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T-lymphocyte function from peripheral blood stem-cell donors is inhibited by activated granulocytes

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Background *PBSC transplant provides 10 times more T cells than BMT. However, the incidence and severity of acute GvHD is similar among recipients of both types of transplants. Studies in mouse models suggest that the similar clinical outcome in BMT and PBSCT is due to differences in the lymphokine profiles.*

Methods *PBMC, PBMC from G-CSF mobilized donors (G-PBMC) and BM mononuclear cells (BM-MC) were analyzed by flow cytometry and ELISA to detect γ-IFN and IL-4 production. Hematoxylin and eosin staining was used to identify morphology and annexin/propidium-iodide was used for apoptosis assays.*

Results *We show decreased production of γ-interferon (85%) and IL-4 (60%) in G-PBMC when compared with either PBMC or BM-MC T cells on* ex vivo *assays. Surprisingly, 85% of fresh G-PBMC is composed of low-density granulocytes (LDG), which undergo apoptosis after 48 h in culture. At this same time, γ-IFN production from G-PBMC T cell was reverted.* In vitro, *G-CSF converts granulocytes into LDGs, able to inhibit T-cell function by H_2O_2 production, and not through immune-deviation towards a Th2-type phenotype.*

Discussion *We show that the estimated numbers of Th1 and Th2 cells infused in BMT and PBSCT do not differ significantly. These findings are discussed with reference to the relatively low incidence of acute GvHD in PBSCT shown in the literature. We suggest that these results might depend on the high number of granulocytes and progenitors infused. The potential use of granulocytes as immunosuppressive short-term therapy is now being investigated by our group using a mouse experimental model.*

Keywords

GvHD, transplantation, Th1/Th2 cells, cytokines, neutrophils.

Introduction

G-CSF is widely used in transplantation for the mobilization of HPC from the BM to the peripheral blood. Recipients of allogeneic G-CSF-mobilized PBSC show a faster hematologic recovery than recipients of BM [1–9]. In PBSCT the number of transferred lymphocytes is 10 times higher than in BMT, one would therefore predict a higher incidence of acute GvHD in the former than in the latter. However, clinical studies have shown that the incidence of acute GvHD is similar in both cases, although small differences cannot be ruled out [2,4–6].

These results suggest a regulatory role for G-CSF on T-cell function — which most probably is not exerted directly, as no receptor for this growth factor has been found on T cells [10,11]. Indirect effects mediated by monocytes have been described in humans, and these cells are present in greater number in G-CSF-treated donors than in untreated controls [11–16]. Stimulation of a Th-2 response [17] by Type 2 DCs, which are present in PBMC from G-CSF mobilized donors (G-PBMC), has been postulated as a possible acute GvHD inhibitory mechanism. Others have reported [18,19] that G-CSF induces an

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immune deviation of the T-cell response towards IL-4 production (Th-2) in mouse models. Since acute GvHD has been associated with a Th-1 response, where γ-IFN is the major cytokine produced [20–23], this would suggest that the relatively low incidence of acute GvHD in patients receiving G-PBMC relates to an excess of Th-2 cells in this product. IL-4 producing cells have been shown to be unable to induce acute GvHD in experimental animals, and to inhibit Th-1 induced disease [24,25]. There is no clear evidence in humans regarding Th1/Th2 cell immune functions of G-PBMC [15,26–28] and there is only one report comparing T cells from G-PBMC with T cells from BM aspirates [28].

In this paper, we show that T-cell activity, measured as γ-IFN and IL-4 production after *ex vivo* activation with phorbol miristate acetate (PMA) and calcium ionophore is inhibited in G-PBMC when compared with BM mononuclear cells (BM-MC) or PBMC T-cells. However, this inhibition is not present when the cells are assayed after 48 h culture with or without allogeneic stimulation in an MLR. The described inhibition is exerted by the LDG, which co-purify with mononuclear cells in the G-PBMC population and die by apoptosis after 48 h *in vitro*. These LDGs are absent from BM-MC and control PBMC populations. The regulatory effects of granulocytes over T-cell function are discussed, as well as the potential use as a short-term immunossupressive therapy.

