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In vivo induction of T-cell hyporesponsiveness and alteration of immunological cells of bone marrow grafts using granulocyte colony-stimulating factor

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A B S T R A C T

Background and Objectives. Granulocyte colony-stimulating factor (G-CSF)-primed bone marrow (G-BM) transplantation is associated with a low incidence of graft-versus-host disease (GVHD) and has been used successfully in patients with human leukocyte antigen (HLA) matched/mismatched donors. This study evaluated the function of T cells and the quantities of immunological cells of G-BM.

Design and Methods. Bone marrow was obtained from fifteen donors by aspiration. Lymphocyte proliferation ability, interferon-γ (IFN-γ) and interleukin-4 (IL-4) secreted by T cells were determined using a monotetrazolium (MTT) assay and sandwich enzyme-linked immunosorbent assay (ELISA), respectively. T-cell subgroups, dendritic cell (DC) subsets, $CD4^+CD25^+$ regulatory T cells and the expression of CD28/CD80/CD86 molecules on monocytes, B and T cells were analyzed using flow cytometry.

Results. G-CSF treatment decreased the quantities of IFN-γ secretion dramatically (p=0.007) and IL-4 moderately (p=0.027), leading to higher ratios of IL-4/IFN-γ (p=0.004). We confirmed T-cell hyporesponsiveness and lower expression of CD28/CD80/CD86 on monocytes, B and T cells. The absolute values of lymphocytes, T cell subgroups, $CD3^+CD4^-CD8^-$ cells, $CD8^+CD28^-$ cells and B cells in bone marrow grafts were similar before and after G-CSF treatment. The number of monocytes per microliter was increased 2.13-fold, while the numbers of $CD4^+CD25^+$ regulatory T cells were unchanged. DC2 were preferentially increased.

Interpretation and Conclusions. Our results suggest that bone marrow T-cell hyporesponsiveness could be induced and that the increase of monocytes and DC2 and the down-regulation of CD28/CD80/CD86 co-stimulatory signals were produced by *in vivo* use of G-CSF; this may be related to the preferential increase of monocytes and DC2 and the down-regulation of CD28/CD80/CD86 co-stimulatory signals.

Key words: bone marrow grafts, granulocyte colony-stimulating factor, co-stimulatory molecules, dendritic cells, T cell

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Recently, granulocyte colony-stimulating factor (G-CSF)-primed bone marrow has been considered for allogeneic hematopoietic stem cell transplantation (HSCT) for patients with hematologic malignancies.[1-5] Morton *et al.* found that G-CSF-primed bone marrow transplantation caused a lower incidence of graft-versus-host disease (GVHD) than did G-CSF-mobilized peripheral blood stem cell transplantation in a human leukocyte antigen (HLA) matched transplant. A randomized study has also showed a very low incidence of grades II to IV acute GVHD when G-CSF-primed bone marrow grafts were used in HLA-matched sibling marrow transplants.[4] Our studies have demonstrated that G-CSF-primed bone marrow transplantation and G-CSF-mobilized peripheral blood grafts without T-cell depletion *in vitro* may be an innovative strategy for patients with HLA-mismatched donors.[2] Most of these data suggest that *in vivo* use of G-CSF may induce the same tolerization of alloreactive T cells in bone marrow grafts as in peripheral blood grafts.

Results from experimental model systems and clinical data indicate that G-CSF induces particular immunological changes in healthy donors. G-CSF mobilization alters the T-cell function of peripheral blood grafts and modulates the balance between Th1 and Th2 immune responses by affecting cytokine production.[6-9] After *in vivo* administration of G-CSF, human and murine peripheral blood T cells have shown reduced cytotoxic activity and proliferative response upon *in vitro* stimulation.[10] Monocytes of peripheral blood from G-CSF-mobilized human donors have also been shown to suppress T-cell alloreactiv-

ity. This suppression may occur through an interleukin-10 (IL-10) dependent mechanism,[11] downregulation of CD28/B7(CD80 and CD86) co-stimulatory molecules signals and/or through the inhibition of IL-12 and tumor necrosis factor-α (TNF-α) release.[6,11] Others also suggest that a modulation of G-CSF-primed peripheral T cells from a Th1 to a Th2 phenotype may be related to the selective mobilization of type 2 dendritic cells upon G-CSF treatment.[12] There is scant information on alteration of bone marrow immunological properties after *in vivo* G-CSF. We hypothesized that G-CSF may exert the same impact on T cells in bone marrow as those in peripheral grafts, which may contribute to overcome HLA barriers after G-CSF-primed, non-T-cell-depleted transplantation and to a lower incidence of GVHD.[2-4]

