($n = 6$) were injected with PBS, PGG β -glucan (9.6 mg/kg), or zymosan (100 μ g per mouse; Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>), and the PB was collected in EDTA-coated tubes after 48 hours of injection. Plasma was diluted 1:5 in PBS, added to plates coated with anti-C3a capture antibody (Ab) (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml), and then incubated for 2 hours at room temperature (RT). The plates were washed four times and then incubated with biotin-labeled anti-C3a detecting Ab (BD Biosciences, San Diego, <http://www.bdbiosciences.com>) for 1 hour at RT. Following additional washes, streptavidin-horseradish peroxidase (HRP) was incubated for 20 minutes. Assays were developed with ABTS 1 Component Microwell Substrate (BioFX, Owings Mills, MD, <http://www.biofx.com>).

In Vitro Cytokine Secretion Assay and Measurement of Proinflammatory Cytokines and Stromal Cell-Derived Factor 1 α

To evaluate effects of PGG β -glucan on cytokine secretion, resident peritoneal macrophages were obtained from 8-week-old female C57Bl/6 mice and seeded in 24-well tissue culture plates at 5×10^5 cells per well in the presence of PGG β -glucan (100 μ g/ml) or lipopolysaccharide (10 μ g/ml). After 3 and 24 hours of incubation at 37°C, supernatants were harvested and measured for the presence of the proinflammatory cytokines tumor necrosis factor- α (TNF- α), IL-6, and monocyte chemoattractant protein-1 (MCP-1). A commercial enzyme-linked immunosorbent assay (ELISA; R&D Systems Inc., Minneapolis, <http://www.rndsystems.com>) was used for cytokine detection. Mouse stromal cell-derived factor 1 α (SDF-1 α) levels from plasma or BM were also measured by ELISA (Quantikine; R&D Systems) according to the manufacturer's instructions.

Measurement of Pro-MMP-9 by ELISA and Zymography

Groups of mice were injected with PGG β -glucan (9.6 mg/kg) or PBS via the tail vein. Mice were sacrificed at 24 hours postinjection. BM cells were harvested and cultured at 500,000 cells per well in serum-free RPMI 1640 medium in 96-well tissue culture plates for 6, 12, 24, or 48 hours. Supernatants were harvested, and the pro-MMP-9 concentration was determined by sandwich ELISA according to the manufacturer's instructions (R&D Systems). The supernatants were also analyzed by zymography as described previously [16]. In brief, supernatants were electrophoresed in a 10% Zymogram Pre-Cast gelatin gel (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>). To remove SDS, the gel was washed four times for 30 minutes each in washing buffer (50 mM Tris-HCl, pH 7.5; 10 mM CaCl₂; 2.5% Triton X-100). The gel was incubated at 37°C overnight with shaking and subsequently stained with Coomassie Blue. The presence of gelatinolytic activity was identified as clear bands on a blue background after destaining. Invitrogen BenchMark prestained ladder (Invitrogen) was used to reveal standard protein molecular weight.

BM Immunohistochemistry and Immunofluorescence Staining Analysis

Paraffin-embedded BM samples were prepared at the pathology core facility of the University of Louisville Health Sciences Center. The BM sections were deparaffinized in 100% xylene and rehydrated stepwise in 100%, 95%, 70%, ethanol. The sections were then washed with distilled water two times, and endogenous peroxidases quenching was performed in 3% H₂O₂ for 10 minutes. The slides were incubated in 3% bovine serum albumin overnight, washed twice with distilled water, and then stained with anti-MMP-9-biotin (clone 7-11C; Santa Cruz Biotechnology Inc., Santa Cruz, CA, <http://www.scbt.com>) for 2 hours at RT. After three washes

with blocking buffer, the sections were stained with streptavidin-HRP (SouthernBiotech, Birmingham, AL, <http://www.southernbiotech.com>) for 1 hour at RT. After additional washes, HRP substrate (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>) was added for 30 minutes at RT. Following an additional three washes, the sections were counterstained with hematoxylin to provide morphological details. For immunofluorescence staining, BM sections were stained with anti-MMP-9-fluorescein isothiocyanate monoclonal antibody (mAb) (clone 7-11C; Santa Cruz Biotechnology) and Ter-119-phycoerythrin (PE) (clone TER-119; BD Biosciences), anti-Gr-1-PE (clone RB6-8C5; BD Biosciences), anti-F4/80-PE (clone 6F12; BD Biosciences), or anti-CD31-PE (clone MEC 13.3; BD Biosciences) mAbs. Fluorescence was analyzed using a Nikon Eclipse confocal microscope (Nikon, Tokyo, <http://www.nikon.com>).

BM Cell Sorting and Real-Time Polymerase Chain Reaction for MMP-9 and Erythropoietin mRNA Levels

Freshly isolated BM cells were immunostained for 30 minutes at 4°C in the presence of anti-CD16/32 mAb Fc receptor blocker (clone 2.4G2; BD Biosciences), fluorochrome-conjugated anti-Ter119 mAb, and anti-CD71 mAb (clone C2; BD Biosciences). Ter119⁺CD71^{high} and Ter119⁺CD71^{low} populations were sorted by MoFlo (Cytomation, Ft. Collins, CO, <http://www.dakocytomation.com>).

To detect MMP-9 mRNA levels by real-time polymerase chain reaction (PCR), RNA was prepared from freshly sorted cells using the RNeasy kit (Qiagen, Valencia, CA, <http://www1.qiagen.com>). Real-time PCR was conducted on a Bio-Rad (Hercules, CA, <http://www.bio-rad.com>) MyIQ single-color real-time PCR system. SYBR Green real-time PCR in conjunction with the following primers was used: for β 2-microglobulin, forward, 5' CAT ACG CCT GCA GAG TTA AGC A 3'; reverse, 5' GAT CAC ATG TCT CGA TCC CAG TAG 3'; for MMP-9, forward, 5' ACC ACC ACC ACC ACC ACA C 3'; reverse, 5' GCC TGC CTC CAC TCC TTC C 3'; and for erythropoietin (EPO), forward, 5' CAT AGA AGT TTG GCA AGG C 3'; reverse, 5' CAG TGA AGT GAG GCT ACG 3'.

BM Chimeras

Recipient WT or MMP-9 KO mice received fully myeloablative preconditioning with a 950-cGy dose of cesium-137 γ -irradiation (MDS Nordion, Ottawa, <http://www.mds.nordion.com>) administered as a single exposure, 8 hours prior to transplant of BM cells. Syngeneic whole BM cells (5×10^6) from WT or MMP-9 KO mice were then administered by i.v. injection in 0.5 ml of M-199 medium containing 20 μ g/ml gentamicin to MMP-9 KO or WT mice, respectively. To confirm the status of donor BM cell engraftment, PB is drawn at day 21 post-transplant, stained with fluorochrome-conjugated mAbs, and then analyzed by flow cytometry.

HPC Transplantation

Recipient SJL (CD45.1) mice received preconditioning irradiation of 800 cGy by a single exposure, 6 hours prior to transplants. Five hundred c-kit⁺sca-1⁺lineage⁻ (KSL) cells from mobilized donor C57BL/6 mice (CD45.2) were administered by i.v. injection. KSL cells were sorted by MoFlo (Cytomation) from the PB of C57BL/6 mice mobilized by PGG β -glucan, G-CSF, or G-CSF plus PGG β -glucan, as described above, and the purity of all sorts was >95%. Thirty days following HPC injection, the animals were characterized for engraftment using flow cytometry to determine the percentage of lineage cells bearing the donor CD45.2 marker using fluorochrome-conjugated mAbs.

Graphing and Statistical Analysis of Data

Differences were evaluated using the two-tailed *t* test function in Microsoft Excel (Microsoft, Seattle, <http://www.microsoft.com>). In comparing statistical significance among multiple groups, one-way analysis of variance was used with Prism 4.0 (GraphPad, San Diego, <http://www.graphpad.com>). *p* values <.05 were considered significant.

