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M-CSF improves protection against bacterial and fungal infections after hematopoietic stem/progenitor cell transplantation

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Myeloablative treatment preceding hematopoietic stem cell (HSC) and progenitor cell (HS/PC) transplantation results in severe myeloid cytopenia and susceptibility to infections in the lag period before hematopoietic recovery. We have previously shown that macrophage colony-stimulating factor (CSF-1; M-CSF) directly instructed myeloid commitment in HSCs. In this study, we tested whether this effect had therapeutic benefit in improving protection against pathogens after HS/PC transplantation. M-CSF treatment resulted in an increased production of mature myeloid donor cells and an increased survival of recipient mice infected with lethal doses of clinically relevant opportunistic pathogens, namely the bacteria *Pseudomonas aeruginosa* and the fungus *Aspergillus fumigatus*. M-CSF treatment during engraftment or after infection efficiently protected from these pathogens as early as 3 days after transplantation and was effective as a single dose. It was more efficient than granulocyte CSF (G-CSF), a common treatment of severe neutropenia, which showed no protective effect under the tested conditions. M-CSF treatment showed no adverse effect on long-term lineage contribution or stem cell activity and, unlike G-CSF, did not impede recovery of HS/PCs, thrombocyte numbers, or glucose metabolism. These results encourage potential clinical applications of M-CSF to prevent severe infections after HS/PC transplantation.

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INTRODUCTION

Severe myeloid cytopenia is a serious complication of radiation or chemotherapy and leads to profound susceptibility to a variety of opportunistic infections. Myeloablative treatments are also required for autologous or allogeneic hematopoietic cell transplantation (HCT), a common medical procedure to treat hematological disorders. Hematopoiesis from the transplanted hematopoietic stem cells (HSCs) and progenitor cells (HS/PCs) occurs with a significant lag phase, in which the patient shows severe myeloid cytopenia and is vulnerable to severe and potentially lethal infections, for example by the clinically important gram-negative bacterium *Pseudomonas aeruginosa* or the fungus *Aspergillus fumigatus*. Despite improved conditioning regimens, better supportive care, and new antimicrobial and antifungal agents, infection remains a cause of death in 8% of autologous HCT and 17–20% of allogeneic HCT recipients (Tomblyn et al., 2009). Antibiotic and antifungal prophylaxis during HCT can also be associated with significant side effects and does not offer complete protection. These concerns are likely to increase with the mounting incidence of multidrug-resistant nosocomial strains (To et al., 1997).

Therefore, there is a clear need for alternative treatments that could offer protection against infections in the most vulnerable early neutropenic phase after HCT. Adoptive transfer protocols of myeloid progenitors have been proposed to improve recovery and resulted in protection against lethal infections of *A. fumigatus* and *P. aeruginosa* in a preclinical model of HCT (BitMansour et al., 2002, 2005). However, cell therapy approaches require complex logistics and rigorous quality control, and their effectiveness and tolerance might be patient specific. Cytokines, such as GM-CSF and G-CSF, have also been considered as a simple and effective way to shorten myeloid cytopenia by enhancing the production and function of immune effector cells (Heuser et al., 2007), but meta-analyses of clinical studies suggest limited benefit against bacterial or fungal infections (Dekker et al., 2006; Sung et al., 2007;

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Abbreviations used: HCT, hematopoietic cell transplantation; HSC, hematopoietic stem cell; HS/PC, HSC and progenitor cell; KSL, Kit$^+$ Sca-1$^+$ Lineage$^-$; rhG-CSF, recombinant human G-CSF; rhM-CSF, recombinant human M-CSF; rmM-CSF, recombinant mouse M-CSF.

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De Santa et al., 2009; Tomblyn et al., 2009). In addition, G-CSF can result in a loss of long-term repopulating activity (de Haan et al., 1995; Bodine et al., 1996; Winkler et al., 2012; Schuettpelz et al., 2014).

M-CSF (or CSF-1) is another important myeloid cytokine with multiple roles in myeloid cell biology (Pixley and Stanley, 2004). It has found surprisingly little clinical applications so far (Metcalf, 2008) but recently gained attention as a potential therapeutic agent (Hume and MacDonald, 2012). We demonstrated before that M-CSF, but not GM-CSF or G-CSF, transiently increased myeloid differentiation of HSCs by direct activation of the myeloid transcription factor PU.1 but did not compromise long-term stem cell activity (Mossadegh-Keller et al., 2013). We therefore reasoned that M-CSF might be effective in ameliorating myeloid cytopenias and in protecting patients from infection after HCT. To test this hypothesis, we used a preclinical mouse model of HS/PC transplantation and infection with lethal doses of the clinically relevant pathogens *P. aeruginosa* or *A. fumigatus*. We demonstrate that M-CSF treatment during transplantation or after infection increased myelopoiesis and resulted in substantially improved survival and decreased pathogen load, even for early infections and single-dose treatments. In contrast, G-CSF showed less efficient cell recovery and no protective activity against the tested pathogens. Our results demonstrate that M-CSF–induced myeloid commitment of HSCs rapidly protects HCT recipients from lethal infection. They also suggest that prophylactic or therapeutic M-CSF treatment during HCT could be both more efficient and have less side effects than commonly used G-CSF and could complement common antifungal or antibacterial drugs.

RESULTS AND DISCUSSION

To study whether M-CSF treatment during HCT stimulated myelopoiesis and had a beneficial effect against infections, we used intraperitoneal injections of *P. aeruginosa* or intranasal instillation with *A. fumigatus* in a preclinical mouse model that mimics potential infection routes in the clinic such as common infections via the gastrointestinal tract or i.v. catheters or inhalation of fungal spores (Latgé, 1999), respectively.

To test the effect of M-CSF on survival after bacterial infection, irradiated and HS/PC-transplanted mice received either three injections of 10 µg of recombinant M-CSF or PBS on the day of HS/PC transplantation (−1 h, 5 h, and 18 h) and were infected with 500 CFU of *P. aeruginosa* 7 d later (Fig. 1 a), a dose that we had established previously to cause 80–90% lethality in untreated transplant-recipient mice. Mice that were treated with baculovirus-produced recombinant mouse M-CSF (rmM-CSF) or with bacterially produced recombinant human M-CSF (rhM-CSF) showed strongly improved survival rates from 15.3% in control to 87.5% in rmM-CSF–treated mice (Fig. 1 b) and to 50% in rhM-CSF–treated mice (Fig. 1 c). All M-CSF–treated mice showed a significant decrease of bacterial load in spleen, lung, heart, and liver 1 d after infection (Fig. 1 d). This effect correlated with increased donor-derived granulocytes and mononuclear phagocytes in the spleen and more monocytes and monocyte-derived macrophages in the liver (Fig. 1 e). Similar effects were observed in uninfected M-CSF–treated mice (Fig. 1 f). M-CSF treatment thus increased myeloid differentiation of transplanted HS/PCs and conferred protection against *P. aeruginosa* infection by reducing bacterial tissue load.

