

Protection against Lethal *Aspergillus fumigatus* Infection in Mice by Allogeneic Myeloid Progenitors Is Not Major Histocompatibility Complex Restricted

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Invasive fungal infections are a leading cause of morbidity and mortality after myelotoxic chemotherapy or radiation exposure. The resulting depletion of myeloid precursors under these conditions appears to be the factor that limits approaches to accelerate immune reconstitution. In a murine model of myeloablation after radiation exposure, we demonstrated that highly purified common myeloid and granulocyte-monocyte progenitors (CMPs/GMPs) accelerated myeloid recovery and, thus, enhanced innate immunity as measured by survival after a lethal challenge with *Aspergillus fumigatus*. Of greatest significance was the demonstration that the protection afforded by CMPs/GMPs was not major histocompatibility complex restricted. Furthermore, the effect of CMP/GMP cellular therapy was additive with that of liposomal amphotericin B treatment. These observations greatly expand the potential donor pool and, thus, the clinical utility of CMP/GMP cellular therapy in patients with myeloid depletion.

Myeloid depletion results in significant susceptibility to bacterial and fungal infections, regardless of whether the depletion occurred as sequelae of therapies for malignancies, exposure to other forms of radiation, or genetic deficiencies. In the case of a myelotoxic insult, the reconstitution of a functional immune system by donor cells is dependent on the de novo regeneration of all hematopoietic lineages from hematopoietic stem cells (HSCs) and lineage-committed progenitor cells. Myeloablative conditioning regimens for hematopoie-

tic cell transplantation (HCT) result in susceptibility to infections, which typically follow a reproducible time pattern correlating with the kinetics of immune reconstitution [1–3]. Invasive bacterial and fungal infections—for example, *Aspergillus fumigatus* infection—remain a major cause of morbidity and mortality, despite the development of new antifungal agents [4–6] and supportive strategies such as granulocyte transfusions [7] or the use of hematopoietic growth factors. Although the numbers of patients undergoing HCT continue to increase, myeloid depletion occurs more routinely in the setting of chemotherapy or radiation injury. The significant morbidity, mortality, and economic costs of neutropenia-associated infections and the sequelae of reductions in chemotherapy dosage in this setting have been well documented. Therefore, we propose that the infusion of myeloid progenitor cells will be a new therapeutic strategy to attenuate susceptibility to a variety of infections associated with neutropenia.

Aspergillus species are ubiquitous fungi that are easily aerosolized and readily reach the lung alveoli after inhalation. The primary defense mechanisms against *Aspergillus* species, therefore, are the alveolar macrophages and the innate immune system of myeloid origin. Recent data demonstrate an important role for dendritic

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Table 1. List of donor-host mouse strain pairings.

Experiment	CMP/GMP donor strain	HSC donor strain	Host strain	CMP/GMP antigen disparity
Engraftment	C57BL/6-CD45.1 (H2 ^b)	None	C57BL/6 (H2 ^b)	Congenic
Engraftment	C57BL/6-CD45.1 (H2 ^b)	None	BALB.B (H2 ^b)	MHC matched
Engraftment	C57BL/6-CD45.1 (H2 ^b)	None	BALB/c (H2 ^d)	MHC mismatched
Infection	C57BL/6-CD45.1 (H2 ^b)	C57BL/Ka-Thy1.1 (H2 ^b)	BALB.B (H2 ^b)	MHC matched
Infection	C57BL/6-CD45.1 (H2 ^b)	B10.D2.Thy1.1 (H2 ^d)	BALB/c (H2 ^d)	MHC mismatched

NOTE. CMP, common myeloid progenitor, GMP, granulocyte-monocyte progenitor; MHC, major histocompatibility complex.

cells and T cell-mediated immunity in the control of *A. fumigatus* infection. Thus, profound multilineage hematopoietic system injury, such as that which occurs during HCT, renders patients particularly susceptible to invasive disease [2, 8]. In our studies, we have focused on improving the early innate immune control of *A. fumigatus* infection.

