G-CSF regulates hematopoietic stem cell activity, in part, through activation of toll-like receptor signaling

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Abstract

Recent studies demonstrate that inflammatory signals regulate hematopoietic stem cells (HSCs). Granulocyte-colony stimulating factor (G-CSF) is often induced with infection and plays a key role in the stress granulopoiesis response. However, its effects on HSCs are less clear. Herein, we show that treatment with G-CSF induces expansion and increased quiescence of phenotypic HSCs, but causes a marked, cell-autonomous HSC repopulating defect associated with induction of toll-like receptor (TLR) expression and signaling. The G-CSF-mediated expansion of HSCs is reduced in mice lacking TLR2, TLR4 or the TLR signaling adaptor MyD88. Induction of HSC quiescence is abrogated in mice lacking MyD88 or in mice treated with antibiotics to suppress intestinal flora. Finally, loss of TLR4 or germ free conditions mitigates the G-CSF-mediated HSC repopulating defect. These data suggest that low level TLR agonist production by commensal flora contributes to the regulation of HSC function and that G-CSF negatively regulates HSCs, in part, by enhancing TLR signaling.

Keywords

G-CSF; hematopoietic stem cell; toll-like receptor; inflammation
INTRODUCTION

In response to infectious or inflammatory stress, pro-inflammatory cytokines shape the innate immune response by enhancing the production and function of immune effector cells. Accumulating evidence shows that certain inflammatory mediators can regulate hematopoiesis at the stem cell level (reviewed in reference 1). While such signals are central to an effective immune response, chronic inflammatory signals may impair HSC function. For example, chronic IFN exposure leads to HSC exhaustion and a reduction in HSC function in transplantation assays. Similarly, long-term LPS-mediated signaling through toll like receptor 4 (TLR4) is associated with significantly impaired HSC repopulating and self-renewal capacities.

The expression of granulocyte-colony stimulating factor (G-CSF) is induced in response to many types of infection. G-CSF is known to play a key role in stimulating neutrophil production and release into the circulation. However, the effect of G-CSF on HSCs is less clear. In addition to myeloid cells, the G-CSF receptor (G-CSFR) is expressed on HSCs, and G-CSFR deficient (Csf3r−/−) mice have impaired HSC function. Moreover, treatment with G-CSF results in a loss of long-term repopulating activity in the bone marrow. Together, these data suggest that G-CSF signals play a non-redundant role in HSC maintenance.

G-CSF is widely used in the clinical setting to mobilize HSCs from the bone marrow to the blood to facilitate harvesting of HSCs for stem cell transplantation. Expression of the G-CSFR on HSCs is not required for their mobilization, indicating that G-CSF acts in non-cell intrinsic fashion to induced HSC mobilization. Indeed, G-CSF treatment is associated with marked changes in the bone marrow microenvironment, including the loss of osteoblasts and reduced CXCL12 expression, both of which have been implicated in HSC maintenance.

The direct and indirect effects of G-CSF on HSCs suggest the hypothesis that the increased G-CSF expression elicited during the immune response may affect HSC function. Herein, we show that G-CSF treatment in mice, while increasing phenotypic HSCs in the bone marrow, results in an increase in HSC quiescence and loss of long-term repopulating activity. Mechanistic studies suggest that G-CSF’s effects on HSCs are mediated in a cell intrinsic fashion, in part, by inducing toll-like receptor signaling in HSCs. Moreover, loss of TLR signaling or suppression of commensal flora, either short-term via antibiotic treatment or life-long via rearing in a germ-free environment, alters baseline and cytokine influenced HSC cycling status and mitigates the G-CSF-mediated expansion of HSCs.

MATERIALS AND METHODS

Mice

C57BL/6J (Ly5.2), C57BL/6 mice (B6.SJL-Ptprc* Pep3b BoyJ) carrying the Ly5.1 allele, MyD88−/− (B6.129P2(SJL)-Myd88tm1.1Defr/J), Tlr4−/− (B6.B10ScN-Tlr4lps-del/JthJ), Tlr2−/− (B6.129-Tlr2tm1Kir/J) and CAG-GFP [Tg(CAG-GFP*) 1Had/J] mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a C57BL/6J background.
Sex- and age-matched mice 6–10 weeks of age were used in accordance with the guidelines of the Washington University Animal Studies Committee, and housed in a specific pathogen-free environment, except germ-free mice, which were raised in the Washington University gnotobiotic facility.

**G-CSF and Antibiotic Administration**

Mice were injected subcutaneously with twice-daily doses of 125µg/kg of recombinant human G-CSF (Amgen, Thousand Oaks, CA) in low-endotoxin PBS with 0.1% BSA (or vehicle alone). For antibiotic treatment, mice were given Ciprofloxacin 100mg/L (Sigma), Polymyxin B 70mg/L (Sigma) and 20g/L sucrose (Sigma) in their drinking water. Stool samples were cultured in Nutrient broth (Sigma) and Brain Heart Infusion (BHI) broth (Sigma) at 37°C under aerobic conditions.