RESULTS

Mobilization of HPC with PGG β -Glucan in the Presence or Absence of G-CSF

To determine the ability of PGG β -glucan to mobilize HPC to the periphery, the time kinetics of HPC mobilization with a single dose of PGG β -glucan were first examined. Mice were given a single dose of 5 mg/kg PGG β -glucan, and CFUs were cultured from PB collected at the times indicated in Figure 1A. Peak CFU mobilization was seen at 24 hours after PGG β -glucan injection (*p* < .05 vs. PBS). The mobilization effect lasted until 48 hours and then declined at 72 hours. Evaluation of differential CFU types demonstrated that PGG β -glucan induced a preferential increase in CFU-GM, CFU-G, or CFU-M counts (data not shown). However, the increase of peripheral white blood cell numbers in PGG β -glucan-treated mice was not definitive (data not shown).

Next, we investigated the dose-response curve of PGG β -glucan-induced HPC mobilization. To this end, mice were given PGG β -glucan at different doses, and PB was collected and plated for CFU at 24 hours after injection. As shown in Figure 1B, the greatest response was seen at the 9.6 mg/kg dose. Interestingly, the higher PGG β -glucan doses (20 and 40 mg/kg) caused less HPC mobilization. Therefore, the dose-response curve of PGG β -glucan is bell-shaped. This has also been observed with other mobilizing agents [17].

G-CSF is now the most commonly used HPC mobilizer. To determine whether PGG β -glucan and G-CSF have potential additive or synergistic mobilization effects, mice were treated on four consecutive days with G-CSF and administered PGG β -glucan (9.6 mg/kg) 48 hours before the last G-CSF injection. PB or BM cells stained for KSL progenitor cells were then assessed by flow cytometry 4 hours after the final G-CSF treatment. Controls included mice treated with PBS alone, PGG β -glucan alone, and G-CSF alone. As shown in Figure 1C–1E, both PGG β -glucan alone and G-CSF alone increased KSL cells in the PB and BM, thus confirming mobilization. In addition, PGG β -glucan showed a collaborative effect with G-CSF (*p* < .05 vs. group treated with G-CSF alone).

PGG β -Glucan-Induced HPC Mobilization Is Independent of C3 Activation, CR3, or Proinflammatory Cytokine Secretion but Increases SDF-1 α Levels in the Plasma

The complement system has recently been implicated in the involvement of HPC homing [18–20], as well as HPC mobilization [21]. To examine whether PGG β -glucan-induced HPC mobilization results in complement activation, the serum C3a level was measured in mice receiving PGG β -glucan. As indicated in Figure 2A, the C3a level was not significantly altered in mice receiving PGG β -glucan or PBS, suggesting that C3 activation is not induced by PGG β -glucan. Previously, we demonstrated that PGG β -glucan is predominately captured and processed by macrophages, followed by an active moiety being released, which primes neutrophil CR3 [8]. We further demonstrated that particulate β -glucan stimulated CR3⁺ HPCs for enhanced engraftment and survival [14]. To explore whether C3 or CR3 is involved in PGG β -glucan-induced HPC mobilization, C3-KO and CR3-KO mice were treated with PGG β -glucan and evaluated for HPC mobilization. As depicted in Figure 2B, the CFU counts did not significantly differ in WT mice versus C3-KO mice or CR3-KO mice.

The release of cytokines or chemokines, such as IL-8, IL-6, macrophage-inflammatory protein-2, and keratinocyte-derived

