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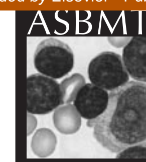

G-CSF–Mobilized Peripheral Blood Mononuclear Cells Added to Marrow Facilitates Engraftment in Nonmyeloablated Canine Recipients: CD3 Cells Are Required

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ABSTRACT

Stable mixed donor/host hematopoietic chimerism can be uniformly established in dogs conditioned with 200 cGy TBI before dog leukocyte antigen (DLA)-identical marrow transplantation and immunosuppressed with a short course of mycophenolate mofetil (MMF) and cyclosporine (CSP) after the transplantation. A further decrease in the TBI dose to 100 cGy or the elimination of MMF in this model results in graft rejection. Here we asked whether the addition of G-CSF–mobilized peripheral blood mononuclear cells (G-PBMC) to marrow grafts would enhance donor engraftment in dogs conditioned with 100 cGy TBI and given postgrafting immunosuppression with CSP alone. Using this model, 7 of 9 dogs given only marrow cells rejected their grafts within 8 to 17 weeks after transplantation. In contrast, the addition of unmodified G-PBMC to marrow grafts resulted in stable mixed donor/host chimerism in 5 of 8 dogs studied (*P* = .06). However, addition of the CD3-depleted fraction of G-PBMC, which contained both CD34 cells and CD14 cells, resulted in engraftment in only 1 of 7 recipients. We conclude that adding G-PBMC to marrow grafts replaced the requirement of MMF and 100 cGy of TBI, and that CD3 cells were required to facilitate engraftment of marrow cells in DLA-identical recipients, whereas the additional CD34 cells present in G-PBMC were not sufficient for this effect.

KEY WORDS

Nonmyeloablative transplants • CD3-depleted • G-CSF • Graft facilitating • Mixed chimerism

INTRODUCTION

Recent studies have indicated that high-risk patients receiving granulocyte colony-stimulating factor (G-CSF)–mobilized peripheral blood mononuclear cells (G-PBMCs) in place of marrow as a source of allogeneic hematopoietic stem cells after myeloablative conditioning regimens experience a survival advantage [1,2] as well as faster platelet and neutrophil recoveries [2-4]. Although the faster engraftment kinetics have been associated with increased numbers of CD34 cells, the biological basis for the survival advantage is less clear. Considerable effort is now focused on both qualitative and quantitative comparisons of these 2 stem cell sources to understand their differences and thereby optimize stem cell products.

These efforts have shown that immunobiologic properties of G-PBMC products differ from marrow grafts [5]. Studies in mice demonstrated that G-PBMC reduces severity of acute graft-versus-host disease (GVHD) [6]. T lymphocytes of dogs treated with recombinant canine G-CSF were found to be hyporesponsive when assayed in the mixed lymphocyte cultures and with concanavalin A [7]. Early reports from human studies showed that despite the approximately 1- to 2-log increase in the number of T lymphocytes present in G-PBMC products, the risk of acute GVHD was no greater with G-PBMC than with conventional HLA-identical sibling marrow grafts [8-10]. Postgrafting immunosuppression could in part explain this finding; however, in vitro studies showed that G-CSF stimulation changed the function of

T cells. Specifically, lymphocytes from G-PBMC products are polarized toward T helper 2 cells [11,12] and are hyporesponsive to alloantigen and mitogen stimulation [13,14].

Additional studies indicated that the impairment of T-cell proliferative responses was associated with decreased induction of the CD28 response complex following T-cell receptor stimulation [15]. This in turn was mediated by the large population of CD14 cells present in G-PBMC grafts [16]. The CD14 cells were found to secrete increased amounts of interleukin-10 (IL-10), which inhibits T-cell proliferation and γ interferon production. The CD14 cells also have reduced expression of HLA-DR, as well as B7.2 (CD86), a costimulatory molecule needed for T-cell activation [17]. Based on these findings, we hypothesized that G-PBMC might provide immunosuppressive effects in vivo that could inhibit both graft-versus-host and host-versus-graft reactions, thereby facilitating alloengraftment after nonmyeloablative hematopoietic stem cell transplantation.