**Peripheral Blood and Bone Marrow Analysis**

Peripheral blood was obtained by retro-orbital venous plexus sampling. Bone marrow hematopoietic cells were isolated by centrifugation of femurs and tibiae at 3,300 × g. Cell counts were determined using a Hemavet (Drew Scientific) automated cell counter.

**Bone Marrow Transplantation**

Bone marrow from G-CSF-treated or control mice (Ly5.2) was mixed with competitor bone marrow (Ly5.1), and a total of 2 million bone marrow cells were injected retro-orbitally into lethally irradiated wild type mice (Ly5.1/Ly5.2). To generate Csf3r<sup>−/−</sup> chimeras, cells from Csf3r<sup>−/−</sup> mice (Ly 5.2) were transplanted with cells from wild type (Ly5.1) mice into lethally irradiated recipients (Ly 5.1/5.2). In some experiments, KLS SLAM cells were isolated using a high-speed cell sorter. For intrafemoral injections, 5 million bone marrow cells were injected with competitor bone marrow intrafemorally into recipients. Recipient mice were conditioned with 1000 cGy from a 137Cesium source. Prophylactic antibiotics (Trimethoprim-Sulfamethoxazole, Alpharma, Baltimore, MD) were given for two weeks post-transplantation. For secondary transplants, bone marrow was pooled from primary recipients and injected into irradiated secondary recipients. For the homing study, KSL cells from CAG-GFP mice were transplanted into irradiated wild type mice along with 1×10<sup>6</sup> wild type whole bone marrow cells and viable (7-amino-actinomycin D−; 7-AAD; eBioscience) recipient marrow was analyzed 16 hours later.

**Flow Cytometry and Cell Sorting**

Bone marrow and peripheral blood was processed for flow cytometry as previously described. (20) Cells were analyzed on a BD flow cytometer or the Gallios flow cytometer (Beckman Coulter) or sorted on a MoFlo high-speed flow cytometer (Dako Cytomation, Fort Collins, CO) or iCyt Reflection cell sorter (Champaign, IL). Data analysis was done using FloJo version 9.3.4 software (TreeStar).

The following antibodies were used to label HSCs (all antibodies were from eBiosciences, except where indicated): anti–mouse Ly6A/E (Sca-1) PerCP-Cy5.5 (clone D7), anti–mouse CD117 (c-Kit) APC–eFluor 780 (clone 2B8), anti–mouse CD34 FITC (clone RAM34), anti–mouse CD16/32 eFluor 450 (clone 93), anti–mouse Flt3 (Flk-2) APC (clone A2F10), anti–
mouse CD48 PE-Cy7 (clone HM48-1), anti-mouse CD150 PE (clone TC15-12F12.2, Biologend, San Diego, CA), anti-mouse CD41 biotin (clone MWRReg30), anti–mouse/human CD45R (B220) biotin (clone RA3-6B2), anti–mouse CD3e biotin (clone 145-2C11), anti–mouse Ly-6G (Gr-1) biotin (clone RB6–8C5), anti–mouse Ter119 biotin (clone TER-119), and streptavidin eFluor 605NC. For the analysis of TLR expression, anti-mouse TLR2 eFluor 450 (clone T2.5), anti-mouse TLR1 biotin (clone TR23) with streptavidin APC-eFluor 780, and anti-mouse TLR13 with goat anti-rabbit IgG (F(ab’)2-specific)- APC (Imgenex, San Diego, CA) were used. Cells were fixed prior to TLR13 staining using the BD cytofix/cytoperm kit (BD) according to manufacturer’s directions. For peripheral blood lineage analyses, the following antibodies were used: anti–mouse CD45.1 (clone A20), anti–mouse CD45.2 (clone 104), anti–mouse/human CD45R (B220; clone RA3-6B2), anti–mouse CD3e (clone 145-2C11), and anti–mouse Ly-6G (Gr-1; clone RB6-8C5).

**Cell Cycle Analysis**

For analysis of cell cycle, bone marrow cells were stained for surface markers as described above, fixed using the BD cytofix/cytoperm kit (BD), blocked with 5% goat serum and stained with mouse anti-human Ki-67 (clone B56; BD Pharmingen). After washing, cells were resuspended in DAPI-containing FACS buffer. For BrdU labeling, bone marrow cells were harvested 12 hours after the final dose of BrdU (4 doses of 1mg every 12 hours, intraperitoneally) and incubated with FITC-conjugated CD48 and CD41, biotinylated-CD150 (clone number TC15-12F12.1, Biologend), and the following panel of PE-Cy5 conjugated lineage markers: CD3, B220, Gr-1, and Ter-119. Streptavidin-PE was then used to detect CD150. Cells were then fixed, permeabilized, incubated with Alexa Fluor® 647-conjugated anti-BrdU antibody (Invitrogen) and analyzed by flow cytometry (BrdU flow kit, BD biosciences, San Jose, CA).