Design and Methods

G-CSF treatment of healthy donors and collection of samples

Bone marrow was obtained by aspiration from fifteen stem cell donors before treatment with recombinant G-CSF (filgrastim; Kirin Brewery Co., Tokyo, Japan) at a dosage of 5 μg/kg/day for 5 consecutive days. The reason for this is that the patients in our institute received transplants using G-CSF-primed bone marrow cells plus G-CSF-mobilized peripheral blood stem cells, which were harvested on day 4 and 5, respectively. G-CSF-primed bone marrow grafts were taken on the 4th day of treatment by aspiration prior to harvest in order to avoid contamination by peripheral blood. Bone marrow mononuclear cells (BMMC) were freshly isolated by Ficoll-Hypaque and incubated in RPMI 1640 (Gibco, BRL) supplemented with 10% fetal calf serum (FCS) (Gibco, BRL) for further analysis. The donors, 8 men and 7 women, provided informed consent and had a median age of 40 years, with a range from 13 to 65 years. Approval for this study was obtained from the Institutional Review Board of the Health Center at Peking University.

Sandwich ELISA

Freshly isolated BMMC were resuspended in RPMI 1640 medium supplemented with 10% FCS in the presence of 10 μg/mL of phytohemagglutinin (PHA, Sigma), then cultured for 48h at 37°C in a 5% CO_2 incubator. Culture supernatants were then harvested and cryopreserved at -30°C until IL-4 and IFN-γ determinations using a sandwich ELISA kit according to the manufacturer's recommendations. Both cytokines were determined simultaneously in parallel ELISA, using the same aliquot of supernatant.

Table 1. Combinations of fluorochrome-labeled monoclonal antibodies (Ab) used for immunophenotyping.

	FITC-Ab	*PE-Ab*	*PerCP-Ab*	*ABC-Ab*
CD28 expression on T cells	CD4	CD8	CD3	CD28
Dendritic cells	CD3 CD14 CD16 CD19 CD20 CD34 CD56	HLA-DR		CD11c
$CD4^+CD25^+$ cells	CD25		CD4	CD3
B7 expression on B cells	CD80	CD86	CD19	
B7 expression on monocytes	CD80	CD86		8CD14

Antibodies were purchased from Becton-Dickinson (San Jose, CA, USA) and PharMingen (San Diego, CA, USA).

Assays of T-cell proliferation

BMMC were cultured in the presence or absence of PHA 10 μg/mL at a concentration of 1×10^5 cells/well in 96-well flat-bottomed microtiter plates. Cells were cultured for 72h. Ten microliters of stock MTT solution (5 mg/mL) (Sigma, St. Louis, MO, USA) were added to each assay well.[13] The plates were incubated at 37°C for 4h and 10% sodium dodecyl sulfate (SDS) (Sigma) was added to all the wells and mixed thoroughly to dissolve the dark blue crystals. After being stored at 37°C overnight to ensure that all the crystals were dissolved, the optical density (OD) values were read on a Microllisa reader, using a test wavelength of 570 nm. The stimulation index (SI) was calculated for each individual experiment as follows:

SI=OD (T-cell responders+PHA)/OD (T-cell responders)

Flow cytometric analysis

Cells were stained without further separation to minimize selective loss. One hundred microliters of bone marrow grafts were incubated with 5~10 μL of combinations of monoclonal antibodies (mAbs) conjugated with fluorescein-isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and peridimin chlorophyll protein (Per-CP) as shown in Table 1. Red blood cells were lysed with fluorescence-activated cell sorter (FACS) solution (Becton-Dickinson). After washing in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide (NaN_3), cells were resuspended in PBS buffer and analyzed by flow cytometry with a FACScalibur system (Becton-Dickinson). A