To test this hypothesis, we used an established canine transplantation model of dog leukocyte antigen (DLA)-identical littermate marrow grafts in which the end point of transplantation, stable mixed donor/host hematopoietic chimerism, can be established after nonmyeloablative conditioning. Specifically, mixed chimerism can generally be achieved using a sublethal exposure of 200 cGy total body irradiation (TBI) before and a combination of the immunosuppressive agents mycophenolate mofetil (MMF) and cyclosporine (CSP) after marrow transplantation. Decreasing the TBI dose to 100 cGy or eliminating the MMF in this model resulted in graft rejection within 12 weeks [18]. Here, we used the canine model to ask whether the addition of G-PBMC to marrow facilitated the establishment of stable mixed chimerism after 100 cGy TBI and using only CSP postgrafting.

MATERIALS AND METHODS

Experimental Animals

Litters of random-bred dogs were raised at the Fred Hutchinson Cancer Research Center (FHCRC) or purchased from commercial kennels licensed by the US Department of Agriculture. The dogs weighed from 6.5 to 16 kg (median, 14.9 kg) and were 6 to 15 months (median, 9 months) old. Dogs were observed for disease for at least 60 days before entering the study. All were immunized for leptospirosis, papillomavirus, distemper, hepatitis, and parvovirus. Research was conducted according to the principles outlined in the *Guide for Laboratory Animal Facilities and Care* (National Academy of Sciences, National Research Council), and the Institutional Animal Care and Use Committee of the FHCRC approved the research protocol. Kennels were certified with the American Association for Accreditation of Laboratory Animal Care. Littermate donor/recipient pairs were chosen on the basis of DLA identity for highly polymorphic major histocompatibility complex (MHC) class I and class II microsatellite markers [19], as well as sequencing for canine DLA *DRB1* genes [20].

Study Design

The day of hematopoietic stem cell grafting was designated as day 0. Marrow for transplantation was collected from the donors on day –30 and cryopreserved as previously described [21]. On days –5 to 0, donors were treated by subcutaneous injection with recombinant canine G-CSF (gift from Amgen, Thousand Oaks, CA) at 10 μcg/kg per day. Leukaphereses were performed on day 0 using arteriovenous shunts and a continuous flow blood separator (CobeSPECTRA, Cobe Laboratories, Lakewood, CO) [7]. On day 0, recipients were given a single dose of 100 cGy TBI delivered at 7 cGy/min either from 2 opposing cobalt-60 sources [22] or a high-energy linear accelerator (Varian CLINAC 4, Palo Alto, CA). Both sources were calibrated to deliver 7 cGy or 9.3 R per minute and were shown to have the same biological effect on granulocyte and lymphocyte counts in dogs that did not receive transplantations. Three groups of recipients were studied. Control recipients in group 1 received marrow cells only. Recipients in group 2 received unmodified G-PBMC in addition to marrow cells. Recipients in group 3 received CD3-depleted G-PBMC in addition to marrow cells. Postgrafting immunosuppression consisted of CSP only, 15 mg/kg twice daily orally, on days –1 to 35. Blood CSP levels were measured on days 7 and 21 to ensure that adequate immunosuppression was achieved in all recipients. CSP levels were measured using the Cyclosporine Monoclonal Whole Blood Assay (Abbott Laboratories, Abbott Park, IL) involving a fluorescence polarization [23]. Dogs with poor compliance to the liquid formulation of CSP (a gift from SangStat Medical, Menlo Park, CA) were given CSP in gel capsules (Neoral, Novartis Pharmaceutical, East Hanover, NJ). All dogs were given standard postgrafting care that included twice-daily oral nonabsorbable antibiotics, neomycin sulfate and polymyxin sulfate combined with systemic enrofloxacin (Baytril), from day –5 until hematopoietic recovery from radiation nadirs occurred. The clinical status of the dogs was assessed twice daily. The end point of the study was the incidence and duration of mixed hematopoietic chimerism. Upon completion of the study, dogs were euthanized, adopted, or transferred to other studies. When euthanized, they underwent complete necropsies with histological examinations of tissue samples.