Methods

Donors and sample processing

BM, blood and apheresis samples were collected from normal volunteers after informed consent. The study was approved by the Institutional Internal Review Board (Comitê de Ética em Pesquisa – INCA). Blood samples were collected before stem-cell mobilization, and apheresis products were obtained 5 days after *in vivo* treatment with G-CSF (Filgrastim, Roche Chemical and Pharmaceutical Products, RJ, Brazil) at a dose of 16 μg/kg/day. All samples were separated by centrifugation over Ficoll density gradient, washed twice in PBS and resuspended in RPMI 1640 supplemented with 10% SFB (Gibco, MD, USA). Mixed lymphocyte cultures utilized RPMI 1640 supplemented with 10% AB human serum. All assays used freshly collected cells.

Detection of surface molecules

Annexin V FITC and anti-CD3-PerCP [all from Becton and Dickinson (B&D), CA, USA] were used. Cells were pre-incubated with PBS 5% human AB serum for 5 min at room temperature. Anti-CD3 or annexin V were added where indicated, and after 15 min the samples were washed in PBS with 5% human AB serum, resuspended in PBS 1% paraformaldehyde for cytometric analyses (Cell Quest software, Facscan, B&D).

T-cell number calculations

T cells were phenotyped as above. Numbers of T cells were calculated as the total numbers of infused nuclear cells/kg of recipient, multiplied by the percentages of CD3 positive cells, obtained by flow cytometric analyses.

Cytospin slides preparation

Cells (10^4) were cyto-centrifugated over slides, followed by hematoxylin and eosin (H&E) staining with Hemacolor Kit (Merck, RJ, Brazil) according to the manufacturer's protocols.

Intracellular lymphokine detection

Low-density cells (LDC) were plated at 2×10^6 cells/well in a 24-well plate with 20 nM PMA, 1 μM ionomycin (Calbiochem, CA, USA) and 3 μM monensin (Sigma Chemical Co, MO, USA) and cultured for 4 h at 37°C, in 5% CO_2. The cells were then stained with CD3-PerCP (B&D), γ-IFN-FITC and IL-4-PE (both from B&D). To stain the samples, LDCs were fixed with paraformaldehyde 2% at 37°C for 5 min. After washing with PBS 5% human AB serum, cells were permeabilized with saponin 0.3% and incubated with the Abs for 30 min at room temperature. After that, the samples were washed again in the presence of saponin and resuspended in PBS 1% BSA for cytometric analyses (Cell Quest software, Facscan, B&D). Specificity controls were performed as previously described [27].

Mixed lymphocyte cultures

A pool of five different EBV-transformed cells [29] treated with Mitomicin C (100 μg/mL) for 30 min at 37°C were used as stimulator cells for MLR. Responder LDCs (10^5) were cultured with 10^5 EBV-transformed cells as stimulators, or each of them alone as controls. After 48 h the supernatants were collected and stored at −80°C until use.

ELISA analyses

ELISA kits for γ-IFN and IL-4 (B&D Pharmingen, CA, USA) were used according to the manufacturer's protocols to determine cytokine production in alloreactions. Duplicates were performed of each sample.

In vitro culture with G-CSF

Total blood was obtained from healthy volunteers. High-density granulocytes (HDG) were obtained as the lower fraction of the Ficoll gradient, counted, and cultured with autologous PBMC at a ratio of 5:1, in the presence of 10 ng/mL G-CSF for 5 h at 37°C. They were then submitted to another gradient, and cells in the low-density fraction were stimulated with PMA and inonomycin for 5 h, as described above.

Catalase

Bovine catalase at a final concentration of 100 and 1000 U/mL (Sigma Chemical Co, MO, USA) was used where indicated.