To determine whether M-CSF also protected against fungal infections, we infected mice 1 wk after irradiation and HS/PC transplantation with $4–6 \times 10^6$ CFU of *A. fumigatus*, a pathogen dose causing 80–90% lethality in irradiated recipients of life-saving HS/PC transplants. Mice treated with rmM-CSF and rhM-CSF during transplantation showed 60% (Fig. 2 b) and 40% survival (Fig. 2 c), respectively, compared with 10% in the PBS-treated control group. Mice treated with rhM-CSF showed a significant decrease in the fungal tissue load when compared with control mice in lungs, liver, and heart 48 h after infection (Fig. 2 d). These results demonstrated that M-CSF treatment also conferred protection against lethal *A. fumigatus* infection by reducing fungal tissue load.

In our assays, both a bacterially expressed bioactive protein fragment of rhM-CSF as well as baculovirus-expressed full-length mouse M-CSF was protective. The somewhat lower activity of rhM-CSF might be explained by incomplete interspecies cross-reactivity, importance of protein glycosylation or sequences outside of the core fragment, or batch or storage variation. Importantly, however, the strong activity of two different independently produced M-CSF preparations indicates that the observed effects are a robust and general property of M-CSF.

In standard clinical practice, G-CSF is the most common cytokine used as a prophylactic or treatment option in HCT protocols. To directly compare the protective effect of M-CSF to G-CSF, we treated mice with rmM-CSF, rhM-CSF, recombinant human G-CSF (rhG-CSF), or control PBS during HS/PC transplantation and infection with *P. aeruginosa*. As observed in the previous experiments, M-CSF–treated mice showed largely improved survival of 70% for rmM-CSF or 45.5% for rhM-CSF compared with 10% in control mice, whereas rhG-CSF–treated mice showed no survival improvement (Fig. 3 a). To investigate the underlying reason for the superior protective effect of M-CSF, we analyzed the activation of the myeloid PU.1 transcription factor as the earliest sign of myeloid lineage commitment in HS/PCs (Mossadegh-Keller et al., 2013). We observed a strong increase in $PU.1^+$ cells 20 h after M-CSF treatment, compared with either PBS controls or G-CSF–treated mice in HSCs, but little effect on multipotent progenitors of PU.1-GFP reporter mice (Fig. 3, b and c). Consistent with our previous observations (Mossadegh-Keller et al., 2013), these results suggested that the improved protective effect of M-CSF against bacterial infection was because of the early and rapid induction of the myeloid commitment of HSCs by M-CSF but not by G-CSF.