Cell Selection

Cell selection was performed using the CliniMACS system (Miltenyi Biotec, Auburn, CA). G-PBMCs were resuspended in Hank's Balanced Salt Solution supplemented with 2% heat inactivated horse serum (HBSS/2% HS) and centrifuged at 200*g* for 6 minutes to remove platelets. For CD3 selection, cells were stained with a canine-specific biotin conjugated anti-CD3ε monoclonal antibody 17.6F9 [24] (kindly provided by Dr. Peter F. Moore, UC Davis, Davis, CA) for 20 minutes at 4°C. After incubation, cells were washed with HBSS/2%HS followed by Miltenyi buffer (phosphate-buffered saline without Ca^{2+} or Mg^{2+} and 1mmol/L EDTA) supplemented with 0.5% biotin-free bovine serum albumin (Sigma, St. Louis, MO) and stained with 100 μL/10^8 cells streptavidin microbeads (Miltenyi Biotec) for 20 minutes. Cells were filtered through a 40 μm PALL filter (PALL Biomedical, Fajardo, PR) prior to being loaded onto the selection column. Depletion program 1.1 was used. Median CD3 depletion efficiency was 1.5 (1.1–2.0) log.

Table 1. *Total Mean CD34, CD3, and CD14 Cell Doses Infused in Each Group*

	Mean Cell Dose					
Group	**CD34, × 10^6/kg ± SD**	**P***	**CD3, × 10^8/kg ± SD**	**P***	**CD14, × 10^8/kg ± SD**	**P***
Group 1: control†	**5.96 ± 3.65**	**—**	**0.31 ± 0.14**	**—**	**0.26 ± 0.1**	**—**
Group 2: +G-PBMC	**7.57 ± 4.68**	**.44**	**1.86 ± 0.53**	**<.0001**	**2.20 ± 0.63**	**<.0001**
Group 3: +CD3 depleted	**12.9 ± 6.03**	**.01**	**0.25 ± 0.09**	**.34**	**1.58 ± 0.32**	**<.0001**

*Two-sample *t* test, results in groups 2 and 3 were compared to those of controls (Group 1).

†The control group received cryopreserved marrow only. Group 2 received unfractionated G-PBMC in addition to cryopreserved marrow. Group 3 received CD3-depleted G-PBMC in addition to cryopreserved marrow. All recipients received 100 cGy TBI for conditioning and were treated with cyclosporine A at a dose of 15 mg/kg orally b.i.d. from day –1 to day 35.

Cell Enumeration

Marrow and G-PBMC products were assessed for CD3, CD34, and CD14 cell content by flow cytometry FACScan (Becton Dickinson, San Jose, CA) on the day of collection. CD3 cells were stained with the fluorescein-conjugated 17.6B3 antibody (provided by Dr. Peter Moore, UC Davis, Davis, CA) that recognizes a distinct CD3 epitope from 17.6F9. CD34 cells were stained with the canine-specific 1H6 antibody [25] (generous gift of Dr. Richard Nash, FHCRC, Seattle, WA). CD14 cells were stained with unconjugated TUK4 (anti-CD14, DAKO, Carpinteria, CA), which cross-reacts with canine monocytes [26]. The absolute numbers of CD3, CD34, and CD14 cells infused were calculated by multiplying the percentage of each cell subset by the total number of nucleated cells quantified by an automated leukocyte counter (Sysmex E 2500, Kobe, Japan). The numbers of infused marrow CD3, CD14, and CD34 cells were corrected by the percentage of cells recovered after thawing.

The total numbers of cells infused from G-PBMC were adjusted in group 2 to maintain a CD3 cell dose of approximately 2×10^8/kg. In the CD3-depleted group, the primary objective was to infuse high numbers of CD34 cells. Therefore, the total cell doses infused in the CD3-depleted group were deliberately higher compared to the calculated doses of non-CD3 cells given to recipients of unfractionated G-PBMC.