Hematopoietic progenitors committed to myeloid lineage development were recently identified in mice and humans and have been named the “common myeloid progenitors” (CMPs), “granulocyte-monocyte progenitors” (GMPs), and “megakaryocyte-erythroid progenitors” (MEPs) [9, 10]. We have demonstrated that the inclusion of congenic committed progenitor cells to the hematopoietic graft could be exploited for protection against the clinically significant pathogens *A. fumigatus* and *Pseudomonas aeruginosa* in the post-HCT setting by accelerating immune reconstitution. Congenic cotransplantation of CMPs/GMPs has been found to rapidly reconstitute a functional pool of mature myeloid cells as early as 4–6 days after transplantation [11]. Moreover, the tissue neutrophil content, and not peripheral-blood neutrophil counts, was found to correlate with protection against lethal challenge with fungal or bacterial pathogens, and depletion of myeloid cells resulted in the abrogation of this protective effect [11]. However, in allogeneic transplantation with increasing donor-host antigen disparities, the barrier to engraftment increases, and graft rejection becomes an issue. Because we had demonstrated that allogeneic common lymphoid progenitors (CLPs) afford protection against cytomegalovirus infection comparable to that afforded by congenic CLPs [11–13], we predicted the protection afforded by allogeneic CMPs/GMPs would also not be major histocompatibility complex (MHC) restricted.

To determine the efficacy and feasibility of cellular therapy with CMPs/GMPs to enhance protection against infection, we assessed the ability and efficiency of CMPs/GMPs to engraft in allogeneic hosts with different donor-host MHC-antigen disparities. Of greater clinical significance, we determined the degree to which CMP/GMP protection against lethal invasive aspergillosis is MHC restricted. Finally, we studied the effects of combining CMP/GMP cellular therapy with liposomal amphotericin B (L-AmB) treatment.

MATERIALS AND METHODS

Mouse strains. All mice were bred and maintained at the animal care facility at Stanford University in accordance with approved guidelines. Donor mice were used at 6–8 weeks of age, and host mice were used at 8–14 weeks of age.

Irradiation. Lethal-dose irradiation was administered (9.5 Gy for C57BL/6 mice and 8 Gy for BALB.B or BALB/c mice) in 2 fractions, 3–4 h apart, using a 200-kV x-ray machine (Philips RT250). Mice were provided with water containing 1.1 g/L neomycin sulfate and 10⁶ U/L polymyxin B sulfate after irradiation.

Isolation of hematopoietic stem and progenitor cell populations. Cells were purified from mouse bone marrow (BM) as described elsewhere [11]. Briefly, HSCs (c-Kit⁺Thy1.1^{lo}Lin[−]Sca-1⁺) were isolated by exclusion of cells expressing lineage markers (CD3, CD4, CD5, CD8, CD45R/B220, Gr-1, Mac-1, and Ter119) and by selection of cells expressing high levels of c-Kit and Sca-1 and low levels of Thy1.1. CMPs (Lin[−]Sca-1[−]c-Kit⁺CD34⁺FcγR^{lo})/GMPs (Lin[−]Sca-1[−]c-Kit⁺CD34⁺FcγR^{hi}) were isolated by exclusion of cells expressing lineage markers (IL-7Rα-chain, CD19, IgM, Thy1.1, Ter119, Gr-1, and CD45R/B220) and Sca-1 and by selection of cells expressing high levels of c-Kit and CD34 and low or high levels of FcγR. Cell sorting was performed on a modified dual-laser fluorescence-activated cell sorter (FACS) (FACSVantage; BD Biosciences) equipped with a 488-nm argon and a 595-nm dye laser (shared user group, Stanford University).

Transplantation. Mice were anesthetized with isoflurane (Abbott Laboratories), and cells were injected into the retro-orbital venous plexus by use of 0.5-mL insulin syringes with 28-gauge needles (Applied Scientific).

Cell counts. Blood samples were analyzed by the Department of Comparative Medicine by use of a CellDYN3500 (Abbott Laboratories). Single-cell suspensions of splenocytes were stained with Turks solution and counted manually by use of a hemocytometer.

Flow-cytometric analysis. Analysis of cellular subpopulations in blood, spleen, and BM was accomplished by depleting erythrocytes via ammonium chloride lysis and then staining with various combinations of antibodies, including Texas

Table 2. Quantitative engraftment analysis of common myeloid and granulocyte-monocyte progenitors (CMPs/GMPs) on day 7 after transplantation in different donor-host pairings.