**Gene Expression Array Analysis**

RNA from KSL SLAM cells from 5–10 mice treated with G-CSF or saline alone was prepared using the RNA XS column kit (Machery-Nagel), amplified using the NuGen Ovation system (NuGen) and hybridized to the MoGene 1.0 ST array. Data normalization using the Robust Multichip Average (RMA) algorithm was made using the Affymetrix Expression Console software. Gene set enrichment was performed using the GSEA software (Broad Institute). Differences in gene expression were determined using Significance Analysis of Microarrays (SAM; Stanford University, Stanford, CA). Expression data will be submitted to Gene Expression Omnibus.

**Statistical Analysis**

Data are presented as mean ± SEM, unless otherwise stated. Statistical significance was assessed using a two-sided Student’s t test or two-sided analysis of variance (ANOVA). Repopulating unit calculations were determined using the equation: RU=[%chimerism of test donor-derived cells] × [# of competitor cells] × 10^{-5}]/ % chimerism of competitor-derived cells. 

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RESULTS

G-CSF Treatment Results in an Expansion of Phenotypic HSCs with reduced repopulating activity

Previous studies have shown that G-CSF treatment leads to a loss of long-term repopulating activity in the bone marrow.\(^{13–15}\) However, it is not clear whether this is secondary to the mobilization of HSCs to peripheral sites or a cell-intrinsic loss of HSC repopulating activity. To address this issue, we quantified HSCs in the bone marrow, spleen and blood following 7 days of G-CSF administration (Figure 1A). HSCs were identified as CD34\(^-\) Flk2\(^-\) CD41\(^-\) Lineage\(^-\) c-Kit\(^+\) Sca-1\(^+\) (KSL) cells or CD150\(^+\) CD48\(^-\) CD41\(^-\) KSL cells. While G-CSF treatment resulted in a 10-fold increase in CD150\(^+\) CD48\(^-\) CD41\(^-\) KSL (KSL SLAM) cells in the spleen (Figure 8F), HSC frequency and absolute numbers were also increased in the bone marrow (Figure 1B–E). To assess the effects of G-CSF on HSC repopulating and self-renewal activities, we performed competitive repopulation assays using whole bone marrow from either control mice or mice treated with G-CSF for 7 days. As expected, the contribution of untreated donor cells to hematopoiesis remained stable for at least 22 weeks following transplantation (Figure 1F). In contrast, despite the increased frequency of phenotypic HSCs, a significant, multi-lineage long-term repopulating defect was observed using bone marrow from G-CSF treated mice (Figure 1F–G). We repeated the competitive repopulation assay using sorted bone marrow CD150\(^+\) CD48\(^-\) CD41\(^-\) lineage\(^-\) cells, with similar results (Figure 1H). To assess HSC self-renewal capacity, secondary bone marrow transplantations were performed. Whereas the contribution of mock treated cells to donor chimerism in secondary recipients was maintained, a marked loss of donor contribution was observed with G-CSF-treated bone marrow (Figure 1I). Thus G-CSF results in the expansion of phenotypic HSCs in the bone marrow with reduced repopulating activity and self-renewal capacity.

To test the possibility that the loss of repopulating activity of G-CSF-treated HSCs is secondary to reduced marrow homing, KSL GFP\(^+\) cells from saline treated or G-CSF treated CAG-GFP mice (ubiquitously expressing GFP) were transplanted into irradiated recipients, and the number of GFP\(^+\) cells in the bone marrow assessed at 16 hours. No difference in the number of KSL cells recovered from the bone marrow was observed (Figure 2A).

G-CSF Treatment Induces HSC Quiescence

HSC quiescence is intimately associated with long-term repopulating activity and self-renewal capacity.\(^{22–24}\) We therefore assessed the cell cycle status of KSL SLAM cells following G-CSF treatment (Figure 3A). As expected, the majority of KSL SLAM cells (67.3 ± 2.5\%) at baseline were quiescent (Figure 3B–C). Consistent with prior studies, we observed increased cycling of HSCs after short-term treatment with G-CSF.\(^{25–27}\) Paradoxically, we show for the first time that HSCs became more quiescent with continued G-CSF treatment, which persisted for 7 days after stopping G-CSF. A similar increase in
quiescence was observed in the CD34− Flk2− CD41− KSL cell population after 7 days of G-CSF (Figure 3D–E). Consistent with these findings, the percentage of CD150+ CD48− CD41− lineage− cells that labeled with 5-bromo-2-deoxyuridine (BrdU) was significantly reduced in G-CSF-treated mice compared with untreated mice (Figure 3F). These data show that G-CSF treatment regulates HSC cycling, initially inducing proliferation but followed by increased quiescence.