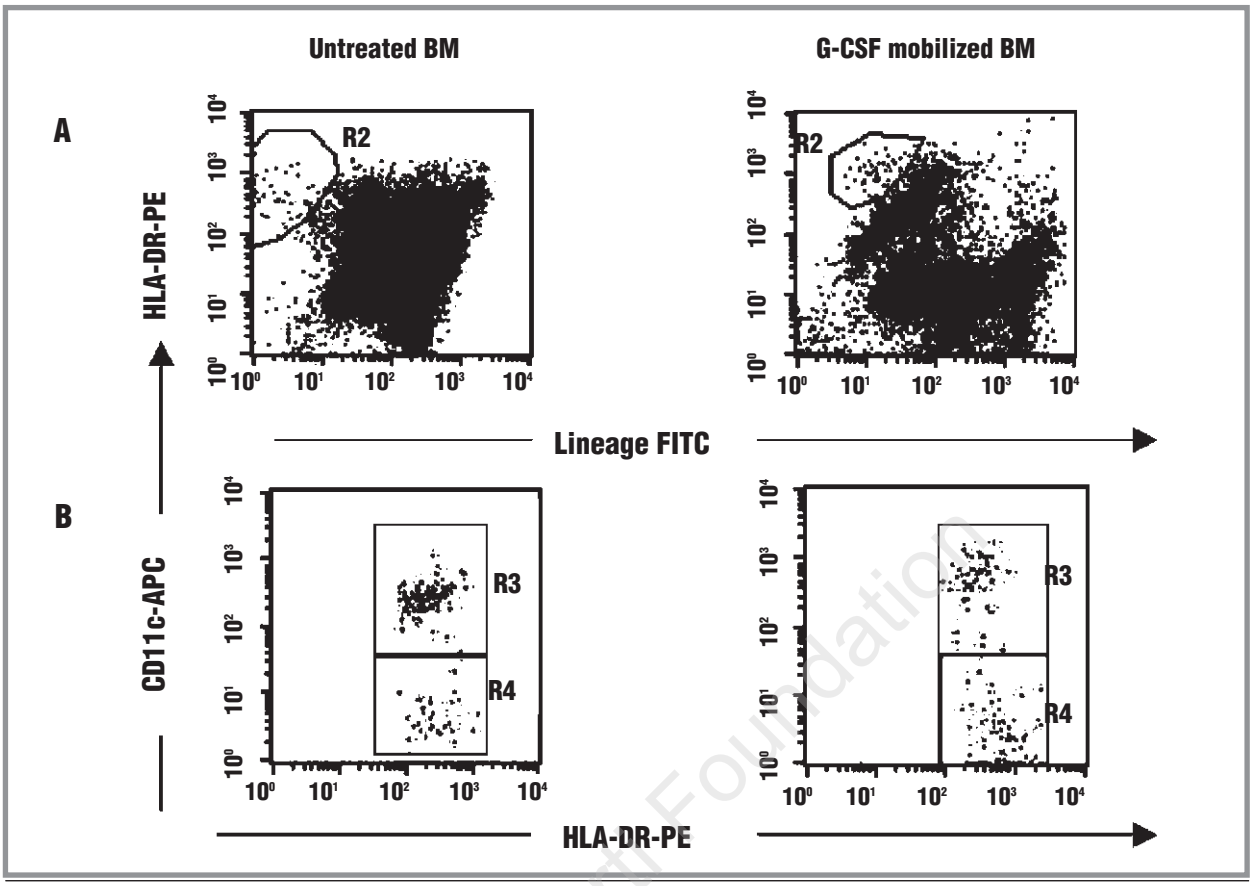

Figure 1. Phenotypic characterization of BM DC before and after G-CSF mobilization. Bone marrow grafts were obtained from the same donor before (left) and after (right) G-CSF treatment. After lysis of erythrocytes, cells were labeled with anti-HLA-DR PE, anti-CD11c-APC, and a mixture of FITC-conjugated mAbs specific for the lineage markers CD3, CD14, CD16, CD19, CD20, CD34, and CD56 expressed on lymphocytes, monocytes, neutrophils, B cells, progenitor cells and natural killer cells (lineage FITC). Cells were then analyzed by 3-color flow cytometry. (A) Dendritic cells (R2) were identified by being positive for HLA-DR and negative for lineage markers. (B) DC1 (R3) were characterized by the expression of CD11c, while DC2 (R4) were negative for CD11c.

minimum of 20,000 nuclear cell-gated events were acquired and analyzed with cellQuest software (Becton-Dickinson). Monocytes, B cells, $CD4^+CD25^+$ regulatory T-cells, T cells subgroups, dendritic cells, and CD28/B7 (CD80 and CD86) expression on T or antigen-presenting cells (APC) were identified. Appropriate isotype controls included FITC-, PE-, APC- and PerCP-conjugated IgG1/2a/2b (Becton-Dickinson). The expression of CD28/B7 costimulatory molecules was analyzed according to the percentage and the mean fluorescence intensity (MFI) of CD28/B7 in monocytes, T and B cells. The overall expression of CD28/B7 was calculated using the following formula:

overall expression = percentage expression of CD28/B7 × MFI

Dendritic cells were identified as positive for anti-HLA-DR-PE and negative for a mixture of FITC-conjugated monoclonal antibodies specific for lineage markers on lymphocytes (CD3), B cells (CD19, CD20), natural killer cells (CD16, CD56), monocytes (CD14), neutrophils (CD16) and progenitor cells (CD34). Anti-CD11c was used to identify DC1 and DC2 (Figure 1) as previously described.[12]

Statistical analysis

Biological data were summarized as the mean±standard deviation (SD). Statistical comparisons were performed using t tests for paired samples. A $p \leq 0.05$ was considered statistically significant.