Cell type, measurement	Normal range	Mean $\pm$ SD		
		Congenic	MHC matched	MHC mismatched
Blood				
Red blood cell count, cells/ μ L ($\times 10^6$)	7–9	7 $\pm$ 3	9 $\pm$ 0	8 $\pm$ 1
Hematocrit, %	39–47	31 $\pm$ 12	40 $\pm$ 2	39 $\pm$ 3
White blood cell count, cells/ μ L	5500–9300	200 $\pm$ 310	91 $\pm$ 79	78 $\pm$ 88
Absolute neutrophil count, cells/ μ L	825–2604	125 $\pm$ 183	60 $\pm$ 47	38 $\pm$ 34
Spleen, no. of cells ($\times 10^5$)				
Total CMP/GMP derived	NA	43.9 $\pm$ 14.0	21.8 ^a $\pm$ 13.1	1.4 ^{b,c} $\pm$ 1.7
Total granulocytes	NA	26.6 $\pm$ 9.3	10.5 ^a $\pm$ 6.5	0.8 ^{b,c} $\pm$ 1.1
CMP/GMP-derived granulocytes	NA	26.6 $\pm$ 9.3	10.5 ^a $\pm$ 6.5	0.8 ^{b,c} $\pm$ 1.1
Bone marrow, % of cells				
CMP/GMP derived	NA	42.9 $\pm$ 16.8	35.3 $\pm$ 16.1	20.1 $\pm$ 10.8
Granulocytes	NA	50.4 $\pm$ 13.5	59 $\pm$ 11.7	71.8 $\pm$ 5.8
CMP/GMP-derived granulocytes	NA	77.4 $\pm$ 8.8	81.3 $\pm$ 5.1	83.6 $\pm$ 5.1

NOTE. CMP/GMP engraftment was significantly lower in major histocompatibility complex (MHC)–mismatched hosts than in congenic or MHC-matched hosts. NA, not applicable.

^a *P* value not significant, congenic vs. MHC matched.

^b *P* = .012, congenic vs. MHC mismatched.

^c *P* = .02, MHC matched vs. MHC mismatched (the significance cutoff for this analysis using the rank sum test was *P* = .017).

Red (TxR) or allophycocyanin (APC)–conjugated anti-CD45.1 (A20.1.7), TxR-conjugated anti-Mac-1 (M1/70), phycoerythrin (PE)–conjugated anti-Gr-1, fluorescein isothiocyanate (FITC)–conjugated anti-Ter119, PE-conjugated anti-MHC class II (I-A/I-E, M5/114.15.2; Pharmingen), and APC-conjugated anti-CD11c (HL3; Pharmingen). Antibody staining and washing was performed using Hanks' balanced salt solution with penicillin/streptomycin (Gibco BRL) and 2% heat-inactivated fetal bovine serum (Sigma). Cells were incubated for 20–30 min at 4°C and then washed twice. Dead cells were excluded by propidium iodide staining. FACS analysis was performed on a modified dual-laser FACS (FACSVantage; BD Biosciences).

Preparation of *Aspergillus fumigatus* conidia and infection.

A conidial suspension of *A. fumigatus* that had caused fatal sinusitis in a patient after allogeneic HCT was prepared as described elsewhere [11]. On either day 3 or day 7 after transplantation, mice were infected intravenously (iv) via the lateral tail vein with 100 conidia *A. fumigatus* in 150 μ L or intranasally with 5×10^5 conidia in 20 μ L of sterile normal saline. Infections were performed by a single individual (J.M.Y.B.) who was blinded to the experimental groups.

Administration of L-AmB. L-AmB (AmBisome; Fujisawa Healthcare) was reconstituted as per the manufacturer's specifications and diluted in 5% dextrose solution (D₅W) to obtain the desired dose. Beginning the day after infection, mice received either 5 mg/kg L-AmB or D₅W iv daily for 15 days.

Culture of target organs. Mice were killed if they exhibited clinical evidence of disease and/or >20% loss of postirradiation body weight. Surviving mice were killed on day 30 after trans-

plantation. Spleen, liver, kidneys, lungs, and brain were harvested, weighed, and then homogenized in 2 mL of Dulbecco's MEM (Gibco BRL). Tissue homogenate was plated on Sabaroud dextrose agar plates, and colonies were counted at 5, 7, and 10 days after plating.