G-CSF Acts in a Cell Intrinsic Fashion to Inhibit HSC Function

To determine whether G-CSF acts in a cell autonomous fashion to regulate HSC function, mixed Csf3r−/− (G-CSFR deficient) bone marrow chimeras were generated by transplanting Csf3r−/− and wild type (Csf3r+/+) cells into irradiated recipients (Figure 4A). After stable hematopoietic reconstitution, mice were treated with G-CSF or saline alone for 7 days. Bone marrow was then transplanted into secondary recipients, and donor chimerism was assessed (Figure 4B). We focused on B cell chimerism, since myeloid Csf3r−/− cells are at a severe competitive disadvantage. A cell-autonomous effect of G-CSF on HSC activity would be expected to result in the selective loss of Csf3r+/+ cells. In recipient mice reconstituted with saline-treated bone marrow, the contribution of Csf3r−/− cells to hematopoiesis progressively decreased, consistent with previous data showing that Csf3r−/− HSCs are at a competitive disadvantage. In contrast, in recipient mice transplanted with G-CSF treated bone marrow, the contribution of Csf3r−/− cells to hematopoiesis remained stable. After 12 weeks, bone marrow was harvested and transplanted into tertiary recipients (Figure 4C). Again, while Csf3r−/− cells from control mice declined over time, those from G-CSF treated donors remained stable. Thus, the selective loss of repopulating activity of wild type HSCs after exposure to G-CSF suggests that G-CSF acts in a cell-intrinsic fashion to inhibit HSC function. Whether the effects of G-CSF on HSC phenotypic expansion and cycling are cell-intrinsic as well requires further study.

G-CSF Treatment Induces Toll-like Receptor Signaling in HSCs

To elucidate the mechanism(s) by which G-CSF affects HSCs, RNA expression profiling was performed on sorted KSL SLAM cells from mice that were treated with or without G-CSF for 36 hours. Using Significance Analysis of Microarrays (SAM) with the criteria of at least a 2-fold change in expression and a false-discovery rate (FDR) of 10% or less, 228 genes were identified as being upregulated upon G-CSF treatment, and 12 were significantly decreased (Supplemental Table 1). Gene set enrichment analysis (GSEA) identified significant enhancement of TLR signaling in G-CSF treated HSCs compared to controls (Supplemental Figure 1). Similarly, enhancement of TLR signaling was evident in HSCs after 7 days of G-CSF treatment, as many of the most significantly upregulated genes have been previously shown to be upregulated upon exposure to LPS or other TLR agonist (Siglec-E, (28) Gp49a, (29) Slfn4, (30) Ltf, (31, 32) and Gcn, (33)) and GSEA revealed significant enhancement of TLR signaling at this time point as well (Figure 5A–B and Supplemental Table 2).

We next asked whether TLR expression itself on HSCs was altered with G-CSF treatment. RNA expression profiling of sorted KSL SLAM cells showed that G-CSF treatment induced expression of Tlr1 and Tlr13, with a trend to increased Tlr2 (Figure 5C). Indeed, cell surface
expression of TLR2 on CD34⁻ Flk2⁻ KSL cells increased after just one day of G-CSF treatment (Figure 5D). We confirmed increased TLR1 surface expression upon G-CSF treatment on this population as well, however TLR13 protein levels by flow cytometry were not altered by G-CSF treatment in HSCs (Supplemental Figure 2).

**TLR2 Signaling Regulates HSC Number in Response to G-CSF**

TLR signaling has been implicated in the regulation of HSC quiescence and repopulating activity. To determine the contribution of TLR signaling to the G-CSF-mediated expansion of phenotypic HSCs, induction of HSC quiescence, and loss of repopulating activity, we analyzed TLR signaling deficient mice. Specifically, we analyzed mice lacking TLR2, as we see increased surface expression of this receptor within 1 day of G-CSF treatment (Figure 5D), and TLR2 functions as a heterodimer with either TLR1 or TLR6, both of which showed increased mRNA expression after G-CSF treatment (Figure 5C). At baseline, the number of phenotypic HSCs in $Tlr2^{-/-}$ mice is similar to wild type controls (Figure 6A). However, the G-CSF induced expansion of HSCs is attenuated in the absence of TLR2 (Figure 6A). Similar to wild type mice, G-CSF is associated with enhanced quiescence in $Tlr2^{-/-}$ HSCs (Figure 6B). To assess the impact of TLR2 signaling on HSC long-term repopulating activity, competitive transplants were performed using bone marrow from saline- or G-CSF-treated $Tlr2^{-/-}$ mice versus untreated wild type mice. Interestingly, a very modest repopulating advantage was observed with $Tlr2^{-/-}$ bone marrow (Figure 6C–D). However, similar to wild type mice, G-CSF treatment of $Tlr2^{-/-}$ mice results in a significant loss of repopulating activity. These data show that TLR2 signaling contributes to the expansion of phenotypic HSCs but is not required for G-CSF induced HSC quiescence or loss of repopulating activity.