Results

IL-4 and IFN-γ produced by lymphocytes in vitro after G-CSF treatment

To determine whether G-CSF could cause bone marrow T cells to produce type 1 or type 2 cytokines, we assessed the amount of type 1 (IFN-γ) and type 2 (IL-4) cytokines in the supernatants of BMMC co-cultured

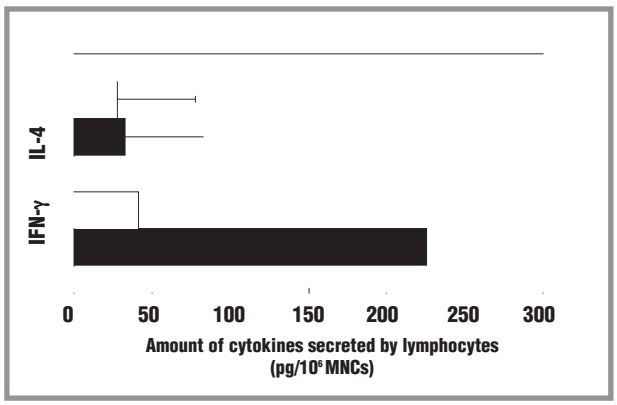

Figure 2. *In vitro* production of IL-4 and IFN-γ by PHA-stimulated lymphocytes before (dark boxes) and after (light boxes) *in vivo* G-CSF treatment (5 μg/kg/day).

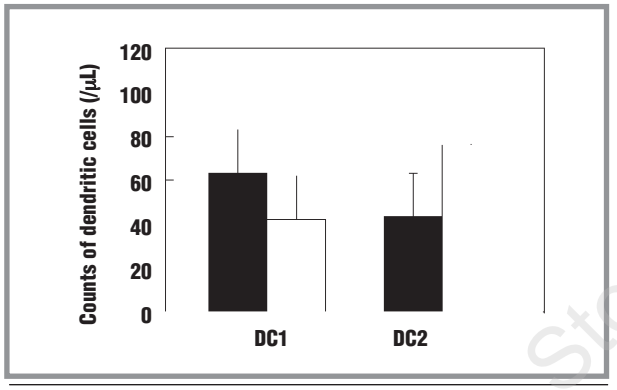

Figure 3. DC subgroups in bone marrow before (black bars) and after (white bars) G-CSF mobilization (5 μg/kg/day).

with PHA as a stimulator (n=12 experiments). The amount of IFN-γ in supernatants was significantly lower after G-CSF treatment (225.19±189.05 pg/10⁶MNC vs. 41.29±17.27 pg/10⁶ MNC; p=0.007). Furthermore, IL-4 production was also reduced (33.36±8.14 pg/10⁶ MNC vs. 27.72±3.92 pg/10⁶ MNC) (p=0.027) (Figure 2). The ratios of IL-4/IFN-γ increased significantly after G-CSF treatment *in vivo* (0.73±0.16 vs. 0.36±0.31; p=0.004). These results suggest that G-CSF polarizes bone marrow T cells from a Th1 to a Th2 phenotype.

Lymphocyte proliferation ability after in vivo administration of G-CSF

Lymphocyte proliferation ability was tested after *in vitro* stimulation by PHA (n=15 experiments). BMMC showed significantly lower proliferative responses after *in vivo* G-CSF treatment than before treatment (after: 0.9±0.14 vs. before: 1.14±0.21, p=0.004), indicating that G-CSF treatment in healthy donors induces hyporesponsiveness of T cells in the bone marrow grafts.

Table 2. Immunological composition of bone marrow grafts before and after G-CSF mobilization (n=15).

	Pre-G-CSF mobilization	*Post-G-CSF mobilization*	*P value*
TNC (×10³/μL)	1.47±0.42	2.74±0.64	<0.00
lymphocytes	2961±1429	2520±747	0.287
T cells (CD3⁺)	1805±992	1514±482	0.301
CD4⁺	919±588	644±211	0.062
CD8⁺	782±328	776±322	0.960
CD8⁺CD28⁻	205±102	239±142	0.409
CD3⁺CD4⁻CD8⁻	91±70	68±32	0.270
CD4⁺CD25⁺	85±76	108±34	0.291
Monocytes (CD14⁺)	433±244	649±299	0.087
*B cells (CD19⁺)	581±264	532±240	0.602