Statistical analysis. The rank sum test was used to compare cell counts, and the log-rank test was used to compare groups in Kaplan-Meier survival analysis.

RESULTS

Allogeneic engraftment. To assess the allogeneic barrier for CMPs/GMPs, we quantitated engraftment of CMP/GMP-derived cells in 3 different donor-host strain pairings (table 1). Lethally irradiated mice received grafts containing CMPs/GMPs from congenic, MHC-matched, or MHC-mismatched donors. Reconstitution was quantitatively assessed in blood, spleen, and BM on day 7 after transplantation. Despite profound peripheral-blood neutropenia in all groups, a high degree of CMP/GMP-derived chimerism was detected in spleen and BM, where the majority of CMP/GMP-derived cells were mature granulocytes (Mac-1⁺Gr-1⁺). The remaining cells were either immature cells, erythroid cells (Ter119⁺), or cells of the dendritic cell lineage (CD11c⁺MHCII⁺). With increasing degrees of donor-host antigen disparity, the engraftment efficiency of CMPs/GMPs decreased. When compared with that in the congenic donor-host pairing, engraftment of allogeneic MHC-matched CMPs/GMPs was not significantly different. However, in the MHC-mismatched donor-host pairing, CMP/GMP engraft-

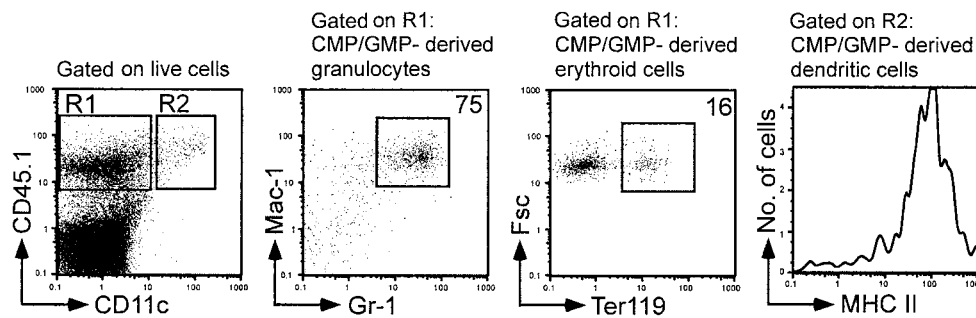


Figure 1. Qualitative engraftment analysis of common myeloid and granulocyte-monocyte progenitors (CMPs/GMPs) on day 7 after transplantation in different donor-host pairings. CMPs/GMPs gave rise to cells of the myelomonocytic, erythroid, and dendritic cell lineages, as is shown by representative fluorescence-activated cell sorting plots in spleen, with a similar distribution in the 3 donor-host mouse strain pairings tested. MHC, major histocompatibility complex.

ment in the spleen was 40-fold less efficient ($P = .012$). Nevertheless, percentages of CMP/GMP-derived cells in the BM of either allogeneic group were not significantly different, identifying the BM as an important site of myelopoiesis from CMPs/GMPs in all groups, regardless of antigen mismatch (table 2 and figure 1).

Protection against lethal *A. fumigatus* infection. The function of allogeneic CMPs/GMPs was assessed by testing their ability to protect against lethal infection with *A. fumigatus* [11]. Lethally irradiated mice received a graft of HSCs alone or HSCs with MHC-matched CMPs/GMPs. Mice were challenged with *A. fumigatus* on day 7 after transplantation. Mice that received grafts containing MHC-matched allogeneic CMPs/GMPs were protected against lethal fungal challenge, compared with mice that received grafts containing HSCs alone ($P = .02$) (figure 2A). To determine whether functional engraftment of CMPs/GMPs was compromised by the allogeneic engraftment barrier, MHC-mismatched allogeneic mice were used as donors. Again, mice that received grafts containing CMPs/GMPs from MHC-mismatched donors were protected against lethal *A. fumigatus* infection ($P < .0001$) (figure 2B). Increasing the dose of transplanted CMPs/GMPs by 4-fold did not further improve the protective effect ($P = .01$) (figure 2C).