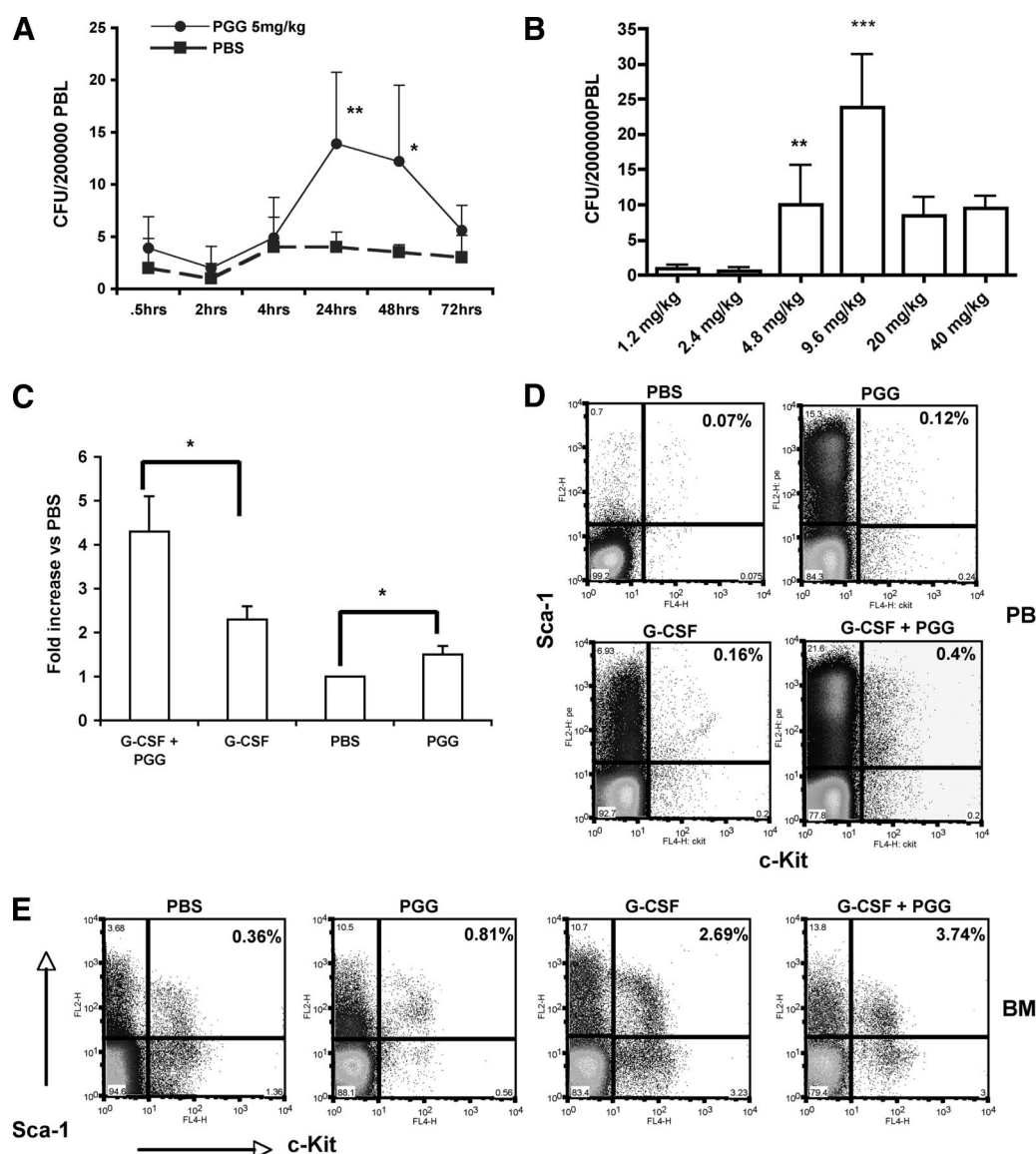


Figure 1. PGG β -glucan-mediated mobilization of hematopoietic progenitor cells (HPCs) with or without G-CSF. (A): Time kinetics of PGG β -glucan-mediated HPC mobilization. C57Bl/6 mice ($n = 5$) received a single i.v. injection of 5 mg/kg PGG β -glucan and were assayed at the indicated time points for mobilization of CFU into peripheral blood (PB) using the MethoCult assay. Mice injected with PGG β -glucan 24 and 48 hrs prior to PBL collection showed significant HPC mobilization compared with other times (*, $p < .05$; **, $p < .01$, compared with PBS). (B): Dose titration curve of HPC mobilization after a single i.v. injection of PGG β -glucan. Results show that a single injection of 4.8 or 9.6 mg/kg revealed significantly greater HPC mobilization compared with PBS (**, $p < .01$; ***, $p < .001$). Mean value $\pm$ SD of duplicates is shown. (C): Fold increase of circulating c-Kit⁺Sca-1⁺Lin⁻ (KSL) cells from PGG β -glucan-, G-CSF-, or PGG β -glucan plus G-CSF-mobilized mice compared with PBS-treated mice. Groups of C57Bl/6 mice ($n = 15$) were mobilized with PGG β -glucan, G-CSF, or PGG β -glucan plus G-CSF. Circulating KSL cells from PB were assessed by flow cytometry (*, $p < .05$). (D, E): Flow cytometry analysis of mononuclear cells derived from PB (D) or bone marrow (E) of PBS control, PGG β -glucan, G-CSF, or PGG β -glucan plus G-CSF-treated mice. Values represent the percentage of KSL cells. Abbreviations: CFU, colony-forming units; G-CSF, granulocyte colony-stimulating factor; hrs, hours; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PGG, poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-glucopyranose.

chemokine, was demonstrated to have a role in HPC mobilization. Therefore, we extensively examined serum cytokine levels in PGG β -glucan-treated animals. Several cytokines, including IL-6, IL-8, TNF- α , MCP-1, GM-CSF, and G-CSF, were measured, and none of these were significantly increased in PGG β -glucan-treated animals (data not shown).

SDF-1 α (CXCL12) is a highly potent chemoattractant both in vitro and in vivo for HPCs, which express its receptor CXCR4 [22, 23]. To investigate whether PGG β -glucan-mediated HPC mobilization induces alteration of SDF-1 α gradients, both plasma and BM from mobilized mice were measured for

SDF-1 α levels. As shown in Table 1, PGG β -glucan significantly increased SDF-1 α levels in the plasma but not in the BM.

PGG β -Glucan Stimulates BM Cells to Secrete MMP-9

To further explore the mechanism of action of PGG β -glucan-induced HPC mobilization, we examined whether protease MMP-9 is involved in this process. Many agents, such as 5-fluorouracil (5-FU) [16], have been described to induce mobilization of HPSC via MMP-9. In addition, studies have demonstrated that mobilization by GRO β requires neutrophils and

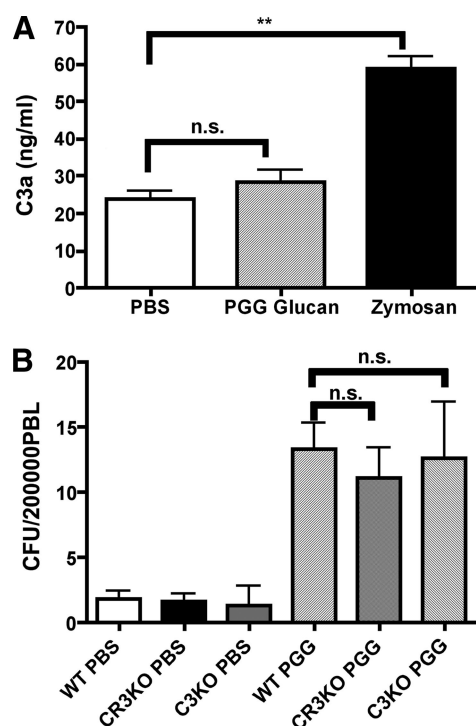


Figure 2. PGG β -glucan-mediated hematopoietic progenitor cell mobilization is independent of complement 3 (C3) activation or complement receptor 3 (CR3). (A): PGG β -glucan does not induce C3 activation. C57Bl/6 mice ($n = 6$) were injected with PBS, PGG β -glucan, or zymosan. Sera were collected 48 hours after treatment and assayed for C3a by enzyme-linked immunosorbent assay. Zymosan treatment-induced C3 activation shown as positive control (**, $p < .01$). (B): C57Bl/6 WT, C3KO, or CR3KO mice ($n = 4$) were given a single dose of 9.6 mg/kg PGG β -glucan. The PBLs were harvested 24 hours later and plated in MethoCult for CFU determination. Results show no significant difference in CFU numbers and WT, C3KO, and CR3KO mice in response to PGG β -glucan treatment. The data shown in this figure are from one representative experiment of two independent experiments performed. Mean value $\pm$ SD of duplicates is shown. Abbreviations: C3KO, complement 3 knockout; CFU, colony-forming units; CR3KO, complement receptor 3 knockout; n.s., not significant; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PGG, poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-glucopyranose; WT, wild-type.

Table 1. Stromal cell-derived factor 1 α (SDF-1 α) protein levels (ng/ml) in plasma and BM

Treatment	Plasma	BM
PBS	2.43 $\pm$ 0.43	2.36 $\pm$ 0.88
PGG β -glucan	4.42 $\pm$ 0.49	2.72 $\pm$ 0.94
	*** $p < .001$	$p > .05$

Cohorts of five C57Bl/6 mice per group per experiment were mobilized by a single injection of PGG β -glucan (9.6 mg/kg) or PBS saline control. After 24 hours, plasma and BM aspirates were collected, and the levels of SDF-1 α were measured by enzyme-linked immunosorbent assay. The results are presented as mean ng/ml values $\pm$ SD. The lower limit of quantitation for SDF-1 α was 0.014 ng/ml. (***, $p < .001$ compared with PBS) Abbreviations: BM, bone marrow; PBS, phosphate-buffered saline; PGG, poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-glucopyranose.

neutrophil-derived active MMP-9 [24]. To this end, BM cells were harvested from PGG β -glucan-injected mice and cultured in the serum-free medium. The supernatants were collected at the different time points and assayed for pro-MMP-9 and active

MMP-9 levels by ELISA and zymography, respectively (Fig. 3A, 3B). Pro-MMP-9 levels were significantly elevated (>10 -fold increase vs. that in BM from PBS-injected mice) after 48 hours of culture and declined in 72 hours of culture. Zymography showed that active MMP-9 levels peaked at 48 hours culture. To further confirm MMP-9 secretion in BM, immunohistochemistry analysis was performed. In PBS-treated animals, there was a small amount of MMP-9 deposition in BM (Fig. 3C). In contrast, massive MMP-9 deposition was observed in BM from PGG β -glucan-treated mice, starting 24 hours after injection (Fig. 3D) and reaching a peak at 48 hours after injection (Fig. 3E). These data suggest that both pro-MMP-9 and MMP-9 are upregulated in BM cells after PGG β -glucan treatment.

To understand the role of MMP-9 in PGG β -glucan-induced HPC mobilization, MMP-9 KO mice were used further. Since commercially available MMP-9 KO mice are on FVB background, we first examined whether PGG β -glucan is capable of mobilizing HPC in this strain of mice. CFU counts in FVB mice injected with PGG β -glucan were comparable to those in C57Bl/6 mice. The time kinetics and dose-response curves were also similar between the two mouse strains (data not shown). Next, WT and MMP-9 KO mice were treated with 9.6 mg/kg PGG β -glucan or in conjunction with G-CSF, and PBLs were harvested and plated for CFU. Strikingly, HPC mobilization induced by PGG β -glucan in WT mice was significantly abrogated in MMP-9 KO mice (Fig. 4A). G-CSF-mediated mobilization was also significantly decreased in MMP-9 KO mice, as previously reported [16]. In addition, the collaborative HPC mobilization was completely abrogated in MMP-9 KO mice (Fig. 4A). Immunohistochemistry analysis revealed that upon PGG β -glucan mobilization, strong MMP-9 deposition occurred in WT mice, whereas no such deposition was detected in BM from MMP-9 KO mice (Fig. 4B). These data suggest that MMP-9 has a critical role in PGG β -glucan-induced HPC mobilization.