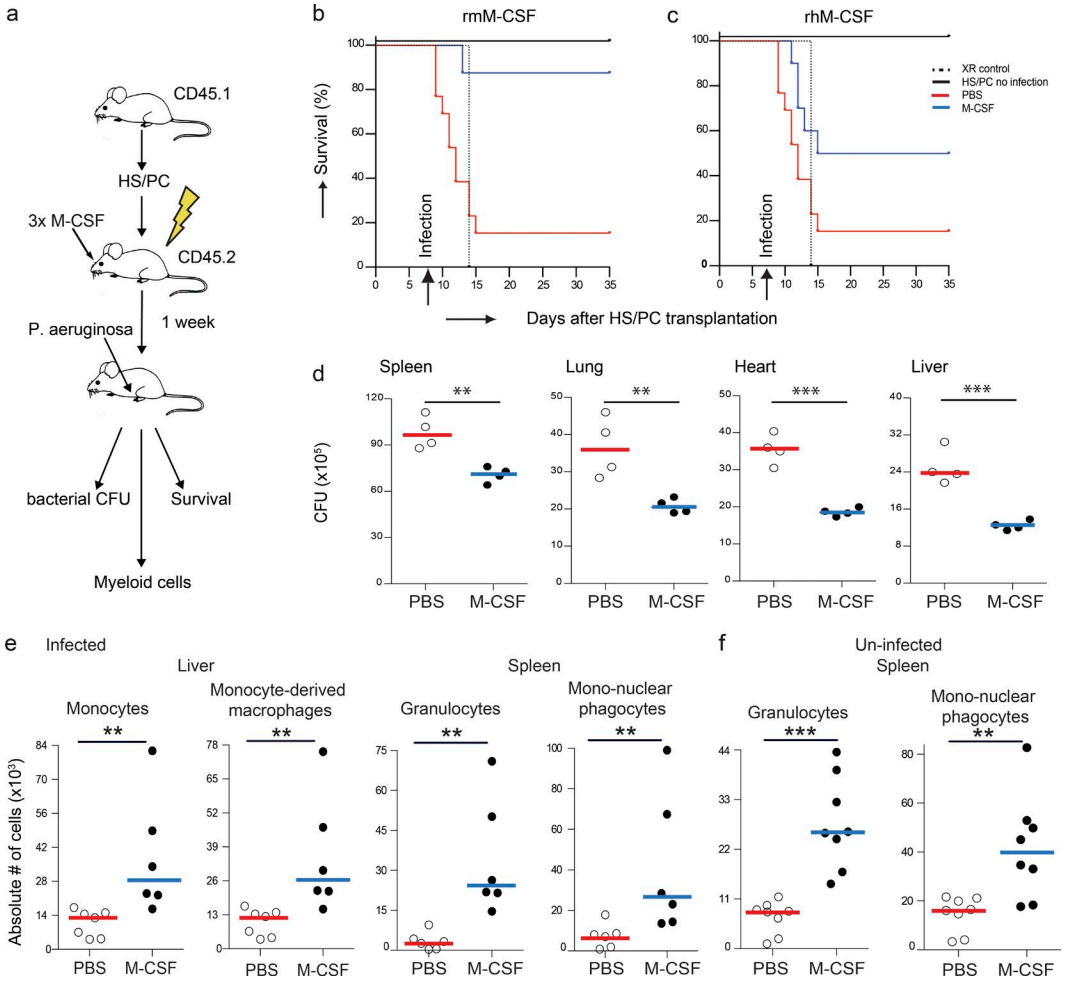


Figure 1. **M-CSF protects against *P. aeruginosa* infection by inducing increased myelopoiesis of transplanted HS/PCs.** (a) Lethally irradiated CD45.2 mice were transplanted with 2,500 CD45.1 HS/PCs, treated with three doses of control PBS or M-CSF during transplantation (−1 h, 5 h, and 18 h), challenged after 1 wk by intraperitoneal injection of 500 CFU of *P. aeruginosa*, and analyzed for bacterial load and myeloid donor cells 1 d after infection or for survival. (b and c) Survival of mice after HS/PC transplantation and bacterial infection (arrow) for PBS control ($n = 13$) or mice treated with three doses of 10 µg of baculovirus-expressed rmM-CSF ($n = 8$; b) or three doses of 10 µg rhM-CSF ($n = 10$; c). Transplanted, not infected (black line; $n = 4$) and irradiated (XR), untransplanted and uninfected control mice (dashed line; $n = 6$) are shown. $P < 0.001$ (rmM-CSF) and $P < 0.05$ (rhM-CSF) by a Mantel-Cox test. (d) Bacterial load in indicated organs 18 h after infection of transplanted mice treated with rhM-CSF or control PBS. (e and f) Median of donor monocytes ($CD11b^+$, $Ly6C^+$) and monocyte-derived macrophages ($CD11b^+$, $Ly6C^+$, $F4/80^{int}$) in the liver or granulocytes ($Ly6G^+$, $CD11b^+$) and mononuclear phagocytes ($Ly6G^-$, $CD11b^+$) in the spleen of PBS control or M-CSF–treated recipient mice 1 d after infection (e) or 9 d after transplantation of uninfected mice (f). Gating strategy is shown in Fig. S1. All data are representative of at least two independent experiments. (d–f) P-values were obtained by Mann-Whitney *U* tests. **, $P < 0.01$; ***, $P < 0.001$.

Pretransplant treatment with M-CSF can also induce the expansion of resident macrophages in HCT recipients (Hashimoto et al., 2011). Theoretically, it was therefore possible that this effect contributed to the protective effect of M-CSF against posttransplant infection. To address this, we treated irradiated but untransplanted mice with rmM-CSF or PBS 1 wk before infection with *P. aeruginosa*. All M-CSF– or PBS-treated mice died within 1–3 d after infection and, thus, 2–3 d before control mice died from radiation-induced bone marrow failure. In contrast and as observed before, HS/PC-transplanted and M-CSF–treated mice showed 83.33% survival (Fig. 3 c). To increase the time period between infection and irradiation-induced death, we also infected mice already 3 d after irradiation and M-CSF treatment (Fig. 3 d). Again, all M-CSF– or PBS-treated mice died 1–3 d after infection and thus 8–9 d before irradiation-induced death, clearly establishing infection as the cause of death. Control mice that had received a HS/PC transplant in addition to M-CSF showed 37.5% survival even for early infection. In the reciprocal experiment, we treated donor mice with PBS or M-CSF before HS/PC isolation and infected recipients 3 d after transplantation of the treated HS/PC graft. Also, in

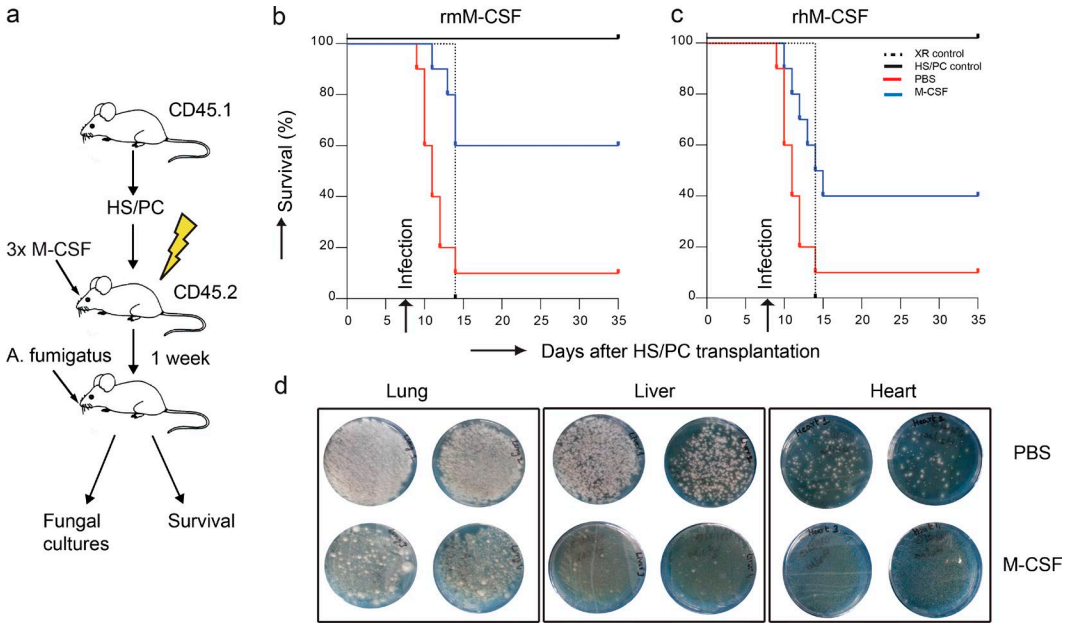


Figure 2. **M-CSF protects against *A. fumigatus* infection after HS/PC transplantation.** (a) Mice were transplanted and treated with M-CSF as described in Fig. 1 a, infected after 1 wk by intranasal instillation of *A. fumigatus* (4–6 × 10^6 CFU), and analyzed for fungal load after 2 d or for survival. (b and c) Survival of mice after HS/PC transplantation and fungal infection (arrow) for PBS control (n = 10) or mice treated with three doses of 10 µg rmM-CSF (n = 10; b) or three doses of 10 µg rhM-CSF (n = 10; c). Transplantation (n = 4) and irradiation (XR) controls (n = 4) are shown. $P < 0.001$ (rmM-CSF) and $P < 0.01$ (rhM-CSF) by Mantel-Cox tests. (d) Fungal load in indicated organs 48 h after infection of transplanted mice treated with rhM-CSF (n = 4) or control PBS (n = 4). Photos of cultures from two representative mice are shown. All data are representative of at least two independent experiments.

this protocol, M-CSF but not PBS treatment of donor cells resulted in improved survival of transplant recipients infected after 3 d, similar to the effect observed for recipient treated mice (Fig. 3 e). Together, these experiments clearly establish that the protective effect of M-CSF against bacterial infection is mediated by its action on donor HS/PCs rather than by resident recipient cells.