In a separate series of experiments, the combination of MHC-mismatched allogeneic CMPs/GMPs and L-AmB treatment was tested. In this model, challenge with *A. fumigatus* on day 3 after transplantation was uniformly lethal, regardless of the cellular composition of the graft. Survival was significantly improved in both groups of mice that received grafts of HSCs alone or HSCs with CMPs/GMPs and concomitant L-AmB treatment ($P = .037$ and $P = .002$, respectively) (figure 2D). However, survival of mice receiving the combination of allogeneic CMPs/GMPs and L-AmB treatment was superior, compared with that of mice receiving L-AmB treatment without CMP/GMP transfer, although the difference did not reach statistical significance ($P = .09$) (figure 2D).

Another series of experiments addressed the question of whether CMPs/GMPs also protected mice after inhaled *A. fumigatus* infection. Inhaled infection represents the biologically more relevant route of infection, whereas the iv route permits better standardization of the inoculum. Again, intranasally infected mice transplanted with CMPs/GMPs had a significant survival advantage over mice transplanted with HSCs alone ($P = .02$) (figure 2E).

In all experiments, *A. fumigatus* was detected in spleen, kidneys, liver, brain, and lungs in mice exhibiting signs of illness. In surviving mice on day 30 after transplantation, no fungus was detected in any organ ($n = 3$ or more mice/group; data not shown). Uninfected control mice from all different graft composition groups were included in all experiments and showed 100% survival, whereas irradiated control mice died within 10–15 days after irradiation ($n = 10$ or more mice/group; data not shown).

DISCUSSION

As expected, CMP/GMP engraftment was quantitatively reduced with increasing donor-host MHC-antigen disparity. Nevertheless, CMP/GMP function, measured as protection against *A. fumigatus*, was conserved in fully allogeneic recipients. When CMPs/GMPs were compared with other early hematopoietic progenitors, studies assessing HSC engraftment in allogeneic hosts showed that 10–60-fold higher numbers of purified HSCs were needed to achieve radioprotection in MHC-mismatched hosts compared with the number required in congenic transplantation [14]. This may be explained by the different biology of the cell populations and the different time frame of the in vivo assays (short-term production of innate effector cells from CMPs/GMPs vs. sustained long-term reconstitution from HSCs). Our observations indicate that proper homing of CMPs/GMPs to their physiological niches in the BM occurs in allogeneic hosts, resulting in a rapid production of functional