Results

Production of cytokines by $CD3^+$ G-PBMC is inhibited in *ex-vivo* assay

BM-MC, PBMC and G-PBMC were separated on Ficoll density gradient and the production of γ-IFN and IL-4 was assessed by flow cytometry in *ex vivo* assays after stimulation with PMA and ionomycin in the presence of monensin for 5 h. Figure 1a shows a typical FACS profile of a donor before and after treatment with G-CSF. Approximately 20% of $CD3^+/\gamma\text{-IFN}^+$ cells and 1% of $CD3^+/IL\text{-}4^+$ cells are present in PBMC before treatment (Figure 1a, left panel). However, after treatment with G-CSF the numbers fell for both cytokines (Figure 1a, middle panels). The inhibition of cytokine production was observed in all of the 10 donors tested (Figure 1b). Interestingly, the relative numbers of $CD3^+/\gamma\text{-IFN}^+$ and $CD3^+/IL\text{-}4^+$ cells in the PBMC and BM-MC are very similar (Figure 1a, right panel). This might be due to blood contamination of BM aspirate. The observed inhibition was not dependent on the assay, since similar results were obtained by measuring the amount of secreted cytokines in supernatants, using ELISA (data not shown).

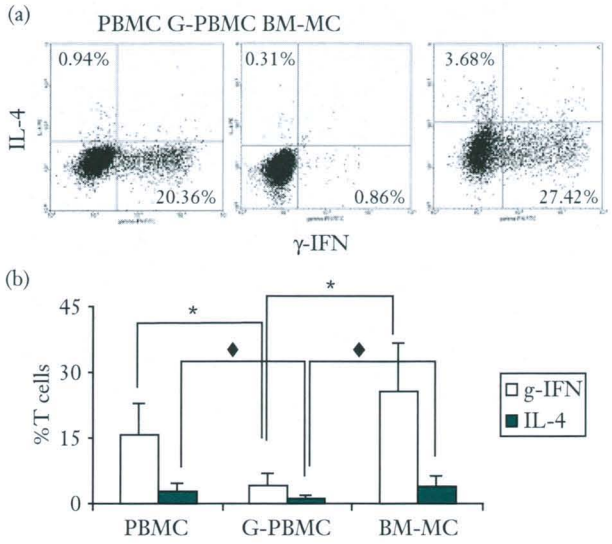

Figure 1. *G-PBMC show inhibited lymphokine production in* ex-vivo *assays. Figure 1a shows FACS profiles for IL-4 and γ-IFN production of T cells obtained from PBMC (left panel), G-PBMC (middle panel) and BM-MC (right panel). Samples were stained with anti γ-IFN FITC, anti IL-4 PE, and anti-CD3 PercP, after 5 h stimulation with PMA and ionomycin, as described above. Data shown was gated on the $CD3^+$ population. Figure 1b summarizes the results obtained from 10 different donors in the PBMC and G-PBMC group and nine for the BM-MC group. $P < 0.001$ for Th1-G-PBMC (*) and $P < 0.05$ for Th2-G-PBMC (●), compared with either PBMC or BMMC, according to Student's* t-*test*

LDG co-purify with the mononuclear cell population in G-PBMC and undergo apoptosis in *in vitro* culture

Since *in vivo* treatment with G-CSF increases the numbers of granulocytes in peripheral blood, we next asked whether there were differences in cell composition of the low density fraction of G-PBMC, PBMC and BM-MC purified by centrifugation in Ficoll density gradient. While PBMC and BM-MC are composed mainly of lymphocytes, monocytes and rare granulocytes (Figure 2a,b and Table 1), the G-PBMC population is mainly composed of LDG, which co-purify with the mononuclear fraction (Figure 2c and Table 1) diluting lymphocytes and blasts. After 48 h of culture, LDGs have the morphological appearance of dead cells (Figure 2d). In fact, > 50% of LDGs are undergoing apoptosis after 48 h, as evidenced by annexin-V binding assays (Figure 2e,f).