**MyD88-dependent TLRs contribute to the G-CSF induced HSC quiescence**

In addition to TLR2, multiple other TLRs are upregulated in HSCs upon G-CSF treatment (Figure 5C and Supplemental Figure 1). We therefore studied $MyD88^{-/-}$ mice to broadly inhibit TLR signaling, as MyD88 is an adaptor protein required for most TLR signaling. In addition, we chose to study $Tlr4^{-/-}$ mice, since TLR4 signals through both MyD88- and TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways, and since TLR4 signaling has been implicated in the regulation of HSC cycling and repopulating activity. At baseline, the number of phenotypic HSCs is similar to wild type controls in both groups (Figure 7A). However, the G-CSF induced expansion of HSCs is abrogated in both $MyD88^{-/-}$ and $Tlr4^{-/-}$ mice. Thus multiple TLRs appear to contribute to phenotypic HSC expansion by G-CSF. At baseline, there is a trend toward decreased quiescence in $Tlr4^{-/-}$ mice (Figure 7B). However, TLR4 signaling is dispensable for induction of HSC quiescence by G-CSF (Figure 7B). In contrast, while the basal cell cycle status of HSCs in $Myd88^{-/-}$ mice is similar to controls, G-CSF induced quiescence is lost (Figure 7B). Collectively, these data show that MyD88-dependent signaling mediates G-CSF-induced HSC quiescence, independent of TLR2 and TLR4.
TLR4 signaling contributes to the G-CSF induced loss of repopulating activity

To assess the impact of TLR4 and MyD88 signaling on long term repopulating activity, competitive repopulating assays were again performed using bone marrow from saline- or G-CSF-treated Myd88−/− or Tlr4−/− mice. Consistent with a prior report, untreated MyD88−/− and Tlr4−/− bone marrow demonstrate a repopulating advantage over wild-type cells (Figure 7C–D). Similar to wild type mice, however, bone marrow from MyD88−/− mice treated with G-CSF showed a significant multi-lineage long term repopulating defect (Figure 7C–D). In fact, G-CSF treatment induced a greater loss of repopulating units in the bone marrow of MyD88−/− mice compared with wild type mice (Figure 7E and Supplemental Table 3). In contrast, although the basal increase in HSC activity was similar in the bone marrow of Tlr4−/− mice and MyD88−/− mice, the loss of multi-lineage long term repopulating following G-CSF was partially mitigated in the absence of TLR4 (Figure 7C–D and Supplemental Table 3). These data suggest that TLR4 signaling contributes to the G-CSF-mediated loss of HSC repopulating activity.

Intestinal Flora Regulate HSCs at Baseline and in Response to G-CSF

We next considered the source of TLR ligand(s) that act on HSCs in the bone marrow. One potential source is commensal bacteria in the intestine. To address this possibility, we treated wild type mice with oral ciprofloxacin and polymyxin B to reduce intestinal flora. After 10 days of antibiotic pre-treatment, mice were treated additionally with G-CSF (or saline alone) and HSC number and cell cycle status assessed (Figure 8A). Stool cultures showed a marked reduction in intestinal flora compared to mice not on antibiotics (Supplemental Figure 3). Given that this antibiotic regimen does not completely remove all commensal pathogens, we also analyzed mice housed under germ-free conditions. The number of phenotypic HSCs in the bone marrow of germ-free and antibiotic treated mice is comparable to control mice reared in a specific pathogen-free environment both at baseline and after G-CSF treatment (Figure 8B). Interestingly, acute suppression of intestinal flora with antibiotics, but not chronic loss of gut microbiota (i.e., germ-free mice), is associated with increased HSC quiescence (Figure 8C). Finally, whereas the long-term repopulating activity of HSCs from antibiotic treated mice is comparable to control mice, HSCs from germ free mice have increased multi-lineage long-term repopulating activity. Moreover, the G-CSF induced suppression of HSC long-term repopulating activity is mitigated in germ free mice (Figure 8D–E).

A previous study suggested that low level endotoxin in the blood from intestinal flora regulates hematopoietic progenitor cell trafficking from the bone marrow.(39) Indeed, we observed reduced HSC mobilization to the spleen in response to G-CSF treatment in antibiotic-treated and germ free mice (Figure 8F). However, mobilization was normal to slightly enhanced in MyD88−/−, Tlr2−/−, or Tlr4−/− mice, suggesting that gut microbiota regulate HSC trafficking in a TLR independent fashion.

