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Cytokine Expanded Myeloid Precursors Function as Regulatory Antigen-Presenting Cells and Promote Tolerance through IL-10-Producing Regulatory T Cells[1]

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The initiation of graft-vs-host disease (GVHD) after stem cell transplantation is dependent on direct Ag presentation by host APCs, whereas the effect of donor APC populations is unclear. We studied the role of indirect Ag presentation in allogenic T cell responses by adding populations of cytokine-expanded donor APC to hemopoietic grafts that would otherwise induce lethal GVHD. Progenipoietin-1 (a synthetic G-CSF/Flt-3 ligand molecule) and G-CSF expanded myeloid dendritic cells (DC), plasmacytoid DC, and a novel granulocyte-monocyte precursor population (GM) that differentiate into class II$^+$,CD80/CD86$^+$,CD40$^-$ APC during GVHD. Whereas addition of plasmacytoid and myeloid donor DC augmented GVHD, GM cells promoted transplant tolerance by MHC class II-restricted generation of IL-10-secreting, Ag-specific regulatory T cells. Importantly, although GM cells abrogated GVHD, graft-vs-leukemia effects were preserved. Thus, a population of cytokine-expanded GM precursors function as regulatory APCs, suggesting that G-CSF derivatives may have application in disorders characterized by a loss of self-tolerance. *The Journal of Immunology,* **2005, 174: 1841–1850.**

Allogeneic stem cell transplantation (SCT)[4] is currently indicated in the treatment of a number of malignant and nonmalignant diseases. However, use of the procedure is limited by its serious complications, the most common of which is graft-vs-host disease (GVHD). Recently, the use of G-CSF-mobilized stem cell grafts has improved rates of immune and hemopoietic reconstitution, reduced transplant related mortality, and improved leukemia eradication after SCT (1). The mechanism by which G-CSF alters T cell function and reduces GVHD remains controversial. G-CSF has been shown to induce Th2 differentiation in donor T cells before SCT, and this been suggested to be a major protective mechanism from GVHD in this setting (2). Although G-CSF may induce Th2 differentiation in SCT donors, similar T cell effects have not been demonstrated in SCT recipients, and we have noted general suppression of responses rather than Th2 differentiation (3). More recently, administration of G-CSF to healthy donors has been shown to increase the number of IL-10-producing T cells (4). G-CSF may also affect dendritic cells (DC) (5), monocytes (6–8), NK cells (9), and NKT cells (10); the possible effect of change in these potential immunomodulatory cells on subsequent GVHD remains unknown.

Progenipoietin-1 (ProGP-1) is a synthetic chimeric molecule that stimulates both G-CSF and Flt-3 ligand receptors and appears to be significantly more potent than the combination of both native cytokines in the expansion of stem cells and APC (11, 12). In these studies we analyzed whether the profound expansion of donor APC in response to ProGP-1 and G-CSF contributed to the suppression of donor T cell responses and the amelioration of GVHD after allogeneic SCT. Unexpectedly, we found that ProGP-1 and G-CSF expand large numbers of granulocyte-monocyte (GM) precursors that act as regulatory APC in donor animals, and these cells, rather than DC, promote transplantation tolerance by the induction of MHC class II-restricted, IL-10-producing T cells.

Materials and Methods

Mice

Female C57BL/6 (B6, H-2^b, CD45.2$^+$), B6 PTORCA Ly-5^a (H-2^b, CD45.1$^+$), B6D2F1 (H-2$^{b/d}$, CD45.2$^+$), BALB/c (H-2^d, CD45.2$^+$), and DBA/1 (H-2^q) mice were purchased from the Animal Resource Center. C57BL/6 IL-10$^{-/-}$ (B6, H-2^b, CD45.2$^+$) mice were supplied by Australian National University, and C57BL/6 class II$^{-/-}$ mice were provided by Walter and Eliza Hall Institute of Medical Research. Mice were housed in sterilized microisolator cages and received normal chow and autoclaved drinking water, which was acidified for the first 2 wk post-bone marrow transplantation.

Cytokine treatment

Recombinant human G-CSF (Amgen) or ProGP-1 (Pfizer) was diluted in 1 μg/ml murine serum albumin in PBS before injection. Mice were injected s.c. with G-CSF (10 μg/animal/day), ProGP-1 (20 μg/animal/day), or diluent once daily from day −10 to day −1.

Bone marrow transplantation

Mice were transplanted according to a standard protocol, as described previously (3). Briefly, on day −1, B6D2F1 mice received 1100 cGy of total body

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[2] J.K.W. is employed by a company (Pfizer) whose (potential) product was studied in the present work.

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[4] Abbreviations used in this paper: SCT, stem cell transplantation; DC, dendritic cell; GM, granulocyte-monocyte precursor population; GVHD, graft-vs-host disease; GVL, graft-vs-leukemia; MLC, mixed lymphocyte culture; ProGP-1, progenipoietin-1; TCD, T cell-depleted; Treg, regulatory T cell.

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irradiation (^{137}Cs source at 108 cGy/min), split into two doses separated by 3 h. Donor spleens were chopped and digested in collagenase and DNase, then whole unseparated spleen cells (10^7 or 7×10^6 T cell-depleted (TCD) splenocytes unless otherwise stated) were injected i.v. into recipients. Animals were monitored daily thereafter. In graft-vs-leukemia (GVL) experiments, grafts were cotransplanted with 4000 P815 leukemia cells of host-type DBA/2 origin (H-2^d). Animals were monitored, and the presence of hind-limb paralysis or characteristic nodular hepatosplenomegaly at postmortem was deemed a leukemic death, as previously described (13, 14). In the absence of these features of leukemia and the presence of GVHD, as defined by clinical scores >4, death was deemed to be due to GVHD.

Assessment of GVHD

The degree of systemic GVHD was assessed by a scoring system that sums changes in five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity (maximum index = 10) (15). Individual mice were graded weekly from 0–2 for each criterion without knowledge of treatment group. Animals with severe clinical GVHD (scores >6) were killed according to institutional ethical guidelines, and the day of death was deemed to be the following day.

mAbs

The following mAbs were purchased from BD Pharmingen: FITC-conjugated Gr1 (RB6-8C5), CD11b (M1/70), CD31 (390), CD45.2 (104), and IgG2a isotype control; PE-conjugated CD3 (2C11), CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD40 (3/23), CD45.1 (A20), CD45R/B220 (RA3-6B2), CD80 (16-10A1), CD86 (GL1), I-A/I-E (2G9), and IgG2b isotype control; and biotinylated CD45.1 and Gr1 and IgG2a isotype control. Streptavidin-PE-Cy5 was from DakoCytomation. Purified mAb against CD3 (KT3), CD19 (HB305), Gr1 (RB6-8C5), Thy1.2 (HO-13-4), Ter119, FcγRII/III (2.4G2), and biotinylated F4/80 were produced in-house.

Cell preparation

DC purification was undertaken as previously described (16). Briefly, low density cells were selected from digested spleen by nycodenz density gradient (1.077 g/l) centrifugation. Non-DC-lineage cells were depleted by coating with rat IgG Abs to B cells (CD19), T cells (CD3, Thy1), granulocytes (Gr-1), and erythroid cells (Ter-119). The coated cells were then removed by magnetic beads coupled to anti-rat IgG (Dynal Biotech). At the end of this procedure, 50–70% of these cell populations were DC (class II$^+$/CD11c$^+$), and 30–50% were GM cells. GM were FACS sorted (Moflo; DakoCytomation) as the negative staining population after staining with CD11c-FITC- and PE-conjugated lineage Abs (B220, CD19, and CD3). At the end of sorting, >98% of the cells were negative for CD11c and lineage markers. DC were sorted as CD11c^{high}B220$^-$ and CD11c^{dim}B220$^+$ populations to >90% purity. For T cell enrichment, after RBC lysis, splenocytes were passed over a nylon wool column and enriched to >85% purity. In CFSE experiments, purified CD45.1$^+$ T cells were incubated with 2 μM CFSE in PBS for 10 min at 37°C, washed twice in culture medium, then transplanted.