Assessment of Engraftment and Chimerism

Marrow engraftment was assessed by sustained recoveries of granulocyte and platelet counts after the postirradiation nadirs, by histologic features of the marrow from biopsy specimens, and by documentation of donor cells in the marrow, lymph nodes, and granulocyte and mononuclear cell populations from the peripheral blood using polymorphic microsatellite markers in a polymerase chain reaction–based assay [27]. The assay has the sensitivity to detect donor or host cells to a level of 1% of the total cell population. Graft rejection was defined as the disappearance of donor cells and exclusive presence of host cells. Blood for chimerism analyses was obtained weekly, and the cells were fractionated by Ficoll-Hypaque gradient centrifugation.

Statistical Analyses

Data were presented as means ± 1 standard deviation. A 2-sample *t* test was used for comparing mean CD34, CD3, and CD14 cell doses among study groups. The log-rank test was used to determine statistical significance of differences between incidence and duration of mixed chimerism.

RESULTS

Cell Composition of the Grafts

Table 1 shows the mean total doses of CD34, CD3, and CD14 cells infused in the 3 groups of dogs studied. Figure 1 shows the doses of CD34, CD3, and CD14 cells given to each recipient. Dogs in group 2 (+ G-PBMC) received significantly more CD3 ($P < .0001$) and CD14 ($P < .0001$) cells than did dogs in the control group. Dogs in group 3 (CD3-depleted) received significantly more CD34 ($P = .01$) and CD14 ($P < .0001$) cells than did the control group, whereas the number of CD3 cells was similar ($P = .34$). The differences in CD3 and CD14 cell doses between the control group and study groups resulted from the addition of cells from unmodified or fractionated G-PBMC. In contrast, the CD34 cell doses were affected both by the variations in marrow CD34 cell numbers and the numbers of CD34 cells added from unmodified or fractionated G-PBMC.

Engraftment

Unmodified Viable G-PBMC Facilitated Engraftment of Marrow Cells. All 9 control dogs in group 1, given marrow only, had initial engraftment as evidenced by mixed donor/host chimerism, with donor contributions ranging from 8% to 25% at week 5 posttransplantation (Figure 2). Subsequently, 7 dogs rejected their grafts between 8 and 17 weeks and survived with complete autologous recovery, whereas 2 dogs achieved stable mixed donor/host chimerism lasting for more than 30 weeks (Table 2). All 8 recipients given unmodified G-PBMC in addition to marrow also showed initial engraftment with 30% to 80% donor granulocytes at week 5. Subsequently, 5 dogs developed stable mixed chimerism for more than 30 weeks, whereas 3 dogs rejected their grafts between 9 and 18 weeks. Log-rank comparison of the duration of mixed chimerism in the control group to that in the G-PBMC group strongly suggested that the addition of unmodified G-PBMC enhanced the development of mixed chimerism ($P = .06$).

CD3-Depleted G-PBMC Fraction Failed To Enhance Engraftment of Marrow Cells.

Seven dogs received, in addition to marrow cells, CD3-depleted G-PBMCs, which contained large numbers of CD34 and CD14 cells. All 7 dogs engrafted. Subsequently, 1 dog developed stable mixed chimerism, whereas 6 rejected