DISCUSSION

Previous studies have established that G-CSF treatment is associated with reduced hematopoietic progenitor activity in the bone marrow. De Haan and colleagues reported that
the number of primitive hematopoietic stem and progenitor cells (HSPCs), as measured by cobblestone area forming cells, was reduced in the bone marrow after G-CSF treatment.(13) Likewise, Bodine et al.(14) reported that the repopulating ability of bone marrow was reduced after 5 days of G-CSF and stem cell factor, and Winkler and colleagues(15) similarly showed a loss of bone marrow repopulating activity from mice treated with 5 days of G-CSF. These studies largely attributed the loss of HSPC activity in the bone marrow to their mobilization. Here, we also observed a marked loss of multi-lineage repopulating activity in the bone marrow after G-CSF treatment, however we show that bone marrow HSC numbers are increased after G-CSF treatment. Consistent with these findings, Grassinger and colleagues,(40) showed that G-CSF treatment for 4 days increases marrow KSL SLAM cells. The disparity between HSC number and repopulating activity may be explained, in part, by the sensitivity of phenotypic HSC markers to inflammatory signals. In other words, the apparent increase in phenotypic HSCs in G-CSF treated mice may reflect altered expression of HSC markers on more committed progenitor populations rather than a true expansion of HSCs. In particular, CD150 is upregulated on multiple hematopoietic cell types in response to TLR signaling.(41–43) Indeed, we find that the increase in CD150+ HSCs is lost in TLR deficient mice (data not shown), implicating TLR signaling in the upregulation of this marker. Less clear is the mechanism for the increase in KSL CD34− Flk2− cells, as CD34 expression is induced by IL-11 and stem cell factor(44), and a prior study(45) found that most G-CSF mobilized HSCs in the blood were CD34+. We show that the G-CSF induced increase in KSL CD34− Flk2− cells is attenuated in TLR deficient mice, raising the possibility that TLR signaling may negatively regulate CD34 expression in HSCs.

Recent studies have implicated TLR signaling in HSCs in the regulation of their quiescence and repopulating activity. HSCs express multiple TLRs,(34) and ex vivo treatment of HSPCs with specific TLR2 or TLR4 agonists induces cycling and myeloid differentiation. Furthermore, chronic in vivo exposure to LPS leads to an expansion of marrow HSCs, increased HSC cycling, and impaired HSC self-renewal and myeloid skewing upon transplantation.(8) Notably, short-term, high-dose LPS treatment was reported to increase HSC cycling and enhance repopulating ability,(46) and thus the specific effects of LPS on HSCs may be dose and/or duration-dependent. Consistent with the idea that TLR signaling on HSCs regulates HSC function, bone marrow from TLR4−/−, TLR9−/− and MyD88−/− mice has a repopulating advantage when transplanted competitively with wild-type marrow.(38) Similarly, we show that under basal conditions, TLR signaling contributes to HSC maintenance, with HSCs from Tlr4−/− mice displaying increased cycling and long-term repopulating activity.

While these previous studies provide compelling evidence that TLR signaling contributes to the regulation of HSCs, several key questions remain unanswered. First, although prior studies establish a role for TLR signaling in the regulation of HSCs during severe infectious stress, the contribution of TLR signaling to HSC maintenance after less severe stress is unclear. Second, it is not clear whether TLR agonists act directly on HSCs or through an inflammatory cell intermediate, although a study by Megias, et al showed that TLR
signaling in HSCs directly induced their myeloid differentiation. Finally, the regulation of TLR expression and activation in HSCs is not known.

We show that G-CSF increases phenotypic HSCs in the bone marrow, induces HSC cycling followed by quiescence, and decreases HSC long-term repopulating activity. Our data strongly implicate TLR signaling in these responses. First, RNA expression profiling suggests that G-CSF induces TLR signaling in HSCs and increases the expression of multiple TLRs on HSCs. Second, TLR signaling contributes to the G-CSF induced expansion of phenotypic HSCs; MyD88 signaling through TLR4 appears to be the major pathway mediating this response. Third, TLR signaling is required for the cell cycle exit of HSCs with prolonged G-CSF treatment. Interestingly, G-CSF induced quiescence is dependent on MyD88 but not TLR2 or TLR4, suggesting that other TLRs may mediate this response. Finally, TLR4 signaling contributes to the G-CSF induced loss of HSC repopulating activity. Based on these findings, it is likely that multiple TLRs contribute to the baseline and cytokine-induced changes in HSCs (see Supplemental Table 4 for a summary of results). Further studies are needed to elucidate the role of specific TLRs in these processes.

There is accumulating evidence that gut microbiota play an important role in the development and regulation of the immune response. Here we provide evidence that gut microbiota also contribute to HSC maintenance in the bone marrow. Most notably, similar to Tlr4−/− mice, long-term repopulating activity is increased in mice housed under germ-free conditions, and G-CSF induced suppression of long-term repopulating activity is attenuated. These observations raise the possibility that low level endotoxin produced by gut microbiota regulates HSCs in the bone marrow by activating TLR4 signaling.