Both Hematopoietic Cells and Nonhematopoietic Cells Contribute to PGG β -Glucan-Induced MMP-9 Secretion

Next, we investigated which cells are responsible for PGG β -glucan-induced MMP-9 production using BM transplantation models. WT or MMP-9 KO mice were lethally irradiated and reconstituted with donor BM cells from MMP-9 KO or WT mice, respectively. After allowing 8 weeks for engraftment, these mice were then administered PGG β -glucan and assayed for HPC mobilization. As shown in Figure 5A, WT mice receiving MMP-9 KO BM mobilized significantly fewer CFU than WT mice ($p < .001$) but mobilized significantly more CFU than MMP-9 KO mice ($p < .05$). Likewise, MMP-9 KO mice receiving WT BM cells mobilized significantly more CFU than MMP-9 KO mice ($p < .001$) but mobilized significantly fewer CFU than WT mice ($p < .01$). These data suggest that both hematopoietic and nonhematopoietic BM cells, possibly BM stromal cells or endothelial cells, contribute to PGG β -glucan-induced MMP-9 release.

To further elucidate possible cell subset(s) producing MMP-9 upon PGG β -glucan stimulation, fluorescein-dye-labeled PGG β -glucan was injected into mice i.v., and BM cells were harvested 24 hours after injection. As shown in Figure 5B, PGG β -glucan was predominately bound to F4/80 $^{+}$ macrophages, Ter-119 $^{+}$ erythroid cells, and CD31 $^{+}$ endothelial cells. Gr-1 $^{+}$ neutrophils, natural killer cells, and CD11c $^{+}$ dendritic cells had a very low binding to PGG β -glucan. To examine whether these cells produce MMP-9 upon PGG β -glucan binding, confocal microscopy analysis was performed. As indicated

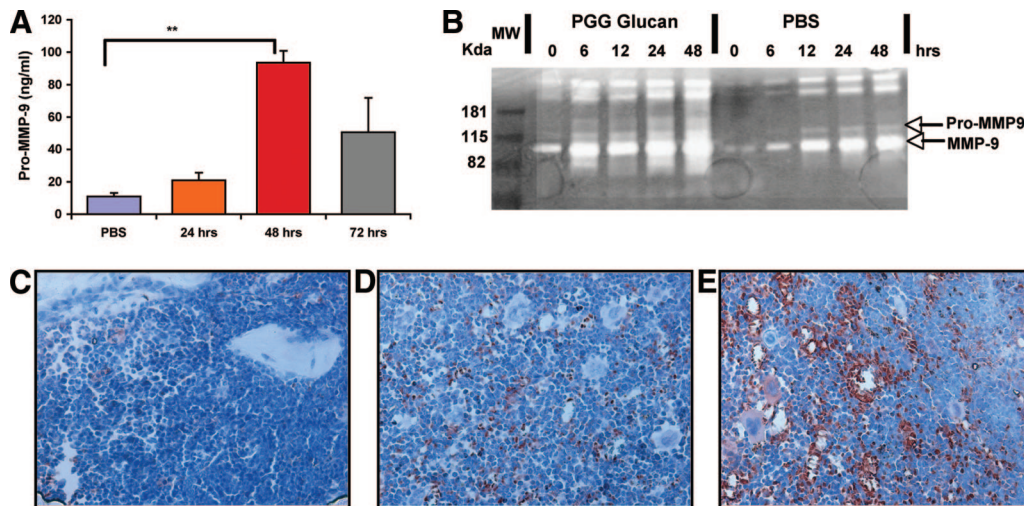


Figure 3. MMP-9 is induced in bone marrow (BM) cells after PGG β -glucan treatment. **(A):** BM cells from C57Bl/6 mice ($n = 5$) administered a single dose of i.v. PGG β -glucan (9.6 mg/kg) or PBS were cultured in serum-free medium. The supernatants were harvested at different time points and assayed for pro-MMP-9 by enzyme-linked immunosorbent assay. The results show that PGG β -glucan-mobilized BM cells secrete pro-MMP-9, peaking at 48 hrs (**, $p < .01$ compared with PBS). **(B):** Supernatants collected at the indicated time points were assayed via zymography for active MMP-9 versus pro-MMP-9. **(C–E):** Immunohistochemistry analysis of BM sections stained with anti-MMP-9 monoclonal antibody demonstrates active MMP-9 deposition, which shows brown staining, in PBS control **(C)** or PGG β -glucan-mobilized BM at 24 hrs **(D)** or 48 hrs **(E)**. Original magnification, $\times 400$. The sections shown in this figure are from one representative BM section of five total BM specimens. The data shown in this figure are one representative experiment of two independent experiments performed. Abbreviations: hrs, hours; Kda, kilodaltons; MMP-9, matrix metalloproteinase-9; MW, molecular weight; PBS, phosphate-buffered saline; PGG, poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-glucopyranose.

in Figure 5C, neutrophils did not appear to be colocalized with MMP-9. Only a fraction of F4/80⁺ macrophages and CD31⁺ endothelial cells appeared to be colocalized with MMP-9. Surprisingly, MMP-9 staining was predominantly colocalized with Ter-119⁺ erythroid cells. These data suggest that Ter-119⁺ erythroid cells are the predominant MMP-9-secreting cells in response to PGG β -glucan treatment.

To further confirm our findings from the confocal microscopy analysis, we sorted BM Ter-119⁺ cells and Gr-1⁺ cells from mobilized mice (Fig. 5D). As shown in Figure 5E, Ter-119⁺ cells, but not Gr-1⁺ cells, secreted significant amounts of MMP-9 upon PGG β -glucan treatment. G-CSF also stimulated Ter-119⁺ cells secreting significant amounts of MMP-9, which is consistent with our previous finding [25]. In addition, a synergistic increase of MMP-9 on Ter-119⁺ cells occurred in response to PGG β -glucan plus G-CSF stimulation. In contrast, Gr-1⁺ cells predominantly secreted MMP-9 after G-CSF mobilization. Erythroblasts in the BM can be further differentiated on the basis of their expression of the transferrin receptor (CD71) [26, 27]. To further delineate which subpopulation(s) is responsible for PGG β -glucan-induced MMP-9 secretion, Ter-119⁺CD71^{bright} (basophilic and polychromatic erythroblasts) and Ter-119⁺CD71^{dim} (orthochromatic erythroblasts) populations were further sorted (Fig. 5D). As depicted in Figure 5F, the Ter-119⁺CD71^{bright} subset predominantly secreted MMP-9 upon PGG β -glucan stimulation. Interestingly, EPO mRNA expression levels were also accordingly upregulated upon PGG β -glucan treatment (data not shown).

HPCs Mobilized by PGG β -Glucan Are Capable of Engraftment and Differentiation

To test the quality of HPCs mobilized by PGG β -glucan, the engraftment and lineage differentiation capabilities were assessed. HPCs mobilized by PGG β -glucan, G-CSF, or both from C57Bl/6 (CD45.2) PB were sorted and transplanted into lethally irradiated recipient SJL mice (CD45.1). As shown in Figure 6A, the percentage of engraftment originated from CD45.2 donors after transplantation was similar among HPCs mobilized from

PGG β -glucan, G-CSF, or PGG β -glucan plus G-CSF. In addition, the engrafted HPCs are capable of differentiating into major lineage cells, as shown in Figure 6B. These data suggest that PGG β -glucan-mobilized HPCs are fully functional and can potentially be used for the clinical application.