T cell depletion

Splenocytes were depleted of T cells by incubation with hybridoma supernatants containing anti-CD4 (GK1.5), CD8 (3.155), and Thy1.2 (HO-13-4) mAbs, followed by rabbit complement, as previously described (3).

Cell culture

The culture medium used throughout was 10% FCS/IMDM (JRH Biosciences). For mixed lymphocyte cultures (MLC), 10^5 nylon wool-purified BALB/c T cells were cocultured in triplicate with various numbers of DC or GM-CSF/IL-4-cultured GM, and pulsed with [^{3}H]thymidine (1 μCi/well) at the time points indicated. Proliferation was determined 16 h later on a Betaplate reader (Wallac). For MLC time-course studies, cultures were set up at a T cell:APC ratio of 10:1. For studies of GM survival and differentiation, GM were cultured for 7 days with or without added murine G-CSF, GM-CSF, or GM-CSF and IL-4 (all at 100 ng/ml). For analysis of IL-10 production, DC and GM were stimulated with LPS (1 μg/ml) and phosphorothioated CpG oligonucleotide (CpG1668; 0.5 μM) for 48 h, and supernatants were assayed for IL-10 by ELISA. For in vitro suppression assays, 5×10^4 nylon wool-purified naive C57BL/6 T cells were cultured with 2.5×10^4 irradiated (2000 cGy) B6D2F1 or B6C3F1 peritoneal F4/80$^+$ macrophages in round-bottom, 96-well plates. Sort-purified splenic T cell subsets (>95% purity; MoFlo) from transplant recipients of control spleen and GM 14 days post-SCT were added to wells as outlined in Fig. 6. After 96 h, the cultures were pulsed with [^{3}H]thymidine (1 μCi/well), and proliferation was determined 16 h later. Phagocytosis assays were performed on sort-purified GM or CD11c^{high} DC (10^6/ml), which were cultured for 18 h in the presence of GM-CSF, IL-3, and 50×10^6 latex beads/ml (1.1 μm polystyrene; Sigma-Aldrich). Cells were washed and imaged, and the frequency of cells having taken up beads and the average number of beads per cell were determined using light microscopy.

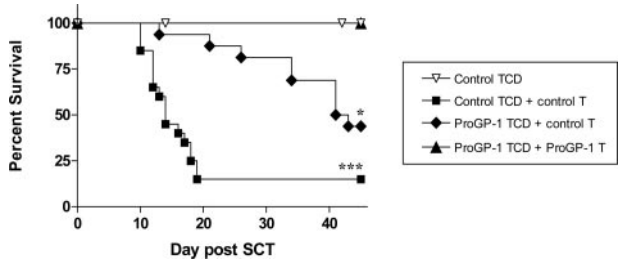


FIGURE 1. The donor non-T cell compartment of spleen from ProGP-1-treated animals regulates GVHD mortality. TCD splenocytes from control- or ProGP-1-treated animals were transplanted in combination with 3×10^6 purified T cells from ProGP-1-treated (ProGP-1 TCD + ProGP-1 T; $n = 5$) or control-treated animals (ProGP-1 TCD + control T, $n = 16$; control TCD + control T, $n = 20$). ***, $p < 0.001$, control TCD + control T vs ProGP-1 TCD + control T; *, $p < 0.05$, ProGP-1 TCD + ProGP-1 T vs ProGP-1 TCD + control T.

Cytokine assays

The Abs used in the IL-2, IFN-γ, IL-10, and IL-4 ELISAs were purchased from BD Pharmingen. All assays were performed according to the manufacturer's instructions. Detection of viable IL-10-secreting CD4 T cells was performed using a mouse IL-10 secretion assay kit (Miltenyi Biotec) as described by the manufacturer. Briefly, after 12-h stimulation with CD3 and CD28 (10 μg/ml), splenocytes were incubated with the IL-10 catch reagent (5 min at 4°C), followed by an IL-10 secretion period (45 min at 37°C). The cells were then washed and labeled with PE-conjugated anti-IL-10 mAb and FITC-conjugated CD4. Cells were analyzed on a FACScan (BD Biosciences) using 7-aminoactinomycin D to exclude dead cells.

Statistical analysis

Survival curves were plotted using Kaplan-Meier estimates and compared by log-rank analysis. The Mann-Whitney *U* test was used for statistical

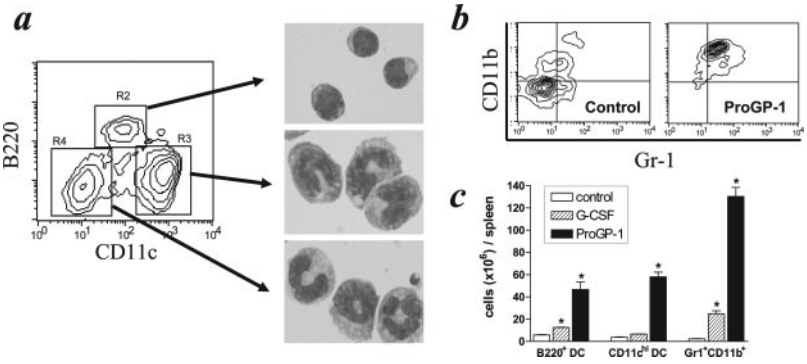


FIGURE 2. Analysis of the donor splenic APC fraction. *a*, The splenic APC fraction from ProGP-1-treated mice contains plasmacytoid DC (R2; CD11c^{dim}B220^{high}), myeloid DC (R3; CD11c^{high}), and a third CD11c$^-$ population (R4). These cells had plasmacytoid (R2) and monocytoid (R3 and R4) morphology. *b*, The CD11c$^-$ fraction was predominantly CD11b$^-$Gr-1$^-$ in control-treated donors and CD11b^{high}Gr-1^{dim} in ProGP-1-treated donors. *c*, The numbers of B220^{high} DC, CD11c^{high} DC, and Gr1$^+$CD11b$^+$ GM cells per spleen were determined after control, G-CSF, or ProGP-1 treatment ($n = 4$–5/group). Results are expressed as the mean ± SE. $p < 0.05$ for all subsets in ProGP-1 vs G-CSF and control, and for B220^{high} DC and Gr1$^+$CD11b$^+$ cells in G-CSF vs control.

analysis of cytokine data and clinical scores. A value of $p < 0.05$ was considered statistically significant.

Results

ProGP-1 pretreatment prevents GVHD in part through effects on the non-T cell compartment

We have recently reported that T cells from ProGP-1-treated animals have a reduced capacity to induce GVHD (3). To examine whether the non-T cell compartment of the ProGP-1-treated stem cell graft also had the capacity to regulate GVHD, ProGP-1 spleen was TCD and supplemented with equivalent numbers of purified T cells from control- or ProGP-1-treated donors. The majority of recipients of TCD spleen and T cells from control-treated B6 donors died by day 20. Surprisingly, TCD splenocytes from ProGP-1-treated donors reduced the GVHD mortality induced by T cells from control-treated donors (Fig. 1). As expected, T cells from ProGP-1-treated donors had a reduced capacity to induce GVHD relative to those from control-treated donors, because the recipients of both TCD spleen and T cells from ProGP-1-treated B6 donors all survived.