There is recent interest in the use of G-CSF “primed” bone marrow as a source of HSCs for clinical stem cell transplantation. While multiple studies have shown that G-CSF treated marrow provides rapid engraftment with lower rates of GVHD than mobilized HSCs,(49, 50) they have not shown a significant difference in transplant-related mortality or long-term survival.(51) Furthermore, our data raise concerns about the use of HSCs from G-CSF-primed bone marrow, as they are predicted to have reduced self-renewal capacity. Whether this translates into late bone marrow failure in recipients of G-CSF primed bone marrow will require further study. Finally, our data suggest that pharmacologic manipulation of TLR signaling may provide a novel strategy to regulate HSC function in vivo.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. G-CSF treatment increases the number of phenotypic HSCs in the bone marrow, but results in a loss of HSC repopulating and self-renewal activity
Bone marrow cells were harvested from mice treated with G-CSF or saline alone (PBS) for 7 days and analyzed by flow cytometry. (A) Representative dot plots showing the gating strategy used to identify phenotypic HSCs in the bone marrow. The frequency (B) and absolute number (C) of KSL CD34+ CD41- Flk2- and the frequency (D) and absolute number (E) of KSL CD150+ CD48- CD41- cells in the bone marrow are shown. Data represent >5 independent experiments and 24–26 mice per condition. Competitive repopulation assays were performed using bone marrow cells from saline- or G-CSF-treated mice (Ly5.2), which was transplanted in a 1:1 ratio with untreated wild-type marrow (Ly5.1).
into lethally irradiated recipients (Ly5.1/Ly5.2). (F) Shown is the percentage of donor (Ly5.2+) leukocytes in the blood. Data represent 6–12 mice per cohort from three pooled experiments. (G) The frequency of donor (Ly5.2+) B cells (B220+), T-cells (CD3e+), and neutrophils (Gr-1high) in the peripheral blood 22 weeks after transplantation for a representative experiment is shown. (H) CD150+ CD48− CD41− lineage- cells were sorted by flow cytometry from the bone marrow. Sixty of these cells (Ly5.1+) were transplanted along with 500,000 competitor (Ly5.1/Ly5.2) bone marrow cells into irradiated recipients (Ly5.2+). Shown is the percentage of donor (Ly5.1+) cells in the blood 20 weeks after transplantation (n=5 mice per condition). (I) Bone marrow cells harvested from primary recipients at 22 weeks as shown in (F) were transplanted into secondary recipients and the percentage of donor (Ly5.2+) leukocytes in the blood at 20 weeks was determined (n=3–4 mice per condition). Data represent the mean ± SEM. **p<0.01; ***p<0.001.
Figure 2. G-CSF treatment does not impair homing to the bone marrow

(A) GFP+ KSL cells (1 × 10^5) were injected retroorbitally into lethally irradiated recipient mice, and viable GFP+ cells in the bone marrow quantified by flow cytometry 16 hours later. Shown is the number of GFP+ KSL cells per 1×10^6 whole bone marrow cells analyzed (n=4 mice per condition). (B) Bone marrow cells from untreated or G-CSF treated mice (Ly5.2) were mixed with an equal number of competitor bone marrow cells (Ly5.1) and injected intrafemorally into irradiated recipients (Ly5.1/5.2). Shown is the percentage of donor (Ly5.2) cells 16 weeks after transplantation relative to the input percentage of donor cells injected intrafemorally (n = 9 from 3 independent experiments). Data represent the mean ± SEM. **p<0.01
Figure 3. G-CSF treatment induces HSC quiescence
Mice were treated with G-CSF or saline alone (PBS) for 12 hours to 7 days and the cell cycle status of HSCs assessed. (A) Shown are representative dot plots gated on KSL SLAM cells showing Ki-67 and DAPI staining. The percentage of KSL SLAM cells in G₀ (B) and S/G₂/M (C) are shown; data from mice 7 and 14 days after completing a 7 day course of G-CSF also is shown (n= 5–20 mice per time point). The percentage of CD34⁻ Flk2⁻ CD41⁻ KSL cells in G₀ (D) and S/G₂/M (E) are shown (n=5–7 mice per time point). (F) Mice were treated with BrdU over the last 48 hours of a 7 day course of G-CSF. Shown is the percentage of BrdU⁺ CD150⁺ CD48⁻ CD41⁻ lineage⁻ cells (n=15–16 mice per group). Data represent the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.
Figure 4. G-CSF acts in a cell intrinsic fashion to inhibit HSC function

(A) Mixed Csf3r<sup>−/−</sup> (G-CSFR deficient) bone marrow chimeras were generated by transplanting a 3:1 ratio of Csf3r<sup>−/−</sup> to wild type (WT) bone marrow cells into irradiated recipients. This resulted in mixed chimeras with approximately 50% Csf3r<sup>−/−</sup> cells (data not shown). After stable hematopoietic reconstitution (6 weeks), mice were treated with G-CSF or PBS for 7 days, and bone marrow was harvested after the last dose and transplanted into secondary recipients. Peripheral blood chimerism for B220<sup>+</sup> cells was assessed starting at 9 weeks (B). After 12 weeks, pooled marrow from secondary recipients was transplanted into tertiary recipients, and peripheral blood chimerism for B220<sup>+</sup> cells was assessed starting at 6 weeks (C). Data are representative of 2 independent experiments, with 5 mice per condition. **p<0.01
Figure 5. G-CSF treatment upregulates TLR expression and signaling