The effects of M-CSF treatment on early recovery of immune competence after HSC transplant might be explained by its activity on HSC commitment. Whereas other myeloid cytokines such as GM-CSF and G-CSF can also stimulate proliferation and differentiation of more mature myeloid progenitors (Hamilton and Achuthan, 2013) and are important at later stages of emergency hematopoiesis (Manz and Boettcher, 2014; Zhao and Baltimore, 2015), M-CSF appears to be unique in its ability to induce myeloid commitment on the HSC level (Sarrazin et al., 2009; Sarrazin and Sieweke, 2011; Mossadegh-Keller et al., 2013). This might explain why M-CSF treatment provides a critical advantage in the early and rapid recovery of myeloid cells and immune competence against bacterial and fungal pathogens. Because M-CSF is massively induced early after infections (Cheers et al., 1988), M-CSF treatment appears to mimic a natural protective mechanism engaging HSCs in the immune response. It will remain to be determined in detail whether the protective effects depend on increased differentiation to the neutrophil or monocyte lineage or both. Some other cytokines and growth factors released during infection and inflammation, including type I and type II interferons, Il-1, IL-6, and TNF, can also act on HSCs (Baldridge et al., 2011; King and Goodell, 2011; Manz and Boettcher, 2014; Mirantes et al., 2014; Zhao and Baltimore, 2015) but mainly influence proliferative responses rather than myeloid differentiation of HSCs or cause HSC exhaustion. The unique activity of M-CSF on HSCs therefore appears to be ideally suited to drive a protective response against infection during HS/PC transplantation.

Because of these considerations, we further characterized the potency of the M-CSF response in various more severe infectious conditions. As shown in Fig. 4 a, rmM-CSF–treated mice infected with *P. aeruginosa* already 3 d after transplantation also showed significantly improved survival of 40% compared with no survival in control or G-CSF–treated mice. M-CSF–treated mice also showed a significant decrease in bacterial load (Fig. 4 b) and increase in donor-derived cells (Fig. 4 c) in the spleen 1 d after early infection. Furthermore, mice treated with only a single dose of 10 µg rmM-CSF during transplantation also showed improved survival of 33.33% in response to infection with *P. aeruginosa* after 7 d compared with 8.33% survival in control mice and no survival in G-CSF–treated mice (Fig. 4 d). Finally, transplanted mice infected after 1 wk with *P. aeruginosa* also showed a significant increase of 60% survival compared with 20% in control mice when rmM-CSF was given after bacterial infection (Fig. 4 e). Collectively, these results demonstrate