Low density fraction of ProGP-1 spleen contains three populations and prevents GVHD

To address whether the protection against GVHD provided by the non-T cell compartment was mediated by a ProGP-1-expanded APC fraction, APC were selected and characterized as described in

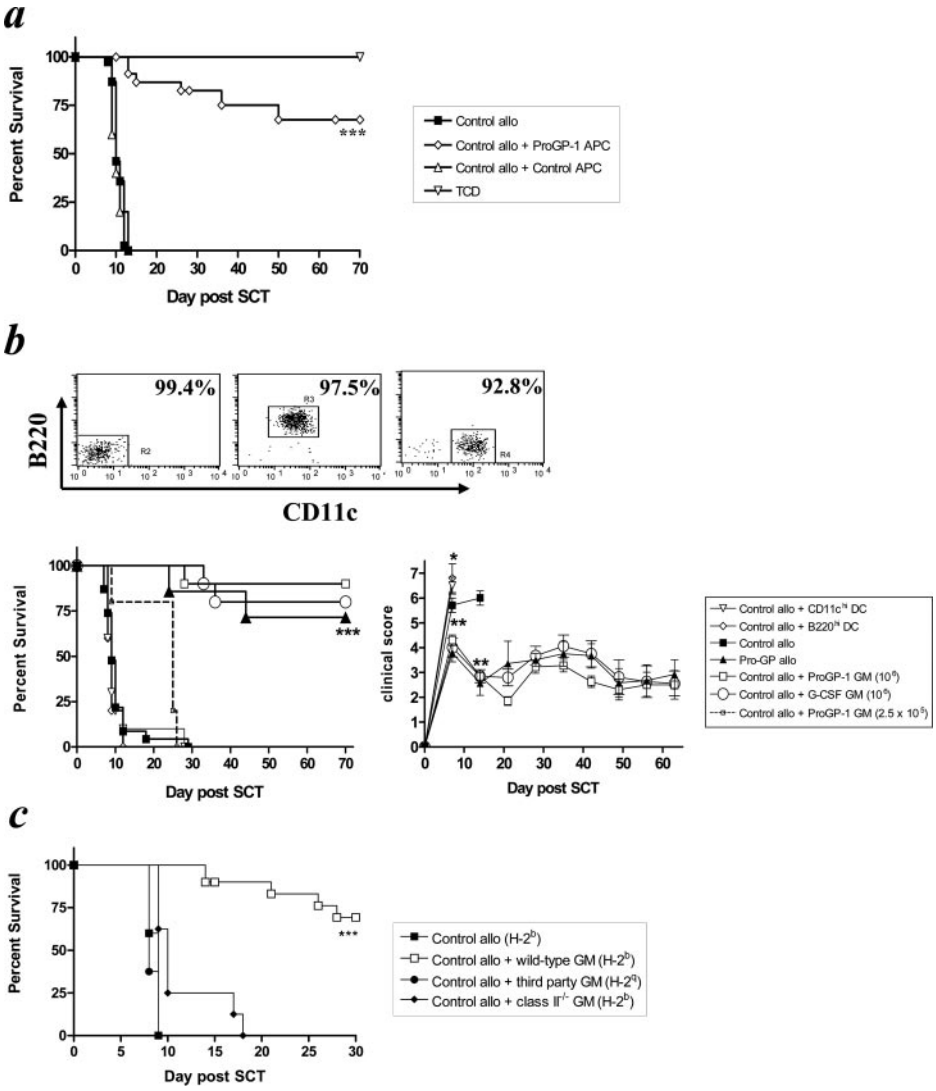


FIGURE 3. Donor GM prevent GVHD in an Ag-specific MHC class II-restricted manner. *a*, Donor APC from ProGP-1-, G-CSF-, or control-treated mice were enriched by density separation and lineage depletion, added (so that 10^6 CD11c^{high} DC were transferred) to splenocytes from control-treated allogeneic B6 animals (Control allo + ProGP-1 APC, $n = 28$; Control allo + Control APC, $n = 5$), and transplanted as described in Fig. 1. Cohorts of GVHD controls received splenocytes without APC (Control allo; $n = 39$), and non-GVHD controls received TCD allogeneic spleen (TCD; $n = 8$). ***, $p < 0.001$, Control allo + ProGP-1 APC vs Control allo and Control allo + Control APC. *b*, Representative plots of sort-purified donor CD11c$^-$B220$^-$, CD11c^{dim}B220^{high}, and CD11c^{high} APC from ProGP-1-treated mice. CD11c^{dim}B220^{high} APC ($n = 5$), 10^6 CD11c^{high} ($n = 10$), or CD11c$^-$ (GM; $n = 10^6$ or 2.5×10^5 cells) APC were added to splenocytes from control-treated allogeneic B6 animals. Cohorts of GVHD controls received unseparated splenocytes from control- or ProGP-1-treated allogeneic donors without APC (Control allo, $n = 13$; ProGP-1 allo, $n = 7$). A cohort of recipients received GM cells from G-CSF-treated donors ($n = 10$). ***, $p < 0.001$, Control allo + ProGP-1 GM and Control allo + G-CSF GM and ProGP-1 allo vs others. GVHD clinical scores were determined weekly as described in *Materials and Methods*. Results are expressed as the mean ± SE. *, $p < 0.05$, Control allo + CD11c^{high} DC and Control allo + B220^{high} DC vs others; **, $p < 0.01$, Control allo vs Control allo + ProGP-1 GM and Control allo + G-CSF GM and ProGP-1 allo. *c*, Animals received splenocytes from control-treated allogeneic B6 supplemented with 10^6 GM from ProGP-1-treated B6 (H2^b; $n = 20$), DBA/1 (H2^q; $n = 8$) or B6 class II$^{-/-}$ (H2^b; $n = 8$) mice. A cohort of GVHD controls received unseparated splenocytes from control donors without GM (Control allo; $n = 10$). ***, $p < 0.001$, Control allo + ProGP-1 B6 GM vs others.

Materials and Methods. Three distinct populations in the APC fraction of ProGP-1-treated spleen were identified: $B220^+CD11c^{dim}$, $CD11c^{high}$, and a third $CD11c^-$ population. These populations comprised 8 ± 2, 38 ± 9, and 44 ± 8% of the APC fraction, respectively (mean ± SD; $n = 5$). Morphologically, these populations exhibited features of plasmacytoid DC, myeloid DC, and GM precursors, respectively (Fig. 2*a*). The precursor population, which we henceforth refer to as GM cells, were uniformly $CD11b^{high}Gr1^{low}$ (Fig. 2*b*). The GM population was not detectable in the control-treated donors, in which the $CD11c^-$ population had the appearance of macrophages as previously described (16). The $CD11c^{high}$ DC populations in control-, G-CSF-, and ProGP-1-treated donors were morphologically and functionally identical (3, 17). The plasmacytoid DC, myeloid DC, and GM cells were expanded 10-, 30-, and 100-fold, respectively, in ProGP-1-treated donors (Fig. 2*c*) relative to control-treated donors. G-CSF resulted in a significant, but more modest, increase in these populations that was intermediate between control- and ProGP-1-treated donors. The GM cells in G-CSF-treated donors were phenotypically identical ($CD11b^{high}$ and $Gr\text{-}1^{low}$) to those in ProGP-1-treated donors, but were present at a lower frequency and absolute number in spleen (Fig. 2*c*) and peripheral blood (data not shown). When the APC fraction from ProGP-1-treated spleen was added to control allogeneic grafts that induced 100% mortality from GVHD, survival was increased to 70% (Fig. 3*a*), and recipients had only moderate clinical features of GVHD. In contrast, the APC fraction from control spleen provided no protection.