KSL SLAM cells were sorted from mice that were treated with PBS or G-CSF for 7 days and RNA expression profiling performed. (A) Shown are all genes significantly upregulated after 7 days of G-CSF. Arrows indicate genes whose expression has previously been reported to be regulated by TLR signaling. (B) Gene Set Enrichment Analysis showed an enrichment of TLR signaling (FDR q-value: 0.15). (C). Shown is the Affymetrix expression values for TLR family members in KLS SLAM cells after 7 days of G-CSF. ***p<0.001. Data represent the mean ± SEM of three independent arrays for each condition. (D) Representative histograms showing increased cell surface TLR2 expression on CD34− Flk2− KSL cells. Data are representative of 5–8 mice from two independent experiments.
Figure 6. TLR2 regulates HSC numbers in response to G-CSF
Bone marrow cells were harvested from \( Tlr2^{-/-} \) mice treated with G-CSF or saline alone for 7 days. Data for wild-type mice treated similarly are shown again for comparison. The number of CD34\(^{-}\) Flk2\(^{-}\) CD41\(^{-}\) KSL cells (A) in the bone marrow is shown. Data represent the mean ± SEM of 7–26 mice. The cycling status of CD34\(^{-}\) Flk2\(^{-}\) CD41\(^{-}\) KSL cells was determined using Ki-67 and DAPI; shown are the percentages of cells in G\(_0\) (B) (n=4–5 mice per group). Competitive repopulation assays were performed to assess repopulating activity in the bone marrow of saline (PBS)- or G-CSF-treated \( Tlr2^{-/-} \) mice. (C) The percentage of donor (Ly5.2\(^{+}\)) leukocytes in the blood over time is shown; data for wild type bone marrow is shown again to facilitate comparison. (D) The frequency of donor (Ly5.2\(^{+}\)) B cells (B220\(^{+}\)), T-cells (CD3\(^{e}\)), and neutrophils (Gr-1\(^{\text{high}}\)) in the peripheral blood 12 weeks after transplantation is shown. The data represent the mean ± SEM of 5 mice per group. *\( p<0.05 \), **\( p<0.01 \), ***\( p<0.001 \), and reflect the comparison to the PBS group.
Figure 7. TLR signaling affects HSC repopulating activity at baseline and in response to G-CSF

Bone marrow cells were harvested from MyD88−/− and Tlr4−/− mice treated with G-CSF or saline alone for 7 days. Data for wild-type mice treated similarly are shown again for comparison. The number of CD34− Flk2− CD41− KSL cells (A) in the bone marrow is shown. Data represent the mean ± SEM of 6–26 mice. The cycling status of CD34− Flk2− CD41− KSL cells was determined using Ki-67 and DAPI; shown are the percentages of cells in G0 (B) (n=4–10 mice per group). Competitive repopulation assays were performed to assess repopulating activity in the bone marrow of saline (PBS)- or G-CSF-treated Myd88−/− or Tlr4−/− mice. (C) The percentage of donor (Ly5.2+) leukocytes in the blood over time is
shown; data for wild type bone marrow is shown again to facilitate comparison. The data represent the mean ± SEM of 4–10 mice per group from 2–3 independent transplants. (D) The frequency of donor (Ly5.2+) B cells (B220+), T-cells (CD3e+), and neutrophils (Gr-1high) in the peripheral blood 22 weeks after transplantation is shown for a representative experiment from each group. In (E), the average repopulating units from 1×10⁶ test donor cells were determined for each group using the equation

\[
RU = \left[ \frac{\%\text{chimerism of test donor-derived cells} \times \# \text{ of competitor cells} \times 10^{-5}}{\%\text{chimerism of competitor-derived cells}} \right]
\]

To determine RU/ bone marrow, this value was then multiplied by the total marrow WBCs/10⁶. *p<.05, **p<.01, ***p<.001, and reflect the comparison to the PBS group.
Figure 8. Commensal flora regulates HSCs at baseline and in response to G-CSF

(A) Bone marrow cells were harvested from wild type mice treated with ciprofloxacin and polymyxin B and then treated with saline alone or G-CSF for 7 days as shown. The number of KSL CD34− Flk2− cells (B) in the bone marrow is shown (n = 5–26 mice); SPF: wild-type mice housed under standard pathogen-free conditions. The cycling status of CD34− Flk2− CD41− KSL cells was determined using Ki-67 and DAPI; shown are the percentages of cells in G0 (C) (n= 4–10 mice per group). (D) Competitive repopulation assays were performed using bone marrow from saline- or G-CSF treated mice. The percentage of donor (Ly5.2+) leukocytes in the blood over time is shown (n = 5–7 mice per group); data for wild type bone marrow is shown again to facilitate comparison. (E) The frequency of donor (Ly5.2+) B cells (B220+), T-cells (CD3ε+), and neutrophils (Gr-1high) in the peripheral blood 12 weeks after transplantation is shown for germ-free mice. (F) The absolute number of KSL SLAM cells in the spleen of mice following treatment with saline alone or G-CSF for 7 days is shown (n= 4–10 mice per group). *p<0.05, **p<0.01, ***p<0.001.

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