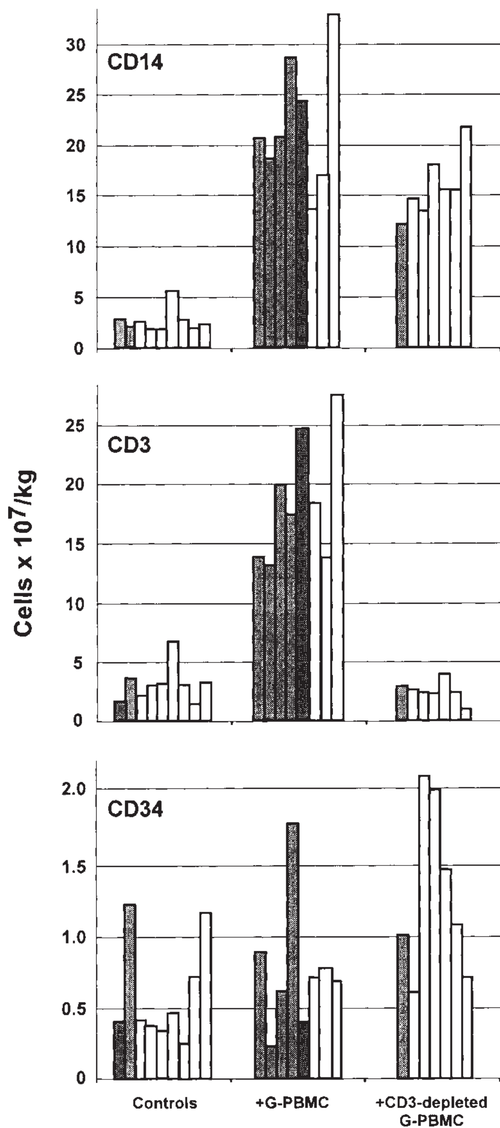

Figure 1. Total number of CD14, CD3, and CD34 cells infused into recipients. The control group received cryopreserved marrow only. The experimental groups received the indicated G-PBMC population (*x* axis) in addition to the cryopreserved marrow. Gray bars indicate dogs that remained stable mixed chimeras; white bars indicate dogs that rejected their grafts. Dogs are listed in the same order in Figures 1 and 2.

their grafts between 8 and 18 weeks after transplantation and survived with complete autologous recovery (Table 2). Comparison of the duration of mixed chimerism between the CD3-depleted and control group showed no significant difference ($P = .70$).

Donor Chimerism

In all dogs studied, the contributions of donor cells to peripheral blood cell populations were greater in the granulocyte than in the mononuclear cell fractions (Figure 2). Donor cells were seen as early as 2 weeks after transplantation, continued to increase until weeks 5 to 6 after transplantation, and then either remained stable in dogs with sustained grafts or began to decline in dogs that subsequently rejected the donor grafts. The percent of donor chimerism at 5 weeks posttransplantation did not predict the development of stable mixed chimerism when all recipients were considered together. However, percent donor chimerism greater than 10% in mononuclear cells or 40% in granulocytes was associated with stable engraftment in group 2 dogs.

DISCUSSION

Previous studies have shown that donor engraftment is achieved uniformly in dogs receiving DLA-identical marrow grafts after myeloablative conditioning without the addition of postgrafting immunosuppression [18]. In contrast, both the type and duration of immunosuppression affected donor engraftment after nonmyeloablative conditioning [18].

This study demonstrates that the composition of the stem cell graft can also affect donor engraftment in nonmyeloablated recipients. The addition of unmodified G-PBMCs to marrow cells enhanced the development of stable mixed hematopoietic chimerism tested in a stringent canine transplantation model. Interestingly, it was previously reported that viable steady-state PBMCs added to DLA-identical marrow grafts in dogs conditioned with 450 cGy did not enhance allogeneic engraftment [28]. This finding suggests that the function and consequently the effect of the G-PBMC product in vivo may indeed be different from steady-state PBMCs consistent with results from in vitro studies [7,13].

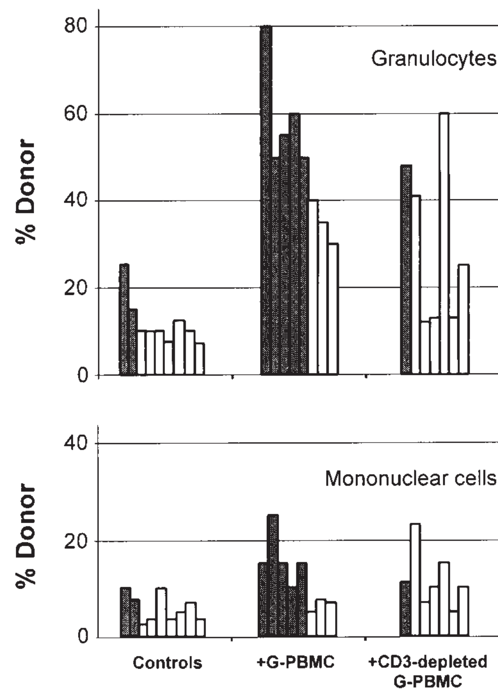

Figure 2. Degree of donor chimerism in peripheral blood granulocytes and mononuclear cells at week 5 after transplantation. Gray bars indicate dogs that remained stable mixed chimeras; white bars indicate dogs that rejected their grafts between weeks 8 and 18 after transplantation. Dogs are listed in the same order in Figures 1 and 2.