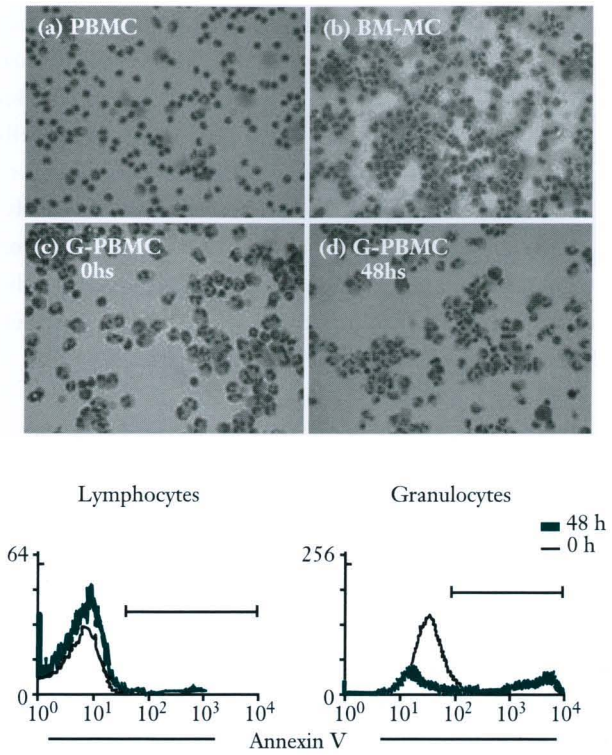

Figure 2. *G-PBMC is composed mainly of LDG, which apoptose after 48 h in culture. All samples shown were passed over a Ficoll gradient as indicated above. Figure 2a–d show cytospin slides of H&E staining, 200 X magnification. Note the difference in cell compositon of PBMC and BM-MC (mainly mononuclear cells) compared with G-PBMC before culture (mainly granulocytes). After 48 h of culture in media only, the great majority of these LDGs apoptose. The cultures were stained for annexin V FITC and PI to address cell death.* ***e and f*** *show the analysis gated on lymphocytes or granulocytes respectively. At 0 h (thin lines), < 1% of the lymphocytes and 7% of LDGs are in apoptosis. After 48 h (thick lines), 3% of the lymphocytes and 51% of the LDGs are dead. (Representative of three experiments.)*

Table 1. Differential counts obtained in the low-density fraction of BM, PB and G-PB

% Cells	BM-MC	PBMC	G-PBMC
Lymphocytes	23 ± 2.8	62 ± 3.5	3 ± 0.5
Monocytes	9.17 ± 0.3	38 ± 4.1	8 ± 0.2
Granulocytes	6.33 ± 0.1	1 ± 0.8	89 ± 3.3
Blasts	61.5 ± 3.2	0	1 ± 0.2

Cytospin slides were prepared with cells obtained from the low density fraction (Ficoll gradient) of the indicated samples. Differential counts were obtained after H&E stain.

Production of cytokines by CD3-G-PBMC T cells is not inhibited after 48 h *in vitro* assays

If LDG are the cells responsible for the inhibition of T-cell function, i.e., IL-4 and γ-IFN production, and since these cells die after a 48 h culture period, the inhibition should not be observed in assays lasting 48 h or longer. We first addressed this issue by resting the cells in culture for 48 h. Indeed, T-cell activity was recovered after this period, correlating with the death of LDGs (Figure 3). This recovery was clear for γ-IFN production, but not for IL-4.

Next, we performed MLR using alloantigen as the stimulating agent instead of polyclonal non-specific stimulation with PMA/ionomycin, and measured γ-IFN in the culture supernatant after 48 h. Proliferative assays, although very useful, are not very informative here, since in G-PBMC and BM-MC cultures, there is a large number of progenitors that grow in culture in response to T-cell cytokines (data not shown) [30,31]. The total number of T cells varies among the three different responder populations (G-PBMC, PBMC and BM-MC), we therefore expressed the MLR results as the production of γ-IFN per $CD3^+$ cell, as determined by flow cytometry, in each of the studied populations.