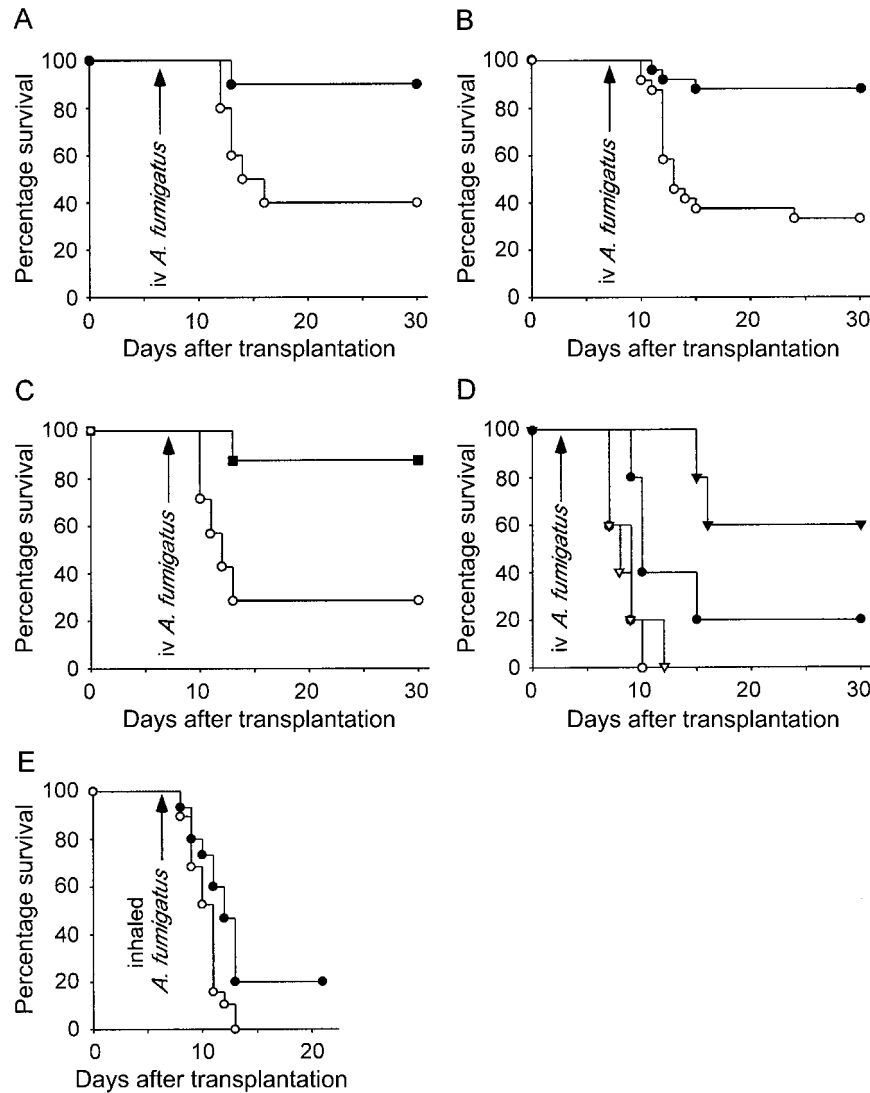


Figure 2. Protection against *Aspergillus fumigatus* infection by allogeneic common myeloid and granulocyte-monocyte progenitors (CMPs/GMPs). A–C, *A. fumigatus* infection on day 7 after transplantation. A, Major histocompatibility complex (MHC)–matched model. Hematopoietic stem cells (HSCs) are from C57BL/Ka-Thy1.1 (H2^b) mice, and CMPs/GMPs are from C57BL/6-CD45.1 (H2^b) mice. Lethally irradiated BALB.B (H2^b) mice were transplanted with 500 HSCs alone (white circles [○]; *n* = 10; 40% survival) or with 500 HSCs, 1 × 10⁴ CMPs, and 2 × 10⁴ GMPs (black circles [●]; *n* = 10; 90% survival). *P* = .02, HSCs and CMPs/GMPs vs. HSCs alone. B, MHC-mismatched model. HSCs are from B10.D2.Thy1.1 (H2^d) mice, and CMPs/GMPs are from C57BL/6-CD45.1 (H2^b) mice. Lethally irradiated BALB/c (H2^d) mice were transplanted with 500 HSCs alone (white circles [○]; *n* = 24; 33% survival) or with 500 HSCs, 1 × 10⁴ CMPs, and 2 × 10⁴ GMPs (black circles [●]; *n* = 25; 88% survival). *P* < .0001, HSCs and CMPs/GMPs vs. HSCs alone. C, MHC-mismatched model, with a high dose of CMPs/GMPs. HSCs are from B10.D2.Thy1.1 (H2^d) mice, and CMPs/GMPs are from C57BL/6-CD45.1 (H2^b) mice. Lethally irradiated BALB/c (H2^d) mice were transplanted with 500 HSCs alone (white circles [○]; *n* = 7; 26.8% survival) or with 500 HSCs, 4 × 10⁴ CMPs, and 8 × 10⁴ GMPs (black squares [■]; *n* = 8; 88% survival). *P* = .01, HSCs and CMPs/GMPs vs. HSCs alone. D, MHC-mismatched model, with *A. fumigatus* infection on day 3 after transplantation and liposomal amphotericin B (L-AmB) or 5% dextrose solution (D₅W) treatment daily for 15 days starting on day 4 after transplantation. HSCs are from B10.D2.Thy1.1 (H2^d) mice, and CMPs/GMPs are from C57BL/6-CD45.1 (H2^b) mice. Lethally irradiated BALB/c (H2^d) mice were transplanted with 500 HSCs alone with D₅W treatment (white circles [○]; *n* = 5; 0% survival) or L-AmB treatment (black circles [●]; *n* = 5; 20% survival) or were transplanted with 500 HSCs, 1 × 10⁴ CMPs, and 2 × 10⁴ GMPs with D₅W treatment (white triangles [▽]; *n* = 5; 0% survival) or L-AmB treatment (black triangles [▼]; *n* = 5; 60% survival). *P* = .037, HSCs alone with L-AmB treatment vs. with D₅W treatment; *P* = .002, HSCs and CMPs/GMPs with L-AmB treatment vs. with D₅W treatment; *P* = .09, HSCs and CMPs/GMPs with L-AmB treatment vs. HSCs alone with L-AmB treatment. E, MHC-mismatched model, with intranasal *A. fumigatus* infection on day 7 after transplantation (5 × 10⁵ cfu/mouse). HSCs are from B10.D2.Thy1.1 (H2^d) mice, and CMPs/GMPs are from C57BL/6-CD45.1 (H2^b) mice. Lethally irradiated BALB/c (H2^d) mice were transplanted with 500 HSCs alone (white circles [○]; *n* = 19; 0% survival) or with 500 HSCs, 1 × 10⁴ CMPs, and 2 × 10⁴ GMPs (black circles [●]; *n* = 15; 20% survival). *P* = .02, HSCs and CMPs/GMPs vs. HSCs alone. The log-rank test was used to compare groups in Kaplan-Meier survival analysis. iv, intravenous.