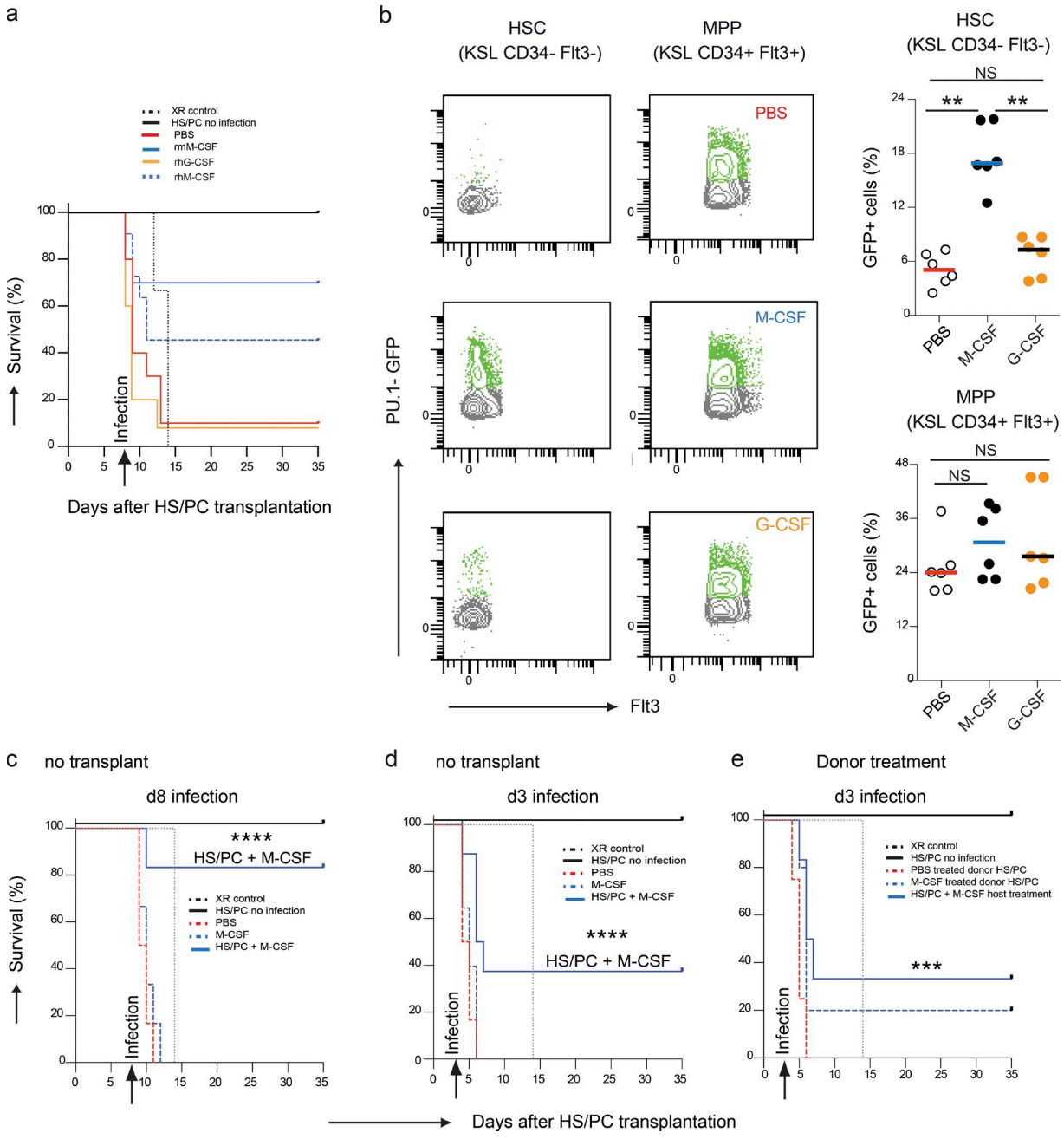


Figure 3. **Specific M-CSF effect on early HSC commitment correlates with better antibacterial protection than G-CSF during HS/PC transplantation.** (a) Mice were transplanted, treated, and infected as described in Fig. 1 a. Survival of mice after transplantation and bacterial infection (arrow) for PBS control (n = 10) or mice treated with rmM-CSF (n = 10), rhM-CSF (n = 11), or rhG-CSF (n = 10) is shown. $P < 0.05$ (rmM-CSF or rhM-CSF) by a Mantel-Cox test. XR, irradiation. (b) Representative FACS profiles and median of GFP expression in HSCs (KSL CD34$^-$ Flt3$^-$) and multipotent progenitors (MPP; KSL CD34$^+$ Flt3$^+$) of PU.1-GFP reporter mice 20 h after control PBS, rhM-CSF, or rhG-CSF injection. **, $P < 0.01$ by a Mann-Whitney U test. (c and d) Survival of nontransplanted lethally irradiated mice treated with three doses of control PBS (n = 6) or 10 µg rmM-CSF (n = 6) after irradiation (6 h, 12 h, and 24 h) and challenged after 8 (c) or 3 (d) d (arrow) by i.p. injection of 500 CFU of *P. aeruginosa*. Control mice were treated identically but transplanted with 2,500 HS/PCs (n = 6). ****, $P < 0.0001$ (rmM-CSF + HS/PC) by a Mantel-Cox test. (e) Survival of mice transplanted with donor HS/PCs from mice treated with three doses (−5 h, −3 h, and −1 h) of 10 µg rmM-CSF (n = 10) or PBS (n = 8) and infected after 3 d (arrow) with 500 CFU of *P. aeruginosa*. Control recipient mice were treated with three doses of 10 µg rmM-CSF (n = 8) during transplantation. ***, $P < 0.001$ (rmM-CSF–treated donor HS/PCs) by a Mantel-Cox test. Transplantation (n = 4) and irradiation controls (n = 4) are shown in all survival analyses. All data are representative of two independent experiments.

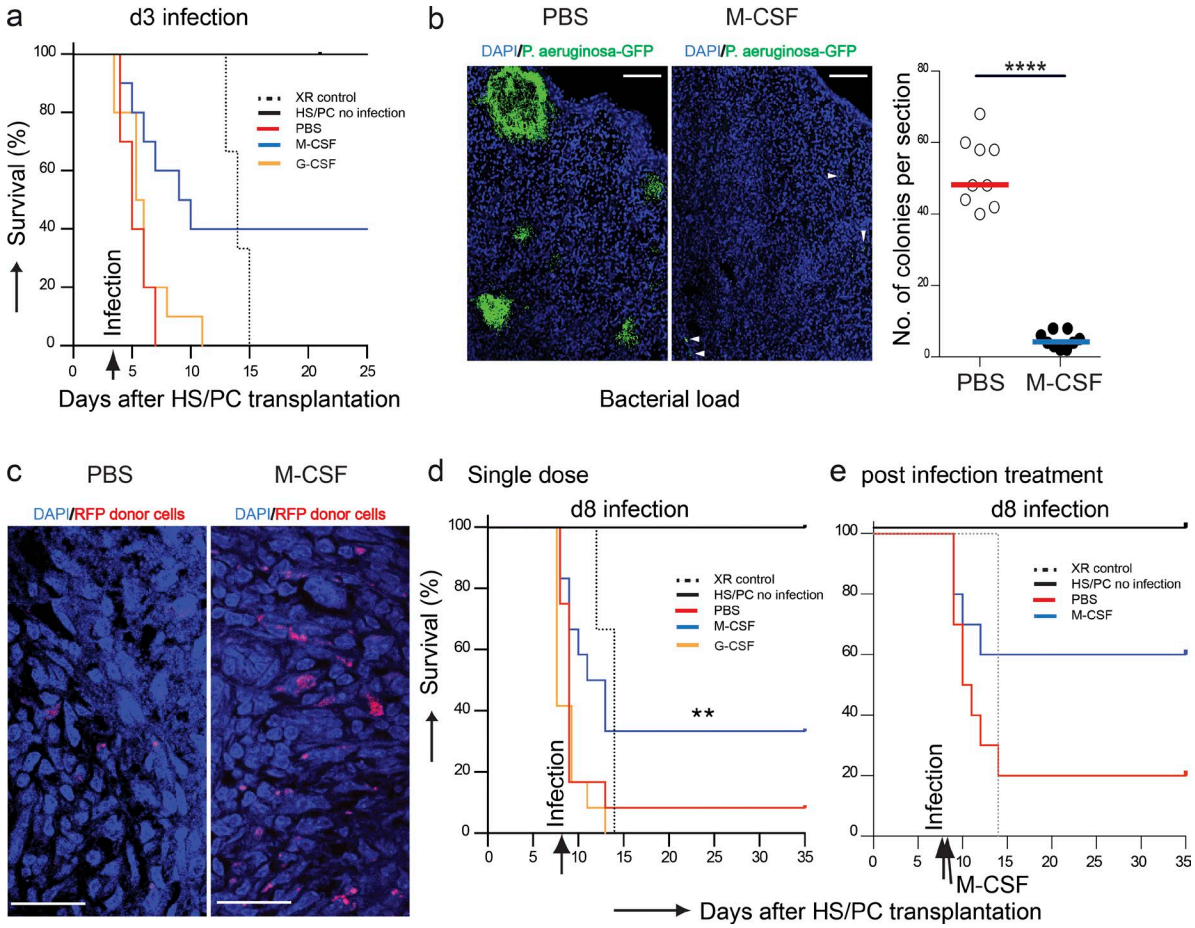


Figure 4. **M-CSF provides antibacterial protection of HS/PC transplant recipients for early infection and single-dose or postinfection cytokine treatment.** (a) Mice were transplanted and treated with PBS, M-CSF, or G-CSF as described in Fig. 1 a but infected after 3 d. Survival of mice after transplantation and bacterial infection (arrow) for PBS control (n = 10) or mice treated with three doses of 10 µg rmM-CSF (n = 10) or rhG-CSF (n = 10) is shown. **, P < 0.01 (rmM-CSF) by Mantel-Cox test. XR, irradiation. (b and c) Confocal images with quantification of bacterial colonies (b) or donor cells (c) in spleens 24 h after early infection on day 3 of transplanted mice treated with rmM-CSF or control PBS. ****, P < 0.0001 by a Mann-Whitney U test. Bars, 50 µm. Arrowheads indicate the maximum colony size in the M-CSF–treated group. (d) Mice were transplanted and infected as described in Fig. 1 a but treated with a single dose of control PBS, M-CSF, or G-CSF during transplantation (5 h). Survival of mice after transplantation and bacterial infection (arrow) for PBS control (n = 12) or mice treated with a single dose of 10 µg rmM-CSF (n = 12) or rhG-CSF (n = 12) is shown. **, P < 0.01 by a Mantel-Cox test. (e) Mice were transplanted and infected as described in Fig. 1 a but treated with three doses (1 h, 3 h, and 5 h) of 10 µg rmM-CSF or PBS after infection. Survival of mice after transplantation and bacterial infection (arrow) for PBS control (n = 10) or rmM-CSF (n = 12) is shown. *, P < 0.05 by a Mantel-Cox test. Transplantation (n = 4) and irradiation controls (n = 4) are shown in all survival analyses. All data are representative of two independent experiments.

that both prophylactic and therapeutic M-CSF as single- or multiple-dose treatment confer protection to HS/PC-transplanted recipients even against early bacterial infection. This identifies M-CSF as a potential attractive treatment option for various clinical needs after HCT.