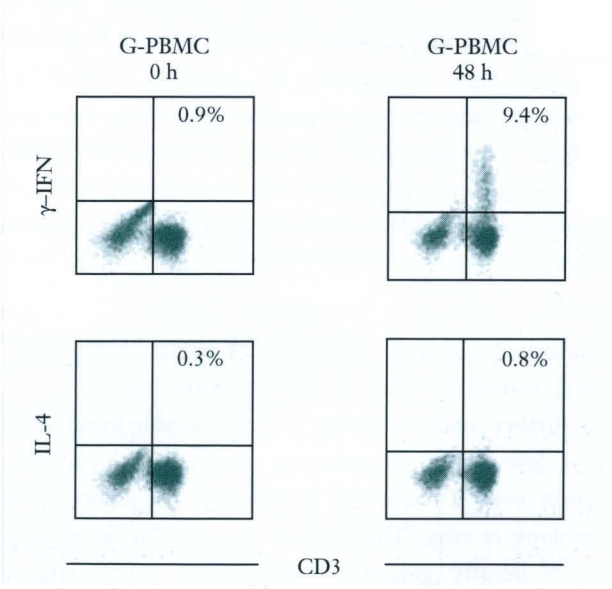

Figure 3. *T-cell activity is restored after 48 h 'rest' culture. G-PBMCs were cultured for 0 h* ***(left panels)*** *or 48 h* ***(right panels)*** *in complete medium before being assayed for lymphokine production. Samples were stained with anti γ-IFN FITC, anti IL-4 PE, and anti-CD3 PercP, as described above, after 5 h stimulation with PMA and ionomycin.*

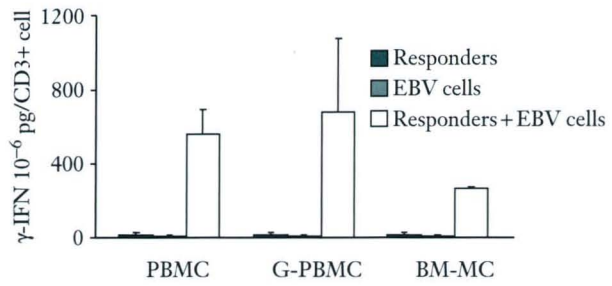

Figure 4. *G-PBMCs are not inhibited for γ-IFN production in MLRs. Cultures were performed as described above. Supernantants were harvested after 48 h and γ-IFN production determined using ELISA. Each sample was stained with CD3-PercP for T-cell counts. The amount of γ-IFN was calculated by dividing the total amount of lymphokines measured by the T-cell number in each culture. Data shown are the mean values for four different donors in each group, and the results are not significantly different: $P > 0.05$.*

Figure 4 shows the results of such experiment. γ-IFN production in response to alloantigenic stimulation is not different in the three groups. These results agree with flow cytometry data, where the frequency of γ-IFN producers, as well as the amount of cytokine produced per T cell, was basically the same in the three groups studied (data not shown). However, if total amounts of γ-IFN is scored, cells from G-PBMC seem to be low producers (data not shown), since the numbers of lymphocytes in this sample are much lower than in BM–MC or PBMC (Figure 2 and Table 1). These results indicate that when long-term read-outs are used and when T cells are specifically stimulated, they can be activated and respond by producing γ-IFN. IL-4 was undetectable in all the groups tested (data not shown).

In vitro treatment with G-CSF turns HDG into T-cell inhibitory low-density granulocytes

To further confirm whether G-CSF was able to turn HDG into low-density granulocytes (LDGs) with inhibitory action over T cells, we investigated the effect of this cytokine *in vitro*. The high-density cellular fraction of a Ficoll density gradient, composed of PMN cells, was treated *in vitro* with G-CSF (10 ng/mL) for 5 h. After treatment, the cells were passed over another density gradient and the low-density fraction recovered (Figure 5a), which was morphologically very similar to G-PBMC (Figure 2c).

To investigate the inhibitory role of these *in vitro*-generated LDGs, HDG were added to PBMCs at a ratio of 5/1 and treated (or not) with G-CSF for 5 h. After another Ficoll separation, T cells were tested for γ-IFN by flow cytometry, as above. *In vitro*-generated LDGs inhibit approximately 50% of the γ-IFN production by T cells (compare Figure 5c middle panel with 5b). This inhibitory effect is very similar to the one observed in the G-PBMC T cells, and specifically dependent on action of G-CSF on the granulocytes, since HDG not treated with G-CSF did not show any inhibitory activity (Figure 5c, left panel) when compared with control T cells (Figure 5b).

The LDG inhibition of γ-IFN production by T-cells is reversed by catalase

Activated granulocytes produce hydrogen peroxide, which is known to inhibit T-cell activation [32]. We tested whether this was the case with *in vitro* and *in vivo*-generated LDGs by using catalase, which breaks H_2O_2 down into water and oxygen. Initially, high-density granulocytes were purified using Ficoll density gradient. These cells were activated *in vitro*, in the presence of G-CSF for 5 h, together with control PBMC, passed over another gradient and stimulated with PMA and ionomycin for cytometric analyses, as shown in Figure 5c. In the presence of catalase, the observed inhibition was clearly reversed (Figure 5c, right panel).