GM cells from ProGP-1- and G-CSF-treated donors prevent GVHD

To elucidate which donor APC subset provided protection from GVHD, we added highly purified cell populations from cytokine-treated donors to control grafts in the proportions present in ProGP-1-treated spleen. Both DC populations increased GVHD severity (clinical scores), whereas GM cells prevented GVHD mortality (Fig. 3*b*). The GM cells from either ProGP-1- or G-CSF-treated donors reduced GVHD clinical scores to the same extent as whole spleen from ProGP-1-treated donors. There was no difference in clinical score or survival in animals receiving GM cells from ProGP-1- or G-CSF-treated spleen, suggesting that cells were functionally equivalent from the two sources (Fig. 3*b*). Lower doses of donor GM cells (2.5×10^5) provided a significant delay in mortality ($p < 0.03$ vs control allografts), but all animals died from GVHD by day 30 (Fig. 3*b*). In additional studies, we addressed whether Ag presentation was indeed a requirement for the protection provided by GM. In these experiments we added purified GM cells from ProGP-1-treated B6 ($H2^b$), DBA/1 ($H2^q$), or B6 class $II^{-/-}$ ($H2^b$) mice to control allogeneic grafts ($H2^b$). As expected, recipients of control allogeneic grafts all died within 10 days, and addition of GM cells from $H2^b$ donors significantly reduced GVHD mortality. In contrast, neither third-party ($H2^q$) nor class $II^{-/-}$ donor-type GM provided long term protection, confirming the requirement for intact APC function for the protection against GVHD afforded by GM cells (Fig. 3*c*).

GM cells augment IL-10 production from donor T cells

GM cells were further characterized by phenotypic analysis to be negative for CD11c, F4/80, and the stem cell markers c-Kit and Sca-1. Weak CD31 expression was detected on <10% of the cells. Class II and the costimulatory molecules CD86 and CD40 were absent, whereas CD80 was weakly positive (Table I). To study the ability to induce and/or inhibit immune responses, GM cells were compared with DC as stimulators in primary MLC. GM cells induced low level proliferation and cytokine production at levels above nonstimulated T cells, but below that induced by DC (Fig. 4*a*). GM cells suppressed allogeneic T cell proliferation induced by DC, although the secretion of IL-2 (Fig. 4*a*) and IFN-γ (data not shown) by responding allogeneic T cells was not inhibited. Interestingly, although donor $CD4^+$ and $CD8^+$ T cell proliferation and expansion during GVHD were not altered in vivo by GM cells (Fig. 4, *b* and *c*), the expansion of $CD4^+CD25^+$ T cells was increased 4- to 5-fold (Fig. 4*d*). When spleen taken from allogeneic transplant recipients 7 days after SCT was stimulated in vitro with CD3 and CD28, a significant increase in IL-10 (and, to a lesser extent, IL-4) was seen in those recipients who also received GM cells (Fig. 4*e*). Furthermore, there was an 8-fold increase in IL-10-producing donor CD4 T cells 7 days after SCT when GM cells were cotransplanted with the graft (Fig. 4*f*).

Table I. *Phenotype of GM before and after differentiation*[a]

	Fresh GM	Cultured GM	Ex Vivo GM
CD11b	+++	+++	+++
Gr-1	+	−	++
F4/80	−	++	−
CD11c	−	+	−
Dec 205	−	−	+
CD3	−	−	−
CD4	−	−	−
CD8	−	−	−
B220	−	−	−
MHC class I	++	+++	+++
MHC class II	−	+	++
CD40	−	−	−
CD80	+	+	+
CD86	−	+	+

[a] GM cells were isolated from ProGP-1-treated donors and phenotyped by flow cytometry fresh, after culture for 7 days in GM-CSF and IL-4, and 7 days after allogeneic transplantation. Intensity of staining was graded as: +, <1 log: ++, 1–2 log: +++, >2 log over baseline.

GM cell induces tolerance only while in an immature state

We next studied the tolerogenic properties of GM cells in relation to their differentiation. After culture in GM-CSF (with or without IL-4) in vitro, >70% of GM cells developed the morphological and phenotypic characteristics of DC with typical veiled cytoplasm and expression of CD11c and MHC class II (Fig. 5*a* and Table I). Survival of GM in vitro was dependent on the presence of GM-CSF and was not supported by G-CSF (Fig. 5*b*). GM cells cultured in GM-CSF exhibited potent APC function, stimulating allogeneic T cell proliferation to a greater level than freshly isolated DC (Fig. 5*c*), and this was associated with rapid expression of CD40 after activation by T cells or LPS (data not shown). To analyze differentiation in vivo, GM cells were analyzed and FACS-sorted for morphological assessment 7 days after SCT (ex vivo GM). At this time point, GM cells represented <1% of total splenocytes and showed monocytic morphology and phenotypic characteristics of APC with up-regulation of MHC class I, class II, CD80, and CD86, although CD40 expression remained absent (Fig. 5*d* and Table I).

We next examined the stage of differentiation at which GM cells were able to induce tolerance. In the first instance we compared the abilities of freshly isolated GM and GM differentiated in vitro in GM-CSF and IL-4 to prevent GVHD lethality. As shown in Fig. 5*e*, freshly isolated GM, but not those differentiated in vitro, induced long term survival, suggesting that protection was afforded only when GM cells were at an immature stage of development. GM cells have the potential to differentiate into DC in vitro (Fig. 5*a*) and in vivo late after SCT (data not shown). Furthermore, the GM cell gives rise to a $CD11b^+Gr\text{-}1^-$ monocyte population in the spleen 7 days after SCT (Fig. 5*f*). To confirm that the GM cell was