DISCUSSION

Previously, PGG β -glucan has been shown to stimulate hematopoiesis after BM injury [11, 22] and to mobilize HPC to the periphery [12]. In a previous study, peak mobilization was seen at 30 minutes after a PGG β -glucan dose of 2 mg/kg, and an additive effect was seen when PGG β -glucan was administered in conjunction with G-CSF [12]. However, the mechanism of action was not elucidated, but it was shown that HPC mobilization induced by PGG β -glucan was not associated with the induction of cytokines or chemokines. Here, we found that peak HPC mobilization occurred at 24–48 hours after a 9.6 mg/kg dose of PGG β -glucan. In addition, a collaborative effect was seen when PGG β -glucan was administered with G-CSF. Moreover, we demonstrated that HPCs mobilized by PGG β -glucan are capable of differentiating into different-lineage cells. The differences found in the dose-response and kinetic studies can be attributed mainly to the different mouse strains used [28].

To date, no mechanism has been elucidated to define how PGG β -glucan mobilizes HPCs. Previous studies demonstrated that particulate β -glucan could be taken up by macrophages that release an active moiety of β -glucan bound to the lectin-like domain of the CR3 on stem cells after bone marrow injury [14]. CR3 priming allowed for accelerated hematopoietic recovery, presumably by enhancing the proliferative ability of progenitor cells [14]. In addition, the complement system has demonstrated an important role in the retention and mobilization of HPCs [18]. However, our results show that soluble PGG β -glucan mobilizes HPCs via a CR3- and C3-independent mechanism. No difference in mobilization was seen in CR3- or C3-deficient mice compared with WT mice. It may be important to examine

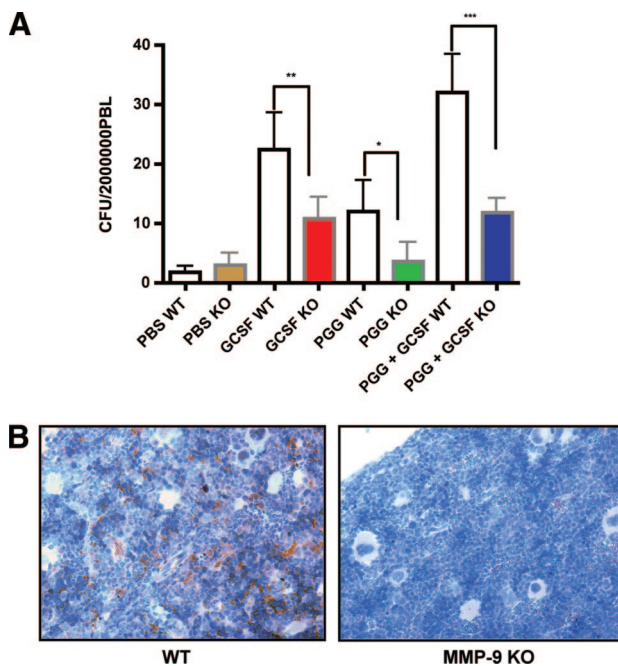


Figure 4. PGG β -glucan-mediated hematopoietic progenitor cell (HPC) mobilization is dependent on MMP-9. (A): FVB WT and MMP-9 KO mice were treated with a single dose of PGG β -glucan (9.6 mg/kg), G-CSF, or PGG β -glucan plus G-CSF, and PBLs were assayed 24 hours later for CFU. The results indicate that PGG β -glucan-mediated, as well as G-CSF-mediated, HPC mobilization is significantly abrogated in MMP-9 KO mice (*, $p < .05$; **, $p < .01$). The collaborative mobilization of PGG β -glucan plus G-CSF is also abrogated in MMP-9 KO mice (***, $p < .001$). (B): Immunohistochemistry analysis of BM sections stained with anti-MMP-9 monoclonal antibody in WT and MMP-9 KO mice after PGG β -glucan treatment. Original magnification, $\times 400$. The sections shown in this figure are representative BM sections of 10 total BM specimens. Abbreviations: CFU, colony-forming units; G-CSF, granulocyte colony-stimulating factor; KO, knockout; MMP-9, matrix metalloproteinase-9; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PGG, poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-glucopyranose; WT, wild-type.

the role of C5 in the PGG β -glucan-mediated mobilization. A recent study demonstrated that C5 plays a critical role in HPC egress, whereas C3 is important in HPC engraftment [29]. In addition, our studies showed that there was no increase in common cytokines known to be active or released during HPC mobilization, such as IL-8, G-CSF, or GM-CSF. Furthermore, after stimulation of PGG β -glucan, the IL-6, TNF- α , and MCP-1 levels were not increased, which is different from the effects induced by the sulfated polysaccharide fucoidan [30, 31]. However, SDF-1 α levels were significantly elevated in PGG β -glucan-mobilized mouse plasma, suggesting that SDF-1-CXCR4 interactions may be involved in HPC mobilization. Thus, PGG β -glucan is unique in its lack of a proinflammatory effect and may offer a clinical advantage over other mobilizing agents.

Proteases such as neutrophil elastase, cathepsin G, and MMP-9 are known to have an important role in HPC mobilization [4]. These proteases are found in the BM environment and can cleave and inactivate a number of proteins essential for the retention of HPCs within the BM. For example, they have been found to cleave the N-terminal domain of CXCR4 on HPCs. In addition, they are capable of degrading SDF-1 α in the BM, thereby altering SDF-1 α gradients [2, 32, 33]. MMP-9 alone has been implicated as having a key mechanistic role in stem cell recruitment and hematopoietic recovery [16]. Increased levels of

MMP-9 have been found after mobilization with G-CSF, IL-8, 5-FU, and GRO β [16, 34–36]. Growth factors and cytokines can upregulate MMP-9 expression in BM stem cells [37]. HPC recruitment by chemotherapy or G-CSF has been shown to be impaired in MMP-9 KO mice [16]. Another study showed that anti-MMP-9 mAbs can also inhibit mobilization induced by IL-8 or GRO β by 90% and mobilization induced by G-CSF by 40% in mice [24, 34]. In our study, we found that after a single PGG β -glucan injection, pro- and active-MMP-9 levels in BM were significantly increased at 48 hours compared with PBS control. Furthermore, the active MMP-9 was significantly deposited on BM 24 hours after administration of PGG β -glucan, and massive deposition occurred at 48 hours. Of significance, selective removal of MMP-9 in KO mice results in significant abrogation of HPC mobilization induced by PGG β -glucan. From these results taken together, we conclude that PGG β -glucan-mediated HPC mobilization is dependent on MMP-9.

MMP-9 is secreted predominantly by granulocytes and macrophages [38, 39]. The enzyme is also secreted by monocytes, lymphocytes, mast cells, keratinocytes, megakaryocytes, early erythroid cells, and fibroblasts upon stimulation [25, 40–46]. Recent studies demonstrate that osteoclasts also secrete MMP-9 [47, 48], as MMP-9 KO mice suffer delayed ossification of growth-plate cartilage [49]. For the most part, neutrophils are generally considered the predominant MMP-9-secreting cells that act in HPC mobilization. Neutrophils have been found to contain abundant MMP-9 [24]. In our study, however, we did not find a significant difference in HPC mobilization between neutrophil-depleted and normal mice (data not shown). Using the BM chimeric model, we demonstrated that both hematopoietic BM cells and nonhematopoietic BM cells contribute to MMP-9 secretion upon PGG β -glucan stimulation. In addition, our studies indicated that hematopoietic BM macrophages bind with PGG β -glucan, but only a fraction of macrophages appear to secrete MMP-9. Surprisingly, hematopoietic erythroid cells are the predominant MMP-9-secreting cells upon PGG β -glucan stimulation. The double-positive cells (Ter-119 $^{+}$ MMP-9 $^{+}$) appear to be in the BM sinusoidal vasculature. In addition, a fraction of nonhematopoietic BM endothelial cells also appear to be capable of binding with PGG β -glucan and producing MMP-9. ELISA and quantitative real-time PCR further confirmed that after PGG β -glucan treatment, MMP-9 expression levels were significantly elevated on early erythroblasts but not on Gr-1 $^{+}$ cells. Therefore, we described a unique and previously underappreciated source of MMP-9 contributing to HPC mobilization upon PGG β -glucan stimulation.