We furthermore evaluated whether increased myeloid lineage commitment after M-CSF treatment affected long-term multilineage contribution of transplanted HS/PCs (Fig. 5 a). We did not detect any difference in the donor cell contribution to the myeloid, B cell, or T cell compartments of peripheral blood (Fig. 5 b) or in myeloid-to-lymphoid lineage ratio (Fig. 5 c) in M-CSF– or G-CSF–treated mice compared with PBS controls. In contrast to G-CSF treatment, M-CSF also did not affect donor-derived myeloid progenitors or multipotent stem and HS/PC populations (Fig. 5, d and e). Finally, secondary transplantation of bone marrow derived from M-CSF–treated mice, including mice that had survived infection, showed robust contribution to all blood lineages (Fig. 5 f). Thus, our data indicated that M-CSF–stimulated myeloid differentiation did not compromise general HSC self-renewal, multilineage differentiation capacity, or stem cell activity and did not lead to HSC exhaustion or HSC mobilization.

Because earlier clinical studies using long-term treatment with M-CSF for different indications had reported mild thrombocytopenia (Garnick and Stoudemire, 1990; Munn et

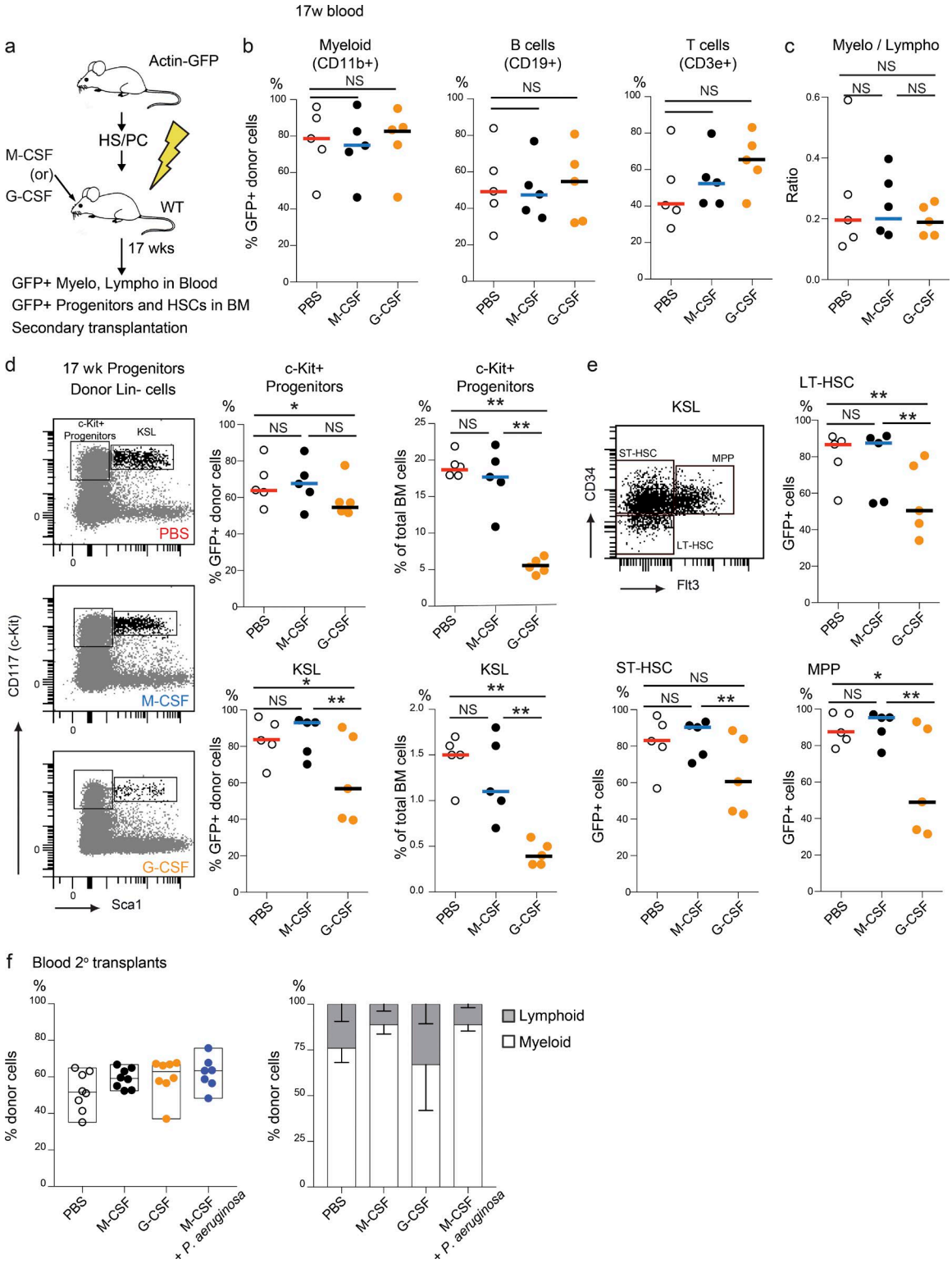


Figure 5. **M-CSF treatment does not compromise HSC self-renewal, multilineage differentiation, or stem cell activity.** (a) Mice were transplanted with actin-GFP HS/PCs and treated with rmM-CSF, rhG-CSF, or PBS as described in Fig. 1 a and analyzed after 17 wk for donor cell contribution in blood and bone marrow. (b and c) Percentage of GFP$^+$ donor cell contribution to CD11b$^+$ myeloid, CD19$^+$ B cells, or CD3e$^+$ T cells in blood of reconstituted mice (b) or expressed as a ratio of CD11b$^+$ myeloid cells to CD19$^+$ lymphoid cells (c). (d) Percentage of GFP$^+$ donor cell contribution and percentage of total bone