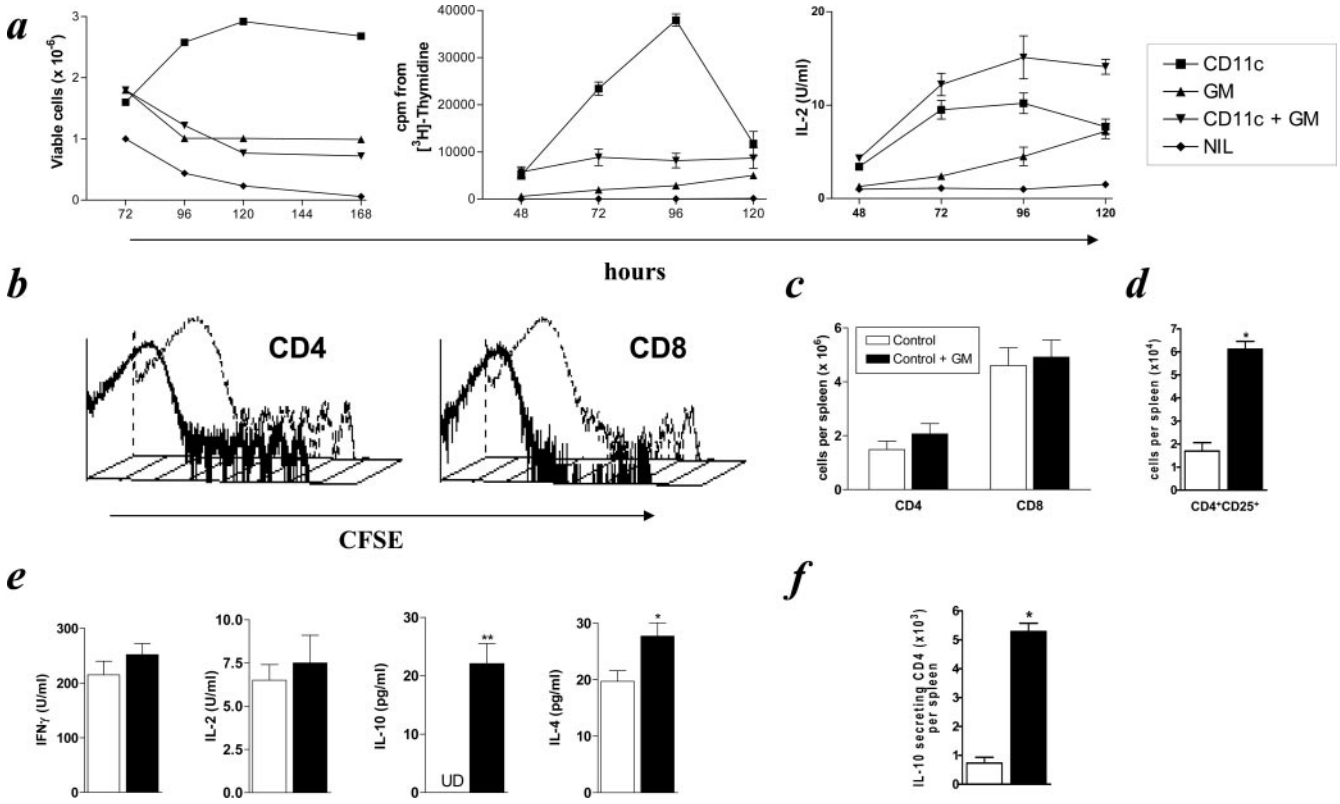


FIGURE 4. Functional analysis of GM cells. Freshly purified GM cells and CD11c^{high} DC were used in isolation or together as stimulators in primary MLC against BALB/c T cells. *a*, T cell survival, proliferation, and IL-2 generation in culture supernatants were determined at the time points indicated. No proliferation or cytokines were detected from stimulators in the absence of allogeneic T cells (data not shown). *b*, GM cells (10^6) from ProGP-1-expanded B6 spleen (CD45.2) were added to 3×10^6 purified CFSE-labeled B6 (CD45.1) T cells and transplanted into lethally irradiated B6D2F1 (CD45.2) recipients. A control group received 3×10^6 CFSE-labeled T cells only. Four days post-SCT, the proliferation of donor T cells was determined by the progressive loss of CFSE staining in the CD45.1$^+$ population. CFSE profiles of donor CD4 and CD8 T cells were identical in the presence (broken line) or the absence (solid line) of GM cells. The expansion of splenic (SP) donor CD4$^+$ and CD8$^+$ T cells (*c*) and CD4$^+$CD25$^+$ T cells (*d*) was assessed 7 days post-SCT (□, control SP; ■, control SP and GM). Results represent the mean ± SE ($n = 9$ and $n = 3$/group in *c* and *d*, respectively). ∗, $p < 0.05$. *e*, Splenocytes (SP) from individual animals were harvested 7 days after allogeneic SCT were stimulated in vitro with CD3 and CD28 (10 μg/ml each), and cytokines were determined in supernatants (□, control SP; ■, control SP and GM). Unstimulated splenocytes from either group did not produce detectable levels of IL-2, IL-10, or IL-4, whereas IFN-γ was 5.9 ± 0.2 U/ml and under the level of detection (UD) in unstimulated splenocytes from control and control plus GM groups, respectively. Results represent the mean ± SE of individual animals ($n = 5$/group). ∗∗, $p < 0.01$; ∗, $p < 0.05$. *f*, Splenocytes from individual animals 7 days after allogeneic SCT (□, control; ■, control and GM) were stimulated overnight in vitro with CD3 and CD28, and the number of IL-10-producing CD4$^+$ T cells was determined by IL-10 capture assay. Results represent the mean ± SE of IL-10-producing CD4$^+$ T cells per spleen ($n = 3$/group). ∗, $p < 0.05$.

indeed an early tri-lineage myeloid progenitor that could also differentiate into cells of the granulocyte lineage, we tracked the differentiation of GM cells into the blood 14 days after SCT. As shown in Fig. 5*g*, at this time GM cells had differentiated into Gr-1^{high} cells with high side scatter, characteristic of granulocytes. Together, these data confirm that the GM cell protects from GVHD at an early stage of differentiation and can give rise to cells of the monocyte/DC lineage and granulocyte lineage.

GM cells prevent GVHD in an IL-10- and T cell-dependent fashion

We have shown that GM cells induce IL-10 production from donor T cells early after SCT. Furthermore, GM cells, but not DC, are a major source of IL-10 in response to LPS and CpG (IL-10 production from equal numbers of GM cells vs CD11c^{high} DC, 3.7 ± 0.3 vs <0.012 ng/ml, respectively). To ascertain whether the high levels of IL-10 produced in vitro by GM cells and/or donor T cells after SCT were causally related to the protection from GVHD afforded by G-CSF and ProGP-1, we transplanted splenocytes from cytokine-treated, wild-type or IL-10$^{-/-}$ B6 donors. As expected, pretreatment of wild-type donors with ProGP-1 and, to a lesser extent, G-CSF significantly increased survival. In contrast, all recipients of splenocytes from G-CSF- and ProGP-1-treated IL-10$^{-/-}$ donors died from GVHD by day 30 (Fig. 6*a*). Recipients of splenocytes from control-treated, wild-type or IL-10$^{-/-}$ donors died at an identical rate within 10–14 days, whereas all recipients of wild-type or IL-10$^{-/-}$ TCD splenocytes survived (data not shown). These data confirm that donor pretreatment with ProGP-1 or G-CSF provides protection through IL-10-dependent inhibition of GVHD. However, IL-10 production by GM cells was not required for protection, because GM cells from ProGP-1-treated IL-10$^{-/-}$ donors provided similar protection from GVHD as those from wild-type, ProGP-1-treated donors (Fig. 6*b*). We therefore asked whether IL-10 from the donor T cell was necessary for the protection afforded by the expanded donor GM cells. In these experiments, GM cells from ProGP-1-treated, wild-type donors were added to wild-type TCD grafts supplemented with either wild-type or IL-10$^{-/-}$ T cells from control-treated donors. The protective effect of donor GM cells was lost if

