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

Prashanth K. Kandalla, Sandrine Sarrazin, Kaweh Molawi, Carole Berruyer, David Redelberger, Anne Favel, Christophe Bordi, Sophie de Bentzmann, and Michael H. Sieweke

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).
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 (Metcalfe, 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 × 10^4 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.
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 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.

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.

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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. 1a. 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 (M-CSF-treated donor HS/PC) 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. 1a 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...
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 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

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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, mice were challenged by intraperitoneal inoculation of conidia in 20–40 µl of sterile PBS. 1 wk after HS/PC transplantation, mice were infected by intranasal dilution in PBS further used for infection studies. 1 wk after 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), FACS Canto, LSR II, and FACS Aria III 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 IA8; 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 described gating strategies was 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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