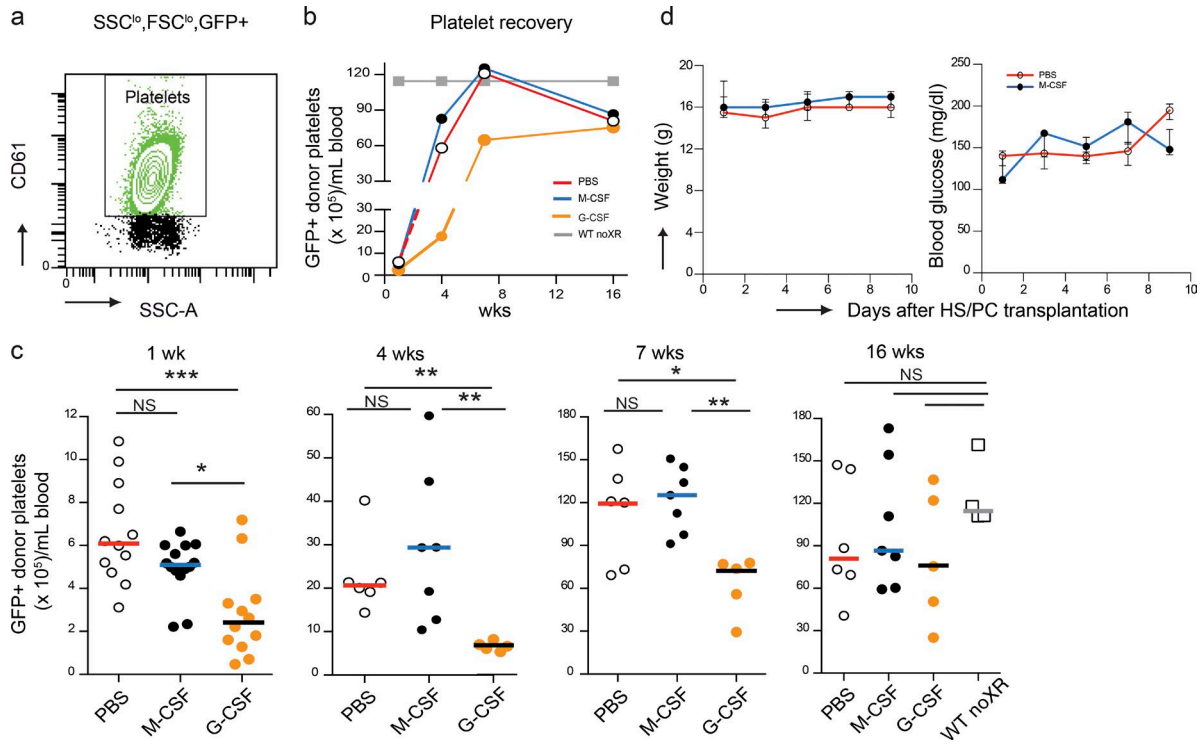


Figure 6. **M-CSF treatment does not affect platelet recovery or blood glucose levels.** (a–c) Mice were transplanted with actin-GFP HS/PCs and treated with rmM-CSF, rhG-CSF, or PBS as described in Fig. 1 a and analyzed for donor side scatter (SSC)^{lo} forward scatter (FSC)^{lo} platelet contribution in the blood shown as time course of median absolute numbers (b) or quantification at indicated time points (c). Non-irradiated (noXR) nontransplanted controls were used as a reference. For 1 wk, * = 0.011 (>0.01 and <0.06). For 7 wk, * = 0.06. (d) Time course of weight and blood glucose levels of mice transplanted and treated with rmM-CSF (n = 6) or PBS (n = 6) as indicated in Fig. 1 a. Error bars represent the median and error with interquartile range. **, $P < 0.01$; ***, $P < 0.001$ by Mann-Whitney U tests. Data are representative of two independent experiments.

al., 1990), we also analyzed platelet recovery after M-CSF treatment. Whereas G-CSF treatment led to a significant delay of recovery with reduced platelet levels between 1 and 7 wk in mice transplanted with actin-GFP HS/PCs, M-CSF treatment showed no disadvantage compared with PBS-treated control mice at any time point, and platelet counts recovered to normal homeostatic levels 16 wk after transplantation (Fig. 6, b and c). Rare cases of M-CSF–treated patients have also been reported with mild hyperglycemia (Bukowski et al., 1994), but we did not detect any adverse effects on blood glucose levels and body weight of M-CSF–treated mice after HS/PC transplantation (Fig. 6 d). The short-term treatments during HCT tested here thus did not result in thrombocytopenia or hyperglycemia and therefore dissipate concerns stemming from earlier clinical studies with long-term continuous high doses of M-CSF treatment.

Our data indicate that M-CSF treatment during HCT appears to have clear advantages over G-CSF both in terms of effective protection against bacterial and fungal infection and reduced side effects on general hematopoietic recovery. In contrast to M-CSF treatment, G-CSF treatment during HS/PC transplantation led to reduced HSC, progenitor, and thrombocyte counts. The ability of G-CSF to induce HSC release into the blood stream that is commonly used for HS/PC isolation by leukapheresis might also have a negative effect on HSC homing and lodging after HCT. Collectively, our findings indicate that M-CSF treatment during HCT should likely be more effective and safer than the commonly used G-CSF.

Although G-CSF does not increase the production of myeloid progenitors from HSCs, it could still have beneficial effects on more mature myeloid cells once these progenitors have been generated by M-CSF stimulation. Furthermore, a recent clinical trial (Wan et al., 2015) reported a benefit of GM-CSF over G-CSF for protection from fungal disease in transplant recipients. Combinatorial or successive treatment

marrow cells for c-kit$^+$ progenitors and HS/PCs (KSL). (e) Percentage of GFP$^+$ donor cell contribution to indicated stem and progenitor cell populations. (f) Percentage of GFP$^+$ donor cell contribution to myeloid and lymphoid cells in blood 7 wk after secondary transplantation. Error bars represent the median with range. *, $P < 0.05$; **, $P < 0.01$ by Mann-Whitney U tests. All data are representative of two independent experiments.

protocols of M-CSF with G-CSF or GM-CSF might therefore offer synergistic effects.

Because M-CSF treatment reduces graft-versus-host disease in allogeneic HCT (Hashimoto et al., 2011; Kimura et al., 2012), it will be important to determine whether it also has a protective effect against infection in this setting, as suggested by earlier retrospective clinical observations (Nemunaitis et al., 1993). In this context, the influence of M-CSF treatment on graft-versus-leukemia effects and on residual malignant cells will also require further investigation. In summary, the clear multiple beneficial effects of M-CSF treatment during HCT on improved graft-versus-host disease and protection against clinically important and often lethal fungal and bacterial infections, combined with the absence of evident negative side effects on reconstitution capacity and hematopoietic differentiation, merits consideration of clinical trials of this cytokine in HCT.