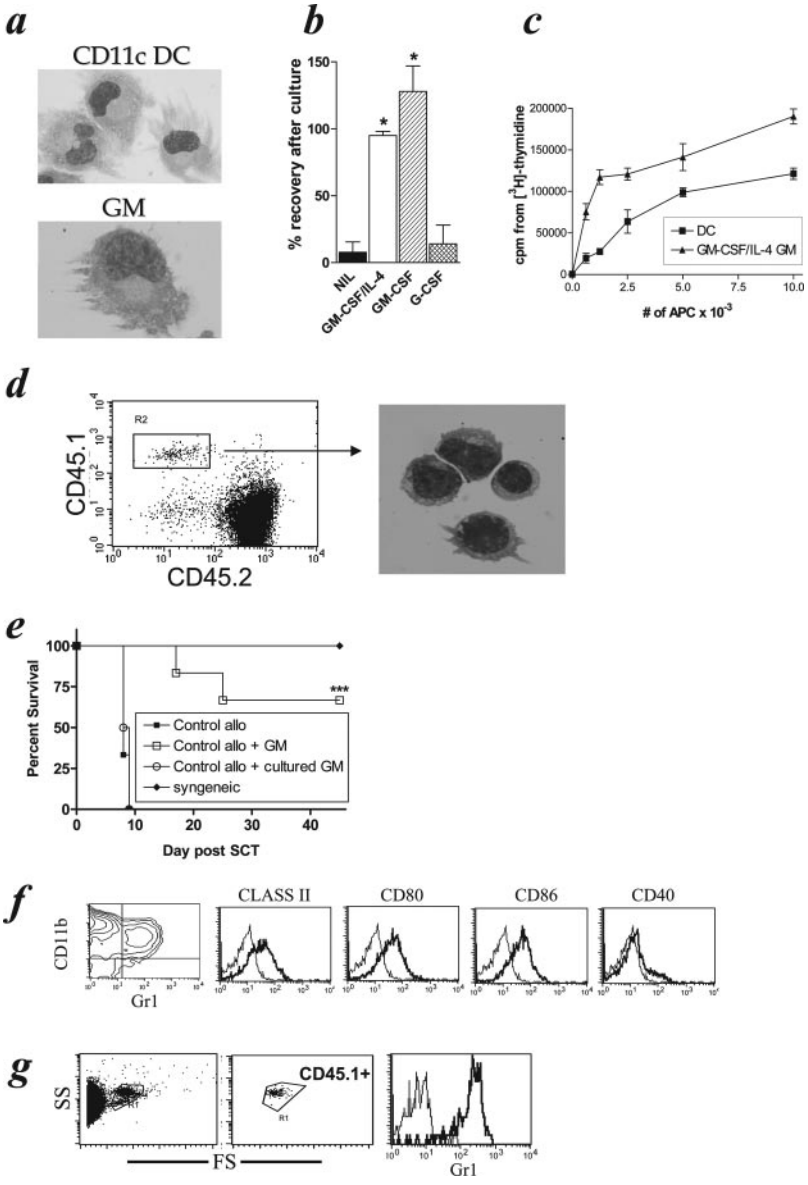


FIGURE 5. ProGP-1-expanded donor GM cells differentiate in vivo during GVHD to CD40$^-$ APC. *a*, Purified GM cells and CD11c^{high} DC from ProGP-1-treated donors were cultured in GM-CSF, and morphology was examined 7 days later. *b*, GM cells were cultured for 7–9 days in GM-CSF and IL-4, GM-CSF alone, G-CSF alone, or no cytokines. The number of viable cells at the end of culture is expressed as a percentage of the number of cells at the beginning of the culture period. *, $p < 0.05$ vs nil and G-CSF. *c*, GM cells were cultured for 7 days in GM-CSF and IL-4 and used as stimulators in a 5-day MLC against BALB/c T cells. Freshly isolated DC from naive animals were used as a positive control. *d*, GM cells from ProGP-1-expanded B6 spleen (CD45.1$^+$) were added to B6 spleen (CD45.2$^+$) and transplanted into lethally irradiated B6D2F1 recipients (CD45.2$^+$). Seven days later, CD45.1$^+$/CD45.2$^-$ cells were FACS-sorted from the spleens of transplant recipients and stained with Giemsa (magnification, ×400). *e*, Allogeneic spleen from control-treated donors (control allo; $n = 6$) was supplemented with 10^6 GM cells freshly isolated from ProGP-1-treated donors (control + GM; $n = 6$) or after culture in GM-CSF and IL-4 for 7 days (control + cultured GM; $n = 6$) as described in *a–c* and transplanted into lethally irradiated B6D2F1 recipients. Non-GVHD controls received splenocytes from control B6D2F1 donors (syngeneic; $n = 3$). ***, $p < 0.001$ vs control allo and control allo + cultured GM. *f*, Lethally irradiated B6D2F1 recipients were transplanted with splenocytes from control-treated B6 (H-2^b, CD45.2$^+$) donors plus 10^6 fresh GM (CD45.1$^+$) cells. Seven days later, the differentiated supplemental population was phenotyped based on CD45.1$^+$/CD45.2$^-$ staining. Thin line, isotype controls; thick line, specific mAb staining. *g*, GM cells (on the basis of CD45.1 expression) were phenotyped in the blood of allogeneic SCT recipients 14 days after SCT. The forward vs side scatter plots demonstrate the size characteristics of ungated peripheral blood (*left*) and back-gating on the CD45.1$^+$ population (*right*). Thin line, isotype controls; thick line, specific mAb staining.

donor T cells were unable to produce IL-10 (Fig. 6, *c* and *d*), confirming that cytokine-expanded GM cells prevent GVHD by the induction of IL-10-producing donor T cells.

GM cells induce regulatory T cells (Treg) specific for host Ag

Because the protection from GVHD afforded by GM cells is IL-10 and T cell dependent, we examined whether GM cells had other characteristics of APC. As shown in Fig. 6*e*, GM cells were highly phagocytic and took up latex beads to a greater extent than DC over a 24-h period in vitro. We next determined whether donor T cell populations expanded in the presence of GM cells had regulatory properties that were specific for host (H-2^d) Ag. To gain sufficient numbers of donor T cell populations to study in suppression assays, we FACS-purified donor T cells from the spleens of SCT recipients cotransplanted with GM cells 14 days after SCT. At this time all recipients of control grafts transplanted without GM cells had died. These purified donor T cell populations were then added in graded numbers to purified naive B6 T cells and stimulators from either B6D2F1 (H-2$^{b/d}$, host) or B6C3F1 (H-2$^{b/k}$, third-party) animals as previously described (18). As shown in Fig. 6*f*, an Ag-specific regulatory capacity was present in the CD4$^+$CD25$^+$ fraction and, to a lesser extent, in the CD4$^+$CD25$^-$ fraction (maximal suppression, 90 and 74%, respectively). As expected by the MHC class II-restricted nature of the tolerance induced by GM cells, no suppressive activity was demonstrable in the CD8$^+$ fraction.

GM cells preserve GVL effects

Donor CD8$^+$ cells are the principle effectors of leukemia eradication after allogeneic SCT (13, 14), and the absence of suppressive activity in this population after tolerance induction by GM cells suggested that GVL effects may be retained. We studied this by the addition of host-type P815 leukemia to the donor inoculum and examined leukemia clearance in allogeneic transplant recipients in the presence or the absence of donor GM cells. As shown in Fig. 7, all (100%) recipients of syngeneic splenocytes died from leukemia by day 22 after SCT. Recipients of allogeneic splenocytes died from GVHD by day 11 after SCT, before the development of leukemia (no evidence of leukemia was seen in these animals at postmortem). In contrast, 68% of recipients of allogeneic splenocytes and GM cells survived the period of observation, and only one of 19 recipients