But what MMP-9 regulates after PPG β -glucan stimulation remains to be determined. One clue comes from the observation that the SDF-1 α in the plasma was significantly elevated in PGG β -glucan-mobilized mice. Sulfated polysaccharide fucoidan and 5-FU also increase plasma levels of SDF-1 α [16, 31, 50]. It is possible that SDF-1 α in the BM is processed and inactivated by MMP-9 as previously described [32]. Therefore, HPSC tethering in the BM niche via the SDF-1-CXCR4 axis is potentially weakened, thereby causing HPSC egress. Alternatively, increased plasma SDF-1 α potentially chemoattracts CXCR4 $^{+}$ HPCs to egress from the BM niche to the periphery. Nevertheless, our study demonstrates that PGG β -glucan-induced HPC mobilization is significantly decreased in MMP-9 KO mice, even though the mobilization is not completely abrogated. This could be due to the compensatory rise in MMP-2 or other gelatinolytic activity seen in MMP-9 KO mice [36].

The collaborative HPC mobilization by G-CSF plus PGG β -glucan was observed in WT mice (Figs. 1, 4) but was abrogated in MMP-9 KO mice (Fig. 4A). Those data reaffirm the critical role of MMP-9 in PGG β -glucan-mediated HPC mobilization. The synergistic mobilization by G-CSF plus PGG

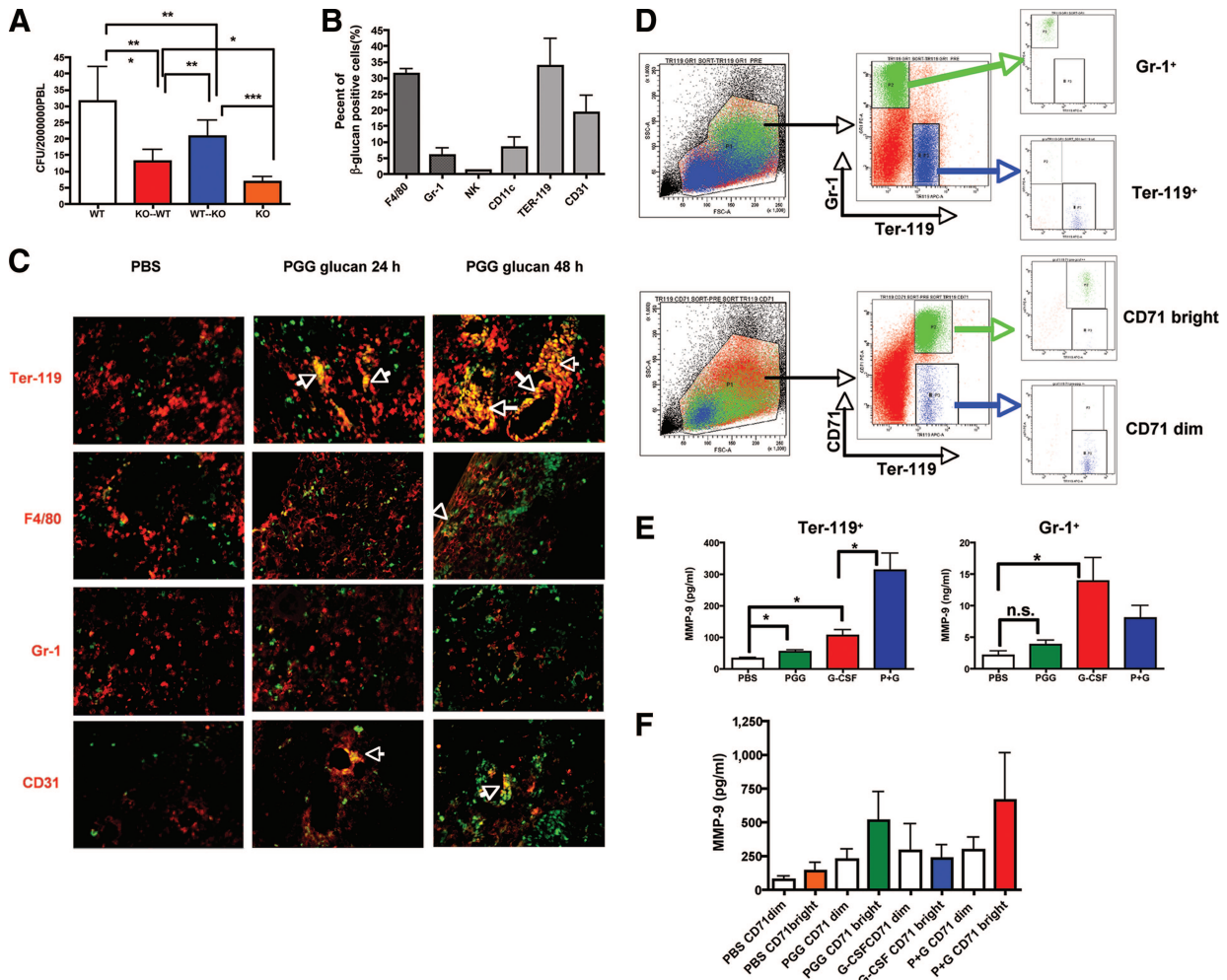


Figure 5. Both hematopoietic and nonhematopoietic bone marrow (BM) cells contribute to MMP-9 secretion upon PGG β -glucan stimulation. (A): BM chimeras. Lethally irradiated (950 cGy) FVB WT or MMP-9 KO mice ($n = 10$) were reconstituted with BM cells from MMP-9 KO or WT mice, respectively. After 8 weeks of engraftment, mice were mobilized with 9.6 mg/kg PGG β -glucan. The PBLs were harvested 24 hours later and assayed for CFU. Mean value $\pm$ SD of duplicates is shown. (B): PGG β -glucan binding to different cell subsets. Mice ($n = 3$) that received fluorescein dichlorotriazine-labeled PGG β -glucan (9.6 mg/kg) were sacrificed after 24 hours of administration. The percentage of β -glucan-positive cells was assessed by flow cytometry. (C): BM sections from mice receiving PGG β -glucan (9.6 mg/kg) administration after 24 or 48 hours or PBS saline control were stained with Ter-119-PE, F4/80-PE, Gr-1-PE, or CD31-PE and costained with fluorescein isothiocyanate-anti-MMP-9 monoclonal antibody (green). Original magnification, $\times 400$. The sections shown in this figure are representative BM sections of 15 total BM specimens. $*, p < .05$; $**, p < .01$; $***, p < .001$. (D): Ter-119⁺ versus Gr-1⁺ cells and Ter-119⁺CD71^{bright} or CD71^{dim} populations were sorted from BM. (E): BM Gr-1⁺ and Ter-119⁺ cells (2×10^5 cells per well) from PBS-, PGG β -glucan-, G-CSF-, and PGG β -glucan plus G-CSF-mobilized mice were cultured in serum-free medium for 24 hours. The supernatants were harvested and assayed for MMP-9 by enzyme-linked immunosorbent assay. $*, p < .05$. (F): The data suggest that the Ter-119⁺ CD71^{bright} population predominately secretes MMP-9 after PGG β -glucan stimulation. The data are combined results from three independent experiments. Abbreviations: CFU, colony-forming units; G-CSF, granulocyte colony-stimulating factor; FSC, forward scatter; h, hours; KO, knockout; KO-WT, knockout-wild-type; MMP-9, matrix metalloproteinase-9; n.s., no significance; NK, natural killer; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PGG, poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-glucopyranose; P+G, PGG plus G-CSF; WT, wild-type; WT-KO, wild-type-knockout.