To confirm the role of granulocytes and their oxidative metabolism on T cells from G-CSF treated donors, we cultured G-PBMC in the presence of catalase. Figure 6 shows that catalase reverses the inhibition of γ-IFN production by T cells in a dose-dependent manner. These results show that G-CSF activates granulocytes, either *in vivo* or *in vitro*, turning them into LDGs which are capable of inhibiting γ-IFN production by T cells.

Equivalent numbers of IL-4 and γ-IFN-producing T cells/kg are infused in PBSCT and BMT

Our results show that G-CSF does not influence T-cell activity directly, but does so by generating a high number of activated granulocytes, which then exert an inhibitory effect on T cells. Based on the results shown in Figure 1b, we estimated the numbers of activated T cells infused in PBSCT and BMT. The relative numbers of activated cells present in BM-MC is much higher (approximately seven times for Th1 and five times for Th2 cells) than the numbers observed in G-PBMC (Figure 1b). However, the absolute number of T cells infused/kg of recipient, is much higher in PBSCT than in BMT (Figure 7a).

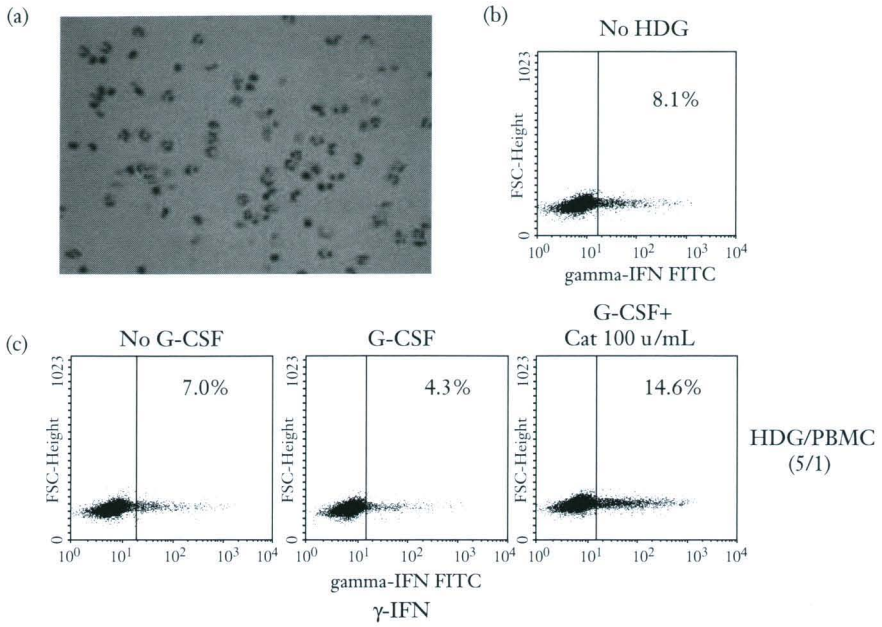

Figure 5. In vitro *activation of granulocytes with G-CSF generates LDGs, which exert an H_2O_2-dependent T-cell inhibition HDG (the lower fraction of a Ficoll gradient) were obtained from a healthy control donor. When treated with G-CSF (10 ng/mL, 5 h) they turn into low-density cells, and can be rescued on the low-density fraction of a Ficoll gradient, as shown (**5a**). High-density cells were added to control syngeneic PBMC at a 5:1 ratio without (no G-CSF) or with G-CSF (10 ng/mL) for 5 h. Cultures were submitted to another Ficoll gradient and the low-density fraction cultured as described for the* ex vivo *assays, in the absence (G-CSF) or presence (G-CSF + cat) of 100 U/mL of catalase (**5c**). Control shows the* ex vivo *assay for the PBMC (without HDG and G-CSF)(**5b**). (The data is representative of three different experiments.)*

Figure 7b shows the absolute number of lymphokine-producing T cells infused/kg of recipient, estimated as the relative number of γ-IFN or IL-4 producing T cells multiplied by the absolute T-cell count. The number of IL-4 or γ-IFN-producing T cells is of the same order of magnitude in PBSCT and BMT, showing no statistically significant difference (see legend to Figure 1). The estimates presented here provide a possible explanation for the similar clinical outcome in both cases, despite the fact that 10 times more T cells are present in the PBSCT than in BMT.