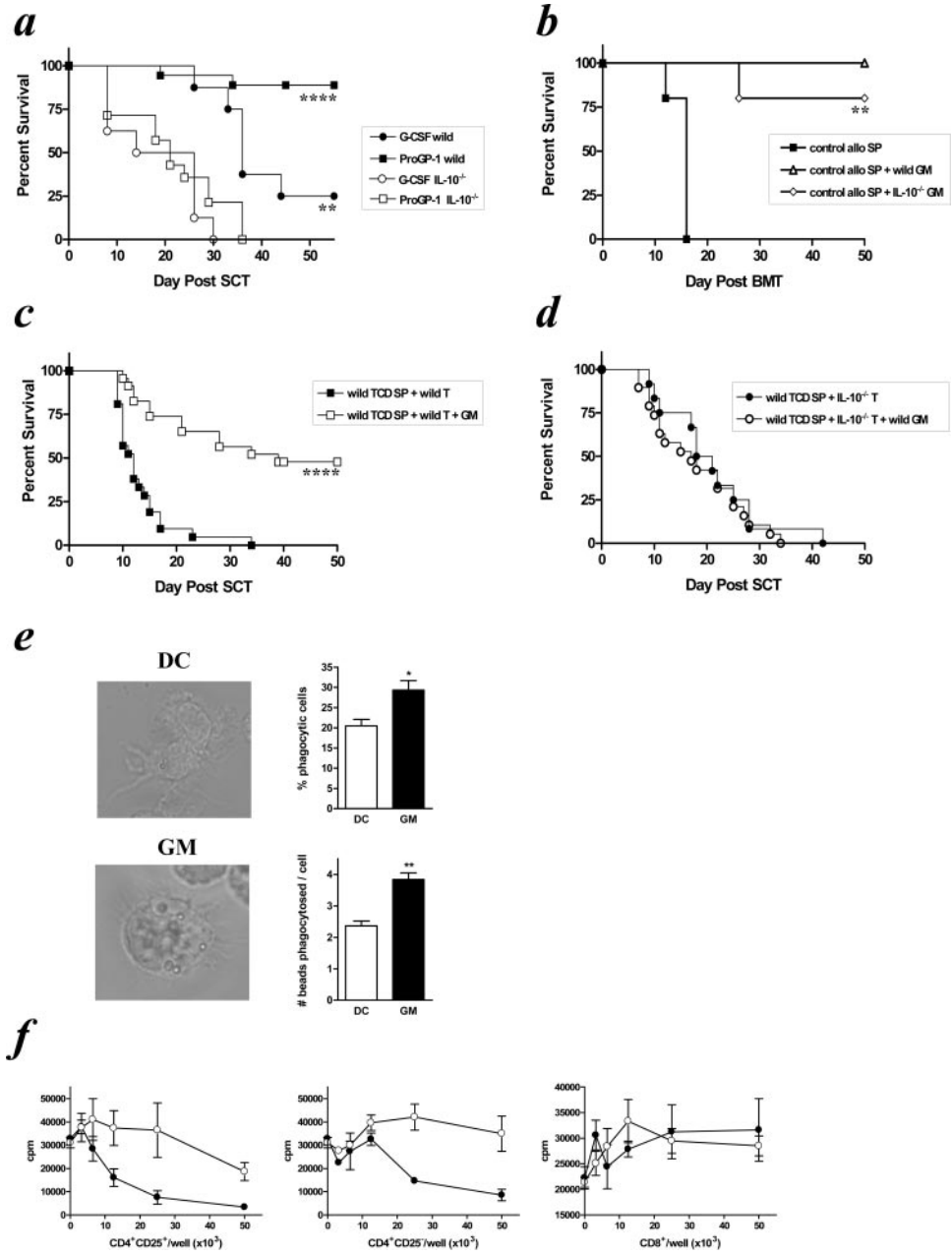
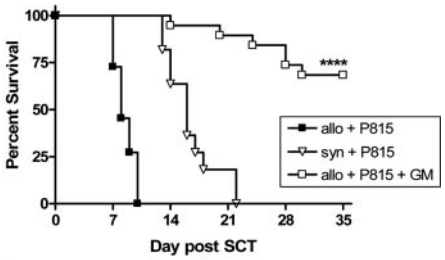


FIGURE 6. Donor GM prevent GVHD mortality in an IL-10- and T cell-dependent fashion. *a*, Donor wild-type or IL-$10^{-/-}$ B6 mice were treated with ProGP-1 or G-CSF. Splenocytes containing equal numbers of T cells were transplanted into lethally irradiated B6D2F1 animals and monitored for survival (n = 8/group in G-CSF arms; n = 14 and 17/group in ProGP-1 IL-$10^{-/-}$ and wild-type allogeneic arms, respectively). All recipients of T cell-depleted splenocytes from ProGP-1- and G-CSF-treated, wild-type and IL-$10^{-/-}$ mice survived (n = 4/group) without features of GVHD (data not shown). Data are pooled from two identical experiments. ****, $p < 0.0001$ and **, $p < 0.01$ for wild-type vs IL-$10^{-/-}$. *b*, Purified donor GM from ProGP-1-treated, wild-type or IL-$10^{-/-}$ B6 mice were added to control allogeneic splenocytes (n = 5/group). GVHD controls received allogeneic spleen from control animals only (n = 5). **, $p < 0.01$ for wild-type and IL-$10^{-/-}$ GM and control allogeneic spleen vs control allogeneic spleen alone. *c*, TCD splenocytes from control-treated, wild-type B6 mice and wild-type B6 T cells were transplanted with or without wild-type GM from ProGP-1-treated, wild-type B6 mice (n = 23 and 21/group, respectively). All recipients of TCD allogeneic spleen alone survived without evidence of GVHD (n = 8; data not shown). ****, $p < 0.0001$. *d*, TCD splenocytes from control-treated, wild-type B6 mice and IL-$10^{-/-}$ B6 T cells were transplanted with or without wild-type GM from ProGP-1-treated, wild-type B6 mice (n = 19 and 11/group, respectively). All recipients of TCD allogeneic spleen alone survived without evidence of GVHD (data not shown). No statistical difference in survival was demonstrable between groups (p = 0.46). *e*, Freshly isolated GM cells and CD11c^{high} DC were cultured overnight in the presence of GM-CSF and IL-4 and imaged (magnification, ×1000) the next morning. The number of cells that had phagocytosed latex beads (per high power field) and the number of latex beads per cell were determined in DC (□) and GM cells (■). **, $p < 0.01$; *, $p < 0.03$. *f*, Lethally irradiated B6D2F1 recipients were transplanted with allogeneic control spleen and GM cells. Donor T cells were FACS-purified 14 days after SCT as $CD4^+CD25^+$, $CD4^+CD25^-$, or $CD8^+$ cells and added in varying numbers to a MLC containing naive B6 T cells and irradiated host-type B6D2F1 (H-$2^{b/d}$; ●) or third-party B6C3F1 APC (H-$2^{b/k}$; ○). T cell proliferation was determined by [^{3}H]thymidine incorporation 4 days later.

(5%) died from leukemia compared with all 11 syngeneic recipients ($p < 0.0001$). Furthermore, there was no evidence of leukemia in long term survivors of allogeneic grafts co-transplanted with GM cells. Thus, GVL effects were preserved after the administration of GM cells despite the attenuation of GVHD.

a



b

	Overall survival	GVHD mortality	Leukemia mortality
Allo + P815	0 %	100 %	n/a
Syn + P815	0 %	0 %	100 %
Allo + P815 + GM	68 %§	26 %§	5 %§

FIGURE 7. Donor GM cells preserve GVL effects. *a*, Overall survival. Splenocytes from control-treated allogeneic (B6) donor mice were combined with host-type P815 leukemia, as described in *Materials and Methods*, and transplanted into lethally irradiated B6D2F1 animals with (allo + P815 + GM; $n = 19$) or without (allo + P815; $n = 11$) purified GM from ProGP-1-treated B6 mice. Non-GVHD controls were transplanted with splenocytes from control-treated B6D2F1 donors and the same dose of host-type leukemia (syn + P815; $n = 11$). All recipients of syngeneic splenocytes transplanted without leukemia survived the period of observation (data not shown). ****, $p < 0.0001$ for allo + P815 + GM vs allo + P815 and syn + P815. *b*, The cause of death (GVHD vs leukemia) was determined as described in *Materials and Methods*. §, $p < 0.0001$ for allo + P815 + GM vs allo + P815 (GVHD deaths) and syn + P815 (leukemia deaths). n/a, not assessable due to early GVHD mortality.