β -glucan could coincide with a synergistic increase of MMP-9. In addition, G-CSF has been shown to induce upregulation of CXCR4 on BM cells [2]. Upregulation of CXCR4 may serve to decrease the sensitivity of HPC to lower SDF-1 α signals. The elevated SDF-1 α levels in the plasma upon PGG β -glucan treatment could therefore synergize with G-CSF to facilitate the egress of HPCs. On the basis of those findings, we propose the mechanism of action of PGG β -glucan-induced HPC mobilization. The i.v.-administered PGG β -glucan is captured by monocytes in the periphery, which then migrate into BM. In the BM, PGG β -glucan predominantly stimulates Ter-119⁺ early erythroblasts or macrophages alone or erythroblast and macrophage aggregates in erythroblastic islands and endothelial cells to secrete MMP-9. MMP-9 could inactivate SDF-1 α in the BM,

and the elevated SDF-1 α in the plasma leads to egress of HPC mobilization. PGG β -glucan may also synergize with G-CSF mobilization, in which synergistically upregulated MMP-9 in BM and CXCR4 expression on HPCs lead to the enhanced HPC mobilization.

SUMMARY

Our study has shown that PGG β -glucan can mobilize HPCs to the PB when used alone and has a collaborative effect when used in combination with G-CSF. This collaborative effect could potentially aid in transforming poor responders or nonresponders to

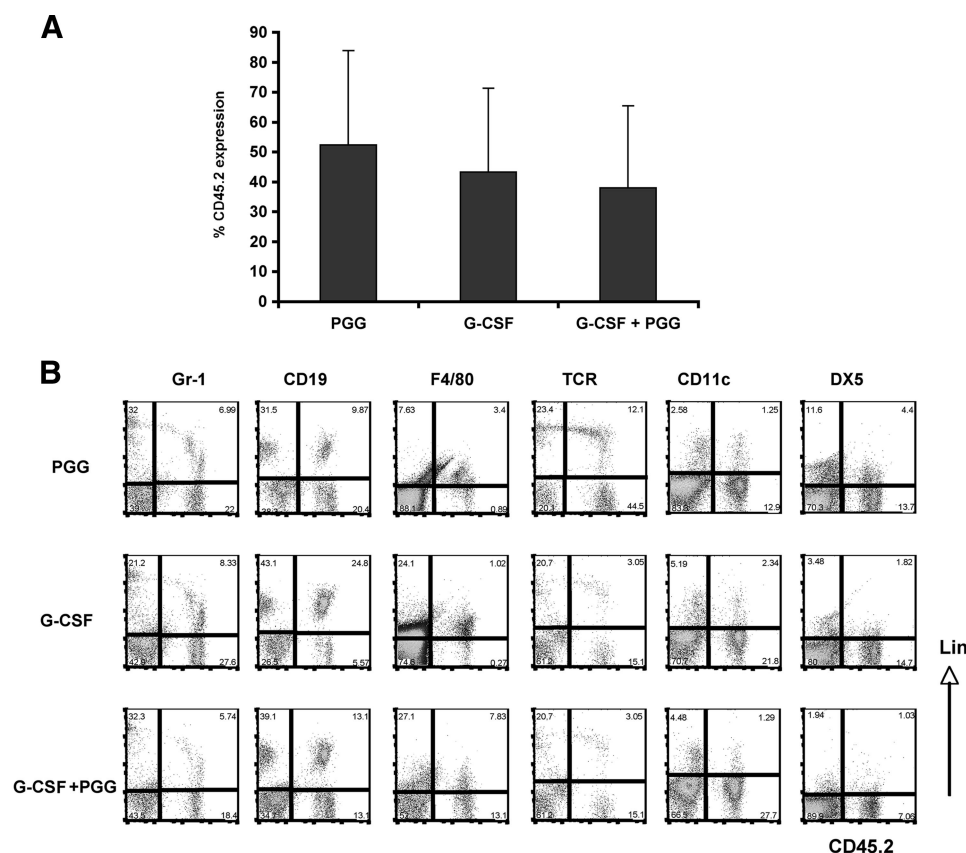


Figure 6. PGG β -glucan-mobilized hematopoietic progenitor cells are capable of engraftment and differentiation. Circulating KSL cells from PGG β -glucan-, G-CSF-, or G-CSF plus PGG β -glucan-mobilized C57Bl/6 (CD45.2) mice were i.v. injected into lethally irradiated SJL mice (CD45.1). Engraftment on day 30 was assessed by flow cytometry (A). (B): Representative experiment of multilineage engraftment from a total of seven mice. The data are from one representative experiment of two separate experiments. Abbreviations: G-CSF, granulocyte colony-stimulating factor; Lin, lineage; PGG, poly-(1,6)- β -D-glucopyranosyl-(1,3)- β -D-glucopyranose.

standard G-CSF mobilization and increase the number and success of transplantations. Because mobilization occurs via a C3- and CR3-independent mechanism, no undue inflammatory response is induced, and therefore, mobilization would eliminate side effects associated with inflammation. Furthermore, the release of MMP-9 from BM erythroid cells, macrophages, and epithelial cells provides a unique and not previously described mechanism for mobilization. Thus, PGG β -glucan offers a safe and novel approach to enhancement of G-CSF mobilization and possibly that of other agents, such as AMD3100 [51].

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

J.Y. has acted as a consultant and has performed contract work for Biothera.

REFERENCES

- To LB, Haylock DN, Simmons PJ et al. The biology and clinical uses of blood stem cells. *Blood* 1997;89:2233–2258.
- Petit I, Szyper-Kravitz M, Nagler A et al. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* 2002;3:687–694.
- Boeve S, Struempel J, Creech S et al. Analysis of remobilization success in patients undergoing autologous stem cell transplants who fail an initial mobilization: Risk factors, cytokine use and cost. *Bone Marrow Transplant* 2004;33:997–1003.
- Winkler IG, Levesque JP. Mechanisms of hematopoietic stem cell mobilization: When innate immunity assails the cells that make blood and bone. *Exp Hematol* 2006;34:996–1009.
- Moncada V, Bolan C, Yau YY et al. Analysis of PBPC cell yields during large-volume leukapheresis of subjects with a poor mobilization response to filgrastim. *Transfusion* 2003;43:495–501.
- Anderlini P, Przepiorka D, Seong C et al. Factors affecting mobilization of CD34+ cells in normal donors treated with filgrastim. *Transfusion* 1997;37:507–512.
- Yan J, Allendorf DJ, Brandley B. Yeast whole glucan particle (WGP) beta-glucan in conjunction with antitumour monoclonal antibodies to treat cancer. *Expert Opin Biol Ther* 2005;5:691–702.
- Li B, Allendorf DJ, Hansen R et al. Yeast beta-glucan amplifies phago-