MATERIALS AND METHODS

Mice

CD45.1 and C57BL/6 mice were obtained from Charles River. 10–14-wk-old sex-matched CD45.2 recipients were reconstituted as previously described (Sarrazin et al., 2009; Mossadegh-Keller et al., 2013) with bone marrow–derived KSL (c-Kit [CD117]$^+$, Sca1$^+$, Lin$^-$) HS/PCs isolated from 6–8-wk-old CD45.1. For in vivo injections, the indicated concentrations of M-CSF and/or sorted cells were injected in 100–200 µl PBS into the retroorbital sinus. For HS/PC transplantation, 2,500 KLS HS/PCs were sorted from CD45.1 mice and mixed with 200,000 cKit$^-$ CD45.2 or cKit$^-$ Terr119$^+$ carrier CD45.2 cells before injection into lethally irradiated (160 kV, 25 mA, and 6.9 Gy) CD45.2 recipient mice. For secondary transplantations, two million donor-derived bone marrow cells were injected per lethally irradiated recipient. After irradiation, all mice were given antibiotics (Bactrim) in drinking water to reduce the chance of opportunistic infection with other pathogens. All mouse experiments were performed under specific pathogen–free conditions. Animals were handled according to protocols approved by the animal ethics committee of Marseille, France, and were performed in accordance to international, national, and institutional regulations.

Isolation of HS/PCs, cKit$^-$, and cKit$^-$ Ter119$^+$ cells

Total bone marrow cells were depleted of mature cells by staining with biotinylated rat anti–mouse lineage antibody cocktail, followed by streptavidin immunomagnetic microbeads (Miltenyi Biotec). Lineage-negative cells were stained with HS/PC markers: anti-CD117–APC-H7 (clone 2B8; BD), anti–Sca-1–PE-Cy5 (clone D7; BioLegend), streptavidin-APC (eBioscience), and LIVE/DEAD Fixable Violet Dead cell dye (Invitrogen) as viability marker. HS/PCs were sorted using FACSAriaIII equipment (BD). For isolating cKit$^-$ carrier cells, whole bone marrow cells were depleted of cKit$^+$ cells by staining with biotinylated anti–mouse CD117 (clone 2B8; BioLegend), followed by streptavidin immunomagnetic microbeads (Miltenyi Biotec). For isolating cKit$^-$ Ter119$^+$ carrier cells, first, cKit$^-$ cells were selected by depleting cKit$^+$ cells, stained with biotinylated anti–mouse Ter119 (clone TER-119; BD), and followed by streptavidin immunomagnetic beads.

M-CSF treatment

Each mouse received three i.v. injections of 10 µg of each cytokine 1 h before HS/PC transplantation and 5–6 h and 18 h after transplantation. rhM-CSF (Novartis), rmM-CSF expressed in baculovirus, and human G-CSF (Neulasta) were used for the study. We thank G. Mouchiroud (Université Claude Bernard Lyon I, Villeurbanne, France) for baculovirus-produced rmM-CSF.

Infection with *P. aeruginosa*

The *P. aeruginosa* PA14 strain was tagged with GFP, as described previously (Koch et al., 2001; Giraud et al., 2011). The GFP-tagged PA14 strain was cultured overnight at 37°C in Luria broth medium, diluted 1:100 in Luria broth medium, and grown for 3 h to reach bacterial exponential phase (3–4 OD_{600nm}). A volume of 100 µl of a bacterial solution of 5×10^3 CFU/ml diluted in PBS was further used for infection studies. 1 wk after HS/PC transplantation, mice were challenged by intraperitoneal inoculation of 500 CFU of bacteria in 100 µl of sterile PBS.

Bacterial tissue load quantification

The infected mice were killed, and the organs (spleen, lungs, liver, and heart) were harvested and weighed. To determine CFU per gram of tissue, serial dilutions of tissue homogenates were prepared in PBS and plated on Pseudomonas Isolation agar (DIFCO), supplemented with appropriate antibiotics, and incubated overnight at 37°C. The colonies were counted after 16–24 h.

Infection with *A. fumigatus*

A. fumigatus (FGSC 1100) was provided by the Centre International de Ressources Microbiennes– Champignons Filamenteux. For each experiment, cultures were grown on malt agar medium (2% malt extract and 2% bacto-agar; DIFCO) for 5 d at 25°C. Conidial suspension was prepared in sterile saline according to a study by BitMansour et al. (2002). 1 wk after HS/PC transplantation, mice were infected by intranasal inoculation of conidia in 20–40 µl of sterile PBS.

Fungal culture of infected organs

The organs (lungs, liver, heart, and spleen) were harvested and weighed, and tissue homogenates were prepared in PBS. The homogenates were serially diluted, and 200 µl of each dilution was plated on Sabouraud dextrose agar (DIFCO). The plates were incubated at 25°C, and pictures were taken after 3–5 d.

Flow cytometry

For FACS sorting and analysis, we used previously described staining protocols (Mossadegh-Keller et al., 2013), published

stem and progenitor cell definitions (Bryder et al., 2006), FACSCanto, LSRII, and FACSAriaIII equipment, and DIVA software (BD), analyzing only populations with at least 200 events. The following antibodies were used for staining cells: anti-CD117 (clone 2B8; BD), anti–Sca-1 (clone D7; BioLegend), anti-CD34 (clone RAM34; BD), anti-CD16/32 (clone 2.4G2; BD), anti-CD11b (clone M1/70; BD), anti-CD19 (clone 1D3; BD), anti-CD3e (clone 145-2C11; BioLegend), anti-Ly6G (clone 1A8; BioLegend), anti-Ly6C (clone HK1.4; BioLegend), anti-CD115 (clone AFS98; eBioscience), anti-CD45.2 (clone 104; BD), anti-CD45.1 (clone A20; BD), anti-B220 (clone RA3-6B2; eBioscience), anti-Ter119 (clone TER-119; eBioscience), and anti-CD71 (clone R17217; eBioscience). LIVE/DEAD Fixable Violet Dead cell dye (Invitrogen) was used as a viability marker. Information describing gating strategies is shown in Figs. S1 and S2.

Immunofluorescence and confocal imaging

Resected spleens were fixed in Antigen-Fix reagent (Diapath) for 2 h at 4°C, washed in phosphate buffer, and dehydrated in a 30% sucrose solution overnight at 4°C. Samples were embedded in TissueTek medium (Sakura), frozen at −80°C, cut into 8-µm–thick sections using a cryostat, and stained overnight at 4°C. The following antibodies were used: chicken anti-GFP (Aves laboratories) detected with donkey anti–chicken–Alexa Fluor 488 (Jackson ImmunoResearch Laboratories, Inc.). Confocal microscopy acquisition was performed on a ZEISS microscope (LSM780) at room temperature.

Glycemia test

Blood glucose was monitored every 2 d with a glucometer (Performa ACCU-CHEK; Roche).

Online supplemental material

Fig. S1 shows gating strategy for donor-derived granulocytes and mononuclear phagocytes from spleens and gating strategy for donor-derived monocytes and monocyte-derived macrophages from livers. Fig. S2 shows gating strategy for quantification of actin-GFP$^+$ HS/PC-derived platelets.

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