Table 2. *Duration of Mixed Chimerism in Dogs Receiving Hematopoietic Stem Cell Grafts From DLA-Identical Littermates After 100 cGy TBI and Post-transplantation Immunosuppression With CSP*

Group	No. Dogs Studied	No. Dogs with Sustained Allografts/No. Studied	Duration of Chimerism, weeks	*P**
Group 1: control†	9	2/9	8, 8, 10, 10, 11, 12, 17, >30, >37	—
Group 2: +G-PBMC	8	5/8	9, 13, 18, >30, >34, >68, >70, >70	.06
Group 3: +CD3 depleted	7	1/7	9, 10, 12, 14, 18, 18, >28	.70

*Log-rank test; results in each study group (groups 2 and 3) were compared to those of controls (group 1). For comparison between groups 2 and 3, $P = .08$.

†The control group received cryopreserved marrow only. The experimental groups received the indicated G-PBMC population in addition to the cryopreserved marrow. All recipients received 100 cGy TBI for conditioning and were treated with cyclosporine A at a dose of 15 mg/kg b.i.d. from day –1 to day 35.

In vitro studies using both canine and human cells indicated that T cells in G-PBMCs are hyporesponsive to alloantigen stimulation [7,16]. Further studies using human cells indicated that T-cell nonresponsiveness could be attributed to the large numbers of monocytes present in the G-PBMC product. These monocytes were also qualitatively different in that they secreted increased amounts of IL-10 and had decreased expression of costimulatory molecules, both of which served to reduce T-cell proliferative responses [17]. Based on these observations, we hypothesized that $CD14^+$ monocytes may facilitate engraftment by reducing both graft-versus-host and host-versus-graft reactions. However, our data indicate that in the absence of CD3 cells, the addition of G-PBMC–derived CD14 cells is not sufficient to facilitate engraftment in this model.

Dogs receiving transplants of marrow plus CD3-depleted G-PBMCs received the largest number of CD34 cells, yet only 1 of 7 of these recipients developed stable mixed chimerism. These data suggest that the larger number of CD34 cells provided by the G-PBMC product is also not sufficient for facilitating engraftment. Clearly, T cells are required; however, whether they alone are sufficient is not addressed by this study.

Clinical studies in humans have documented the role of T cells in the engraftment of allogeneic cells after myeloablative hematopoietic stem cell transplantations [29,30]. We have also shown that after myeloablative conditioning, T cell depletion of donor grafts in DLA-identical dogs was associated with graft rejection [31,32]. The results of the current study showed that the number of T cells is also important to engraftment in the nonmyeloablated recipients. It is believed that donor T cells facilitate engraftment by eliminating or suppressing host lymphocytes [33]. However, which subset of human or canine T cells contains the engraftment-facilitating cells is not yet clearly determined [34-37]. T lymphocytes capable of facilitating engraftment without causing GVHD have been identified in the mouse model as the Tc type 2 subset of $CD8^+$ cells [38,39].

In the current study, all 3 groups contained dogs that rejected their grafts and dogs that developed stable mixed chimerism. Intragroup differences in the number of CD3, CD14, and CD34 cells do not explain these findings. The results of blood CSP monitoring (data not shown) indicated that there were no significant differences in the degree of recipients' pharmacological immunosuppression. Therefore, other factors in addition to T-cell number, such as the degree of disparity in minor histocompatibility antigens or yet-undefined differences in the cell composition of the graft, may also influence donor cell engraftment in nonmyeloablated recipients.

We conclude that the cell composition of hematopoietic stem cell grafts appears to affect donor cell engraftment in nonmyeloablated canine recipients. The addition of G-PBMCs to cryopreserved marrow grafts replaced the requirement of MMF and 100 cGy of TBI to achieve stable mixed chimerism. G-CSF–mobilized T cells were required, whereas additional CD14 and CD34 cells were not sufficient.

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