Discussion

Our results indicate an *in vivo* decrease in the number of cells producing both lymphokines, γ-IFN and IL-4, in G-PBMC when compared with PBMC using a methodology

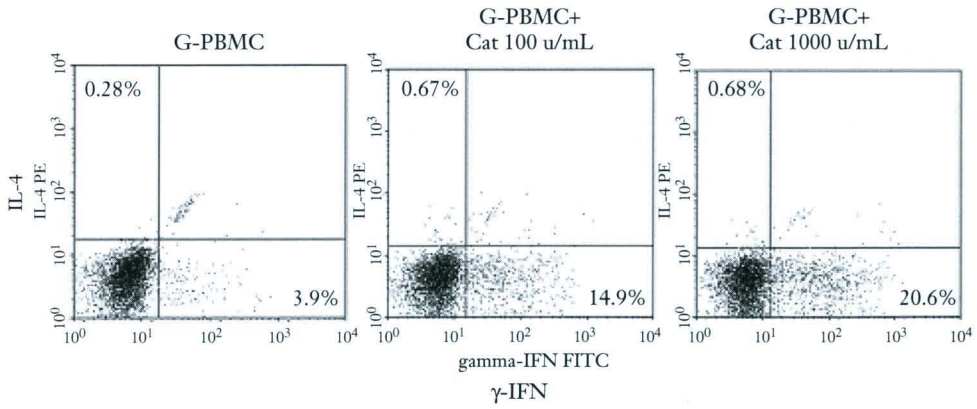

Figure 6. Ex vivo *inhibition of lymphokine production by G-PBMC T cells is reverted by catalase. G-PBMC were cultured in the absence (left panel) or presence of catalase, (100 U/mL-middle panel or 1000 U/mL-right panel). After the routine 5 h culture with PMA and ionomycin, the samples were stained for the indicated cytokines and anti-CD3. Data shown is gated on $CD3^+$ cells and is representative of three different donors.*

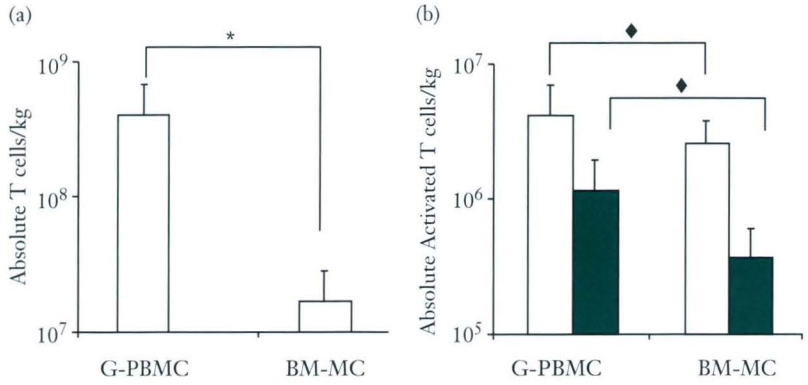

Figure 7. *Estimated numbers of activated T cells present in G-PBMC and BM-MC inoculum. Absolute numbers of T cells infused per kilogram of recipient for each product (**a**) were obtained after CD3 and CD34 staining and cytometric analysis as described. Relative numbers of γ-IFN (□)- and IL-4 (■)-producing T cells from the same donors were obtained from* ex vivo *assays with PMA and Ionomycin (Figure 1**b**) and multiplied by the absolute numbers depicted in (**a**). The result, absolute numbers of activated T cells infused per kg, is shown in (**b**). * Significant with $P < 0.05$ and ◆ is not significant.*

that provides the best estimate of the numbers of *in vivo* activated cells [23,24,33]. The data shown suggest that the inhibition occurs *in vivo*, and agree with several reports in human studies, which used different assays [11,13,15,33–35].