myeloid cells. This timely, limited production is sufficient to result in a durable augmentation of the myeloid tissue pool during the critical neutropenic period and results in the ability to mount an effective immune response against *A. fumigatus* regardless of whether the mice are challenged iv or intranasally.

CMPs/GMPs have been identified and isolated in human BM [10] and mobilized peripheral blood (J.M.Y.B., unpublished data). Our data support the postulation that CMP/GMP cellular therapy in patients would be more feasible and effective than granulocyte transfusions. A single infusion of CMPs/GMPs results in the sustained enrichment of myeloid cells in vivo for a period of 1–2 weeks, compared with that resulting from infusion of granulocytes that have a half-life of several hours. The therapy could be administered on a weekly schedule, without the need for daily granulocyte transfusions. Additionally, in contrast to mature granulocytes, myeloid progenitors survive freezing and thawing. More recently, we showed that a single infusion of CMPs/GMPs could overcome chemotherapy-induced neutropenia. 5-Fluorouracil (5-FU)-treated neutropenic mice transplanted with congenic CMPs/GMPs had greater protection against lethal *A. fumigatus* infection than did 5-FU-treated mice without CMP/GMP transfer [15]. Thus, the potential utility of this cellular therapy may be extended to patients with myeloid depletion due to chemotherapy or radiation. Furthermore, the observation that this protection is not MHC restricted expands the pool of donors.

Empiric or prophylactic antifungal therapy is a standard clinical practice in high-risk neutropenic patients; thus, it was important to determine the influence of early empiric antifungal therapy concomitant with this cellular therapy. In combination with L-AmB, the protective effect of CMPs/GMPs was increased as early as day 3 after transplantation. At this time point, CMP/GMP transfer alone could not rescue mice from lethal infection, most likely because mature CMP/GMP-derived cells were not yet reconstituted. Moreover, early empiric therapy alone, without the additional complement of myeloid progenitors, also failed to protect the mice. This series of experiments was not designed to compare cellular versus pharmacologic prophylaxis. The results show, however, that survival after cellular therapy exceeded that seen in the most common practice of early empiric antifungal therapy alone. Addition of L-AmB treatment could bridge the time until the expansion of the CMP/GMP-derived granulocyte population occurs.

These results demonstrate that infusion of allogeneic CMPs/GMPs after myeloablative irradiation protects mice against challenge with a lethal dose of *A. fumigatus*. The fact that this protection is not MHC restricted is of great potential clinical significance, since progenitors collected from unrelated donors, analogous to the infusion of other blood products, may be used as a cellular therapy. Furthermore, the additive effects of L-AmB

or granulocyte colony-stimulating factor with CMPs/GMPs supports a complementary role for this cellular therapy with current use of antifungal agents in neutropenic patients. In conclusion, the fact that CMP/GMP protection is not MHC restricted increases the clinical feasibility and applicability of myeloid progenitors as cellular therapy to decrease susceptibility to infections during periods of neutrophil depletion.

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