Discussion

We have shown that donor treatment with the myeloid growth factors ProGP-1 and G-CSF attenuates GVHD by expanding a $CD11b^{high}Gr\text{-}1^{low}$ regulatory APC population within the donor. The freshly isolated GM population has poor stimulatory capacity, is dependent on GM-CSF in culture for survival, and differentiates in vivo to express high levels of MHC class I and II and the costimulatory molecules CD80 and CD86, but does not express CD40. Although these APC produce high levels of IL-10, their ability to induce transplantation tolerance is instead due to the induction of IL-10-secreting, class II-restricted, Treg.

Myeloid suppressor cells have been described in a number of murine models, including chemotherapy- and radiotherapy-induced myelosuppression (19, 20), tumor-bearing mice (21, 22), and immunization (23). These cells have been described as $CD11b^{high}Gr\text{-}1^{high}CD31^{+}$ and can suppress immune responses in vitro through the production of NO (20, 24). Although GM expanded by ProGP-1 inhibited alloreactive proliferative T cell responses to DC in vitro, there was no inhibition in vivo, suggesting that NO is not a major operative mechanism responsible for the prevention of GVHD by these cells. Furthermore, NO production in vivo is known to be associated with severe lethal GVHD, and its inhibition reduces tissue damage and mortality (25). It is therefore unlikely that NO production by GM cells in these studies had any protective effect on GVHD.

A role for DCs in the development of peripheral tolerance through the induction of Treg has been supported by several recent studies. The ability of myeloid DCs to induce immunity or tolerance is linked to maturation, RelB activity, and CD40 expression (26–30). Immature DCs generated from murine BM induce T cell unresponsiveness in vitro and prolong cardiac allograft survival (29). Immature myeloid DCs induce $CD4^{+}$ Treg in vitro and $CD8^{+}$ Treg in vivo, both of which produce high levels of IL-10 and low levels of IFN-γ, but no IL-4 (26, 27). Various drugs, cytokines, and inhibitors of NF-κB have been shown to inhibit myeloid DC maturation (28, 31–35). DCs generated in the presence of these agents alter T cell function in vitro and in vivo, including promotion of allograft survival (32, 36). Interestingly, Sato et al. (37) recently described the generation of regulatory host type DC (by differentiation in the presence of IL-10) that induce transplant tolerance via the induction regulatory $CD4^{+}CD25^{+}$ T cells. These regulatory DC have a similar costimulatory phenotype to GM cells, but are required to express host MHC to promote tolerance (i.e., direct Ag presentation). We speculate that donor GM cells are less mature and retain greater Ag uptake and processing capability so that they can effectively represent host Ag in the early post-transplant period and thus operate via indirect Ag presentation. Thus, regulatory DC may be defined by the expression of MHC and CD80/CD86 in the absence of CD40 and appear capable of inducing tolerance in models of both autoimmune arthritis and GVHD (30, 37). Of importance, GM cells expanded by ProGP-1 express this regulatory phenotype in vivo, and they induce IL-10-producing Treg that prevent GVHD in an MHC class II-restricted fashion. Plasmacytoid DC can also induce regulatory T cells (38). However, GM cells are phenotypically, morphologically, and functionally distinct from this DC subset, in that they express neither CD11c nor B220 and retain pluripotent differentiation capacity. Therefore, the current data support the idea that G-CSF and ProGP-1 expand a population of $CD40^{-}$ APC with similar regulatory properties to DC generated in the presence of NF-κB inhibitors in vitro. Although IL-10 is able to inhibit NF-κB activation, and the cells secrete high levels of IL-10, this is not required for their suppressive activity, because IL-10-deficient GM also protect against GVHD. The data imply that factors intrinsic to the GM cells other than IL-10 maintain the blockade of NF-κB signaling while these cells are immature, despite the inflammatory setting of GVHD. In contrast, the expression of host Ag and CD80 and CD86 expression by maturating GM cells in vivo is probably responsible for the augmented expansion of $CD4^{+}$ Treg in a similar fashion to that described for DC (39).

The mechanism by which donor-derived GM cells induce regulatory IL-10-producing T cells is likely to involve the indirect presentation of host ($H\text{-}2^{d}$) peptides within the context of donor MHC class II, because the majority of protection afforded by GM cells was lost in the absence of donor class II (Fig. 3*c*). Thus, GM cells may present host peptides to donor T cells after transplantation in the absence of CD40-CD40L engagement. Similar APC have been previously demonstrated to induce IL-10-producing regulatory donor T cells (30, 37). The ability of CD40L inhibition to promote transplant tolerance and regulatory T cells is consistent with this concept (40). In addition, donor GM cells may alter the function of other donor or host accessory cells to promote Treg expansion. Finally, donor GM cells may also express membrane or soluble factors that act directly or indirectly to promote regulatory function in T cells. G-CSF and ProGP-1 also prevent GVHD when they are administered to the transplant donor by modulating donor T cell function before exposure to alloantigen (3). This may occur through direct effects on the T cell or indirectly via the GM cells described in this report. However, the ability of GM cells to prevent GVHD by inducing IL-10 from donor T cells that have never been exposed to G-CSF or ProGP-1 supports the hypothesis that GM cells are the major determinant of altered T cell function in response to these cytokines. Furthermore, treatment of transplant recipients (but not the donor) with G-CSF does not prevent GVHD (41), as would be expected if G-CSF were exerting direct effects primarily on the T cell. Because the administration of G-CSF after

SCT would not be expected to expand GM until hemopoietic recovery, effects on donor T cell activation may be minimal or even detrimental in this setting.

Host APC are critical in the induction of GVHD (42) and GVL (43, 44), and it has been suggested that donor NK cells may eliminate host APC and thereby prevent GVHD while also directly mediating the effects of GVL (45). Recently, donor APC have also been shown to amplify the effects of GVHD, but not GVL (44). However, the relative contributions of particular donor APC subsets to this process have not been previously described, and our data suggest that the relative ability of a donor APC population to regulate (e.g., GM cells), rather than augment, GVHD (e.g., mature DC) is a reflection of the particular APC's maturity, consequent costimulatory profile, and ability to uptake and present host Ag. Human studies have demonstrated that G-CSF expands plasmacytoid DC, which can induce Th2 differentiation in vitro (5), and this has been suggested as a mechanism by which G-CSF can prevent acute GVHD. Although murine $CD11c^{dim}/B220^{high}$ DC are also expanded after G-CSF and ProGP-1 treatment, this population of cells did not provide protection from GVHD in isolation. G-CSF is known to expand monocytes in normal donors that are capable of inhibiting T cell responses in vitro, in part through IL-10 production (7, 8). Furthermore, exogenous IL-10 is capable of ameliorating GVHD in appropriate physiological dosage (46, 47). Our data suggest that expanded GM cells within SCT donors are an important cell responsible for the promotion of transplant tolerance after stem cell mobilization with G-CSF or ProGP-1 and may be the principle means by which these cytokines separate GVHD and GVL.

Allogeneic stem cell transplantation with G-CSF-mobilized stem cells represents a major advance in the management of hemological malignancies due to the increased separation of GVHD and GVL compared with that following traditional bone marrow transplantation. Despite this advance, GVHD remains a major limitation of allogeneic SCT, and additional improvements to the safety of this procedure are required. The data presented in this report provide a novel mechanism by which myeloid growth factors prevent GVHD through the induction of IL-10-secreting T cells and provide a rationale for the optimization of cytokine administration and stem cell collection for allogeneic SCT. In addition, the data provide impetus for the study of G-CSF and ProGP-1 in autoimmune diseases and solid organ transplantation, where the induction of IL-10-secreting regulatory T cells is highly desirable in controlling disease progression and graft rejection.

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