Inhibitory effects have been described mediated by monocytes involving IL10, IL12 or NO production [11,13–15,33]. In our case, monocytes do not seem to play a major role since reversal of T-cell function is observed in either the presence or absence of adherent cells (data not shown). Moreover, *in vivo* experimental models have shown that host APC and not donor APCs, are responsible for triggering T cells into the graft versus host reaction [36].

Analysis of the low-density fraction population purified in Ficoll gradient, which was initially assumed to be mononuclear cells, showed striking differences pending the origin of the studied populations. Control peripheral blood or BM low-density cells (which we called PBMC and BM-MC respectively) are composed mainly of lymphocytes and monocytes, with very few granulocytes. In contrast, low-density cells from G-CSF treated donors (G-PBMC) are mainly (approximately 85%) granulocytes.

Schimielau *et al.* have shown that *in vivo*-activated granulocytes inhibit *in vitro* T-cell function in a peroxide-dependent way, which can be reversed in the presence of catalase [32]. They showed the presence of LDG, which co-purify with mononuclear cells in advanced cancer patients, and attributed the immune suppression observed in this population to the presence of LDGs. Our results, although in a different model system, point in the same direction.

Reversal of T-cell function, shown as γ-IFN production, was observed after 48 h culture either in the absence of stimuli, or in the presence of allogeneic stimulation. At this same time-point, at least half of the low-density granulocytes had undergone apoptosis. Others [13,15,34,35] have shown inhibition of T-cell proliferation and lymphokine production by G-PBMC when compared with PBMC in MLRs. In fact, if we do not express our results as γ-IFN production per T cell, a decrease in this T-cell activity is observed in bulk MLR cultures (data not shown). However, IL-4 is not detected in the assays mentioned above. This could result from a higher cell sensitivity to the inhibitory effects of granulocytes, and/or reflect the fact that IL-4, besides being produced at lower levels, takes longer than γ-IFN to appear after T-cell activation [37–39]. One way or another, in our experimental system G-CSF does not enrich for Th2 cells, contrasting with the results established in the mouse models [18,19]. This immune-deviation might depend on the presence of DC2(17) cells associated with longer assays.

Despite the higher numbers of T cells infused in PBSCT in comparison to BMT, the actual numbers of active cells, from either Th1 or Th2 phenotype, do not differ between the two groups. The relatively low

incidence of acute GvHD in PBSC transplant recipients [1,6] can be explained by the proportionally small numbers of Th1 cells found within this product.

We should consider that PBSC grafts carry high numbers of activated granulocytes and also high numbers of committed myeloid precursors. When injected *in vivo*, we can postulate that the injected activated granulocytes will inhibit allo-specific T cells at the site of the GvHD target tissues to which both cell-types are recruited. On the other hand, besides inhibiting T-cell activity through the production of peroxides, granulocytes might prime T cells for apoptosis [40]. However, this last possibility seems unlikely since our *in vitro* results show only a small number of apoptotic lymphocytes in G-PBMC cultures (Figure 2f), not different from non-stimulated cultures [41,42].

Maintenance of the inhibitory effect for a short, but relevant period of time post-transplant, could result from precursors present in the G-PBMC that, once transplanted, will generate more granulocytes. The activation of these newly generated granulocytes will be mediated by inflammatory cytokines and LPS [43,44], instead of exogenous G-CSF.

Using activated granulocytes infusion or G-CSF might be useful to diminish GvHD in situations where the numbers of allogeneic T cells are not as high as in PBSCT. In fact, a recent report by Morton [5] showed that recipients of BMT whose donors received G-CSF prior to harvesting had a lower incidence of acute and chronic GvHD (G-BMT). The chronic GvHD in G-BMT is milder than with PBSCT. Since in BMT the absolute T-cell counts are already smaller than in PBSCT, the inhibitory effect of G-CSF activated granulocytes over a smaller number of T cells, could explain these results. Accordingly, a recent comparison of acute GvHD incidence in G-CSF-treated BMT donor versus untreated BMT donors showed a lower incidence and severity of acute GvHD in the former [45].

Our results open the possibility of using activated granulocytes or G-CSF as a cell-therapy tool, to inhibit undesirable immune reactions in immunodeficient hosts. We are currently investigating this possibility in experimental models.

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