- cyte killing of iC3b-opsonized tumor cells via complement receptor 3-Syk-phosphatidylinositol 3-kinase pathway. *J Immunol* 2006;177:1661–1669.
- 9 Hong F, Hansen RD, Yan J et al. Beta-glucan functions as an adjuvant for monoclonal antibody immunotherapy by recruiting tumoricidal granulocytes as killer cells. *Cancer Res* 2003;63:9023–9031.
 - 10 Patchen ML, MacVittie TJ. Hemopoietic effects of intravenous soluble glucan administration. *J Immunopharmacol* 1986;8:407–425.
 - 11 Patchen ML, Vaudrain T, Correia H et al. In vitro and in vivo hematopoietic activities of Betafectin PGG-glucan. *Exp Hematol* 1998;26:1247–1254.
 - 12 Patchen ML, Liang J, Vaudrain T et al. Mobilization of peripheral blood progenitor cells by Betafectin PGG-Glucan alone and in combination with granulocyte colony-stimulating factor. *STEM CELLS* 1998;16:208–217.
 - 13 Thornton BP, Vetvicka V, Pitman M et al. Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). *J Immunol* 1996;156:1235–1246.
 - 14 Cramer DE, Allendorf DJ, Baran JT et al. Beta-glucan enhances complement-mediated hematopoietic recovery after bone marrow injury. *Blood* 2006;107:835–840.
 - 15 Broxmeyer HE, Cooper S, Kohli L et al. Transgenic expression of stromal cell-derived factor-1/CXC chemokine ligand 12 enhances myeloid progenitor cell survival/antiapoptosis in vitro in response to growth factor withdrawal and enhances myelopoiesis in vivo. *J Immunol* 2003;170:421–429.
 - 16 Heissig B, Hattori K, Dias S et al. Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 2002;109:625–637.
 - 17 Salleri C, Montuori N, Ricci P et al. In vivo activity of the cleaved form of soluble urokinase receptor: A new hematopoietic stem/progenitor cell mobilizer. *Cancer Res* 2006;66:10885–10890.
 - 18 Ratajczak J, Reca R, Kucia M et al. Mobilization studies in mice deficient in either C3 or C3a receptor (C3aR) reveal a novel role for complement in retention of hematopoietic stem/progenitor cells in bone marrow. *Blood* 2004;103:2071–2078.
 - 19 Ratajczak MZ, Reca R, Wysoczynski M et al. Transplantation studies in C3-deficient animals reveal a novel role of the third complement component (C3) in engraftment of bone marrow cells. *Leukemia* 2004;18:1482–1490.
 - 20 Reca R, Mastellos D, Majka M et al. Functional receptor for C3a anaphylatoxin is expressed by normal hematopoietic stem/progenitor cells, and C3a enhances their homing-related responses to SDF-1. *Blood* 2003;101:3784–3793.
 - 21 Molendijk WJ, van Oudenaren A, van Dijk H et al. Complement split product C5a mediates the lipopolysaccharide-induced mobilization of CFU-s and haemopoietic progenitor cells, but not the mobilization induced by proteolytic enzymes. *Cell Tissue Kinet* 1986;19:407–417.
 - 22 Aiuti A, Webb IJ, Bleul C et al. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* 1997;185:111–120.
 - 23 Ma Q, Jones D, Borghesani PR et al. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A* 1998;95:9448–9453.
 - 24 Pelus LM, Bian H, King AG et al. Neutrophil-derived MMP-9 mediates synergistic mobilization of hematopoietic stem and progenitor cells by the combination of G-CSF and the chemokines GRObeta/CXCL2 and GRObetaT/CXCL2delta4. *Blood* 2004;103:110–119.
 - 25 Ratajczak J, Majka M, Kijowski J et al. Biological significance of MAPK, Akt and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. *Br J Haematol* 2001;115:195–204.
 - 26 Socolovsky M, Nam H, Fleming MD et al. Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. *Blood* 2001;98:3261–3273.
 - 27 Liu Y, Pop R, Sadegh C et al. Suppression of Fas-FasL coexpression by erythropoietin mediates erythroblast expansion during the erythropoietic stress response in vivo. *Blood* 2006;108:123–133.
 - 28 Roberts AW, Foote S, Alexander WS et al. Genetic influences determining progenitor cell mobilization and leukocytosis induced by granulocyte colony-stimulating factor. *Blood* 1997;89:2736–2744.
 - 29 Reca R, Cramer D, Yan J et al. A novel role of complement in mobilization: Immunodeficient mice are poor granulocyte-colony stimulating factor mobilizers because they lack complement-activating immunoglobulins. *STEM CELLS* 2007;25:3093–3100.
 - 30 Sweeney EA, Priestley GV, Nakamoto B et al. Mobilization of stem/progenitor cells by sulfated polysaccharides does not require selectin presence. *Proc Natl Acad Sci U S A* 2000;97:6544–6549.
 - 31 Sweeney EA, Lortat-Jacob H, Priestley GV et al. Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: Involvement in mobilization of stem/progenitor cells. *Blood* 2002;99:44–51.
 - 32 McQuibban GA, Butler GS, Gong JH et al. Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1. *J Biol Chem* 2001;276:43503–43508.
 - 33 Delgado MB, Clark-Lewis I, Loetscher P et al. Rapid inactivation of stromal cell-derived factor-1 by cathepsin G associated with lymphocytes. *Eur J Immunol* 2001;31:699–707.
 - 34 Pruijt JF, Fibbe WE, Laterveer L et al. Prevention of interleukin-8-induced mobilization of hematopoietic progenitor cells in rhesus monkeys by inhibitory antibodies against the metalloproteinase gelatinase B (MMP-9). *Proc Natl Acad Sci U S A* 1999;96:10863–10868.
 - 35 Kollet O, Shvitiel S, Chen YQ et al. HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. *J Clin Invest* 2003;112:160–169.
 - 36 Pelus LM, Fukuda S. Peripheral blood stem cell mobilization: The CXCR2 ligand GRObeta rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. *Exp Hematol* 2006;34:1010–1020.
 - 37 Janowska-Wieczorek A, Marquez LA, Nabholz JM et al. Growth factors and cytokines upregulate gelatinase expression in bone marrow CD34(+) cells and their transmigration through reconstituted basement membrane. *Blood* 1999;93:3379–3390.
 - 38 Hibbs MS, Hoidal JR, Kang AH. Expression of a metalloproteinase that degrades native type V collagen and denatured collagens by cultured human alveolar macrophages. *J Clin Invest* 1987;80:1644–1650.
 - 39 Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000;14:163–176.
 - 40 Iwata M, Pillai M, Ramakrishnan A et al. Reduced expression of inducible gelatinase B/matrix metalloproteinase-9 in monocytes from patients with myelodysplastic syndrome: Correlation of inducible levels with the percentage of cytogenetically-marked cells and with marrow cellularity. *Blood* 2007;109:85–92.
 - 41 Johnatty RN, Taub DD, Reeder SP et al. Cytokine and chemokine regulation of proMMP-9 and TIMP-1 production by human peripheral blood lymphocytes. *J Immunol* 1997;158:2327–2333.
 - 42 Bond M, Fabunmi RP, Baker AH et al. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: An absolute requirement for transcription factor NF-kappa B. *FEBS Lett* 1998;435:29–34.
 - 43 Wilhelm SM, Collier IE, Marmer BL et al. SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J Biol Chem* 1989;264:17213–17221.
 - 44 Tanaka A, Arai K, Kitamura Y et al. Matrix metalloproteinase-9 production, a newly identified function of mast cell progenitors, is down-regulated by c-kit receptor activation. *Blood* 1999;94:2390–2395.
 - 45 Heissig B, Rafii S, Akiyama H et al. Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization. *J Exp Med* 2005;202:739–750.
 - 46 Majka M, Janowska-Wieczorek A, Ratajczak J et al. Stromal-derived factor 1 and thrombopoietin regulate distinct aspects of human megakaryopoiesis. *Blood* 2000;96:4142–4151.
 - 47 Kollet O, Dar A, Shvitiel S et al. Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med* 2006;12:657–664.
 - 48 Kollet O, Dar A, Lapidot T. The multiple roles of osteoclasts in host defense: Bone remodeling and hematopoietic stem cell mobilization. *Annu Rev Immunol* 2007;25:51–69.
 - 49 Vu TH, Shipley JM, Bergers G et al. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 1998;93:411–422.
 - 50 Irhimeh MR, Fitton JH, Lowenthal RM. Fucoidan ingestion increases the expression of CXCR4 on human CD34+ cells. *Exp Hematol* 2007;35:989–994.
 - 51 Broxmeyer HE, Orschell CM, Clapp DW et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med* 2005;201:1307–1318.