**n: 10, TNC: total nucleated cells. Data are expressed as the counts of cells/μL (mean±SD). Paired t tests were used for the statistical analysis.*

Effect of G-CSF treatment on bone marrow cell counts

The absolute quantities of immunological cells per microliter of bone marrow were evaluated before and after G-CSF treatment (Table 2). The number of total nucleated cells (TNC) increased greatly after G-CSF treatment. There were no differences in the absolute cell counts of lymphocytes, CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, B cells, CD4⁺CD25⁺ T-regulatory cells, CD3⁺CD4⁻CD8⁻ and CD8⁺CD28⁻ T-suppressor cells, whereas there were 2.13-fold more monocytes after G-CSF treatment (Table 2). Therefore, the monocyte T-lymphocyte ratio was significantly higher in bone marrow after G-CSF treatment (0.27±0.12) than before (0.15±0.04, p=0.007).

To determine whether the T-cell hyporesponsiveness resulted from a preferential increase of T-suppressor cells, we analyzed CD3⁺CD4⁻CD8⁻ and CD8⁺CD28⁻ T-suppressor cells. The percentages of CD3⁺CD4⁻CD8⁻ and CD8⁺CD28⁻ T-suppressor cells within the T-cell population were not different in bone marrow samples after and before G-CSF treatment (10.29±7.19% vs. 7.56±3.72%; p=0.172) and (2.77±1.06% vs. 3.31±2.59%; p=0.462), respectively. These findings demonstrate that the absolute number of the majority of the cell populations, including lymphocytes, CD3⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, CD3⁺CD4⁻CD8⁻ and CD8⁺CD28⁻ T-suppressor cells, B cells and CD4⁺ CD25⁺ T-regulatory cells, was unchanged, but the number of monocytes was actually doubled (Table 2).

Decrease of DC1 but not DC2 after G-CSF treatment

In our studies the absolute number/μL of DC2 increased after G-CSF treatment (after: 76±72/μL vs. before: 43±32/μL; p=0.091), the absolute number of

DC1 decreased slightly (42±24/μL vs. 63±48/μL; p=0.130), while the ratio between absolute counts of DC1 and DC2 was lower after G-CSF treatment than before (0.85±0.93 vs. 2.27±2.21, p=0.029). The DC2/T-lymphocyte ratio was 2.74-fold higher after G-CSF treatment (3.02± 2.68) than before (1.69±1.57, p=0.049). These data suggest that DC2 were preferentially increased by *in vivo* administration of G-CSF (Figure 1 and Figure 3).

Expression of CD28/B7 co-stimulatory molecules on T cells and antigen-presenting cells

Several lines of evidence indicate that CD28/CD80/CD86 co-stimulation plays a significant role in T-cell alloreactivity.[14] Therefore, we investigated changes of CD28/B7 co-stimulatory molecules on T cells and APC. Data are expressed as the percentage of $CD28^+$ cells within T cells, or the percentage of $CD80^+CD86^+$ cells within APC, and as the mean fluorescence intensity (MFI). Because the changes of CD28/B7 cannot be fully described by the percentage and MFI, the overall expression of CD28/B7 was introduced (see the section above on flow cytometric analysis).

Compared with bone marrow grafts before G-CSF treatment, bone marrow primed with G-CSF showed overall expression of CD28 on $CD3^+$, $CD4^+$ and $CD8^+$ T cells (p=0.042, 0.014 and 0.042, respectively) (Figure 4C). The MFI of CD28 expression on $CD4^+$ T cells was also significantly lower after G-CSF treatment (p=0.016) (Figure 4b), whereas the percentages of CD28 expression on T cells was not affected by 4 days of treatment with G-CSF (Figure 4A). The percentages, MFI and overall expression of B7 (CD80/CD86) on $CD19^+$ cells primed by G-CSF were not different from those in unprimed bone marrow (Figure 5A, 5B, 5C). In contrast, the percentages, MFI and overall expression of CD86 molecules on monocytes were significantly lower in G-CSF-primed bone marrow (p=0.03, $p \leq 0.001$ and $p<0.001$), respectively (Figure 6). The percentages of $CD19^+CD80^+CD86^+$ and $CD14^+$ $CD80^+CD86^+$ cells within total nucleated cells decreased significantly after *in vivo* G-CSF treatment (0.22±0.09% vs. 0.06±0.03%, $p \leq 0.001$) and (0.34±0.22% vs. 0.16±0.09%, p=0.021), respectively. There was very little CD80 expression on $CD14^+$ cells. Therefore, the expression of CD28/B7 molecules on T cells and antigen-presenting cells was lower after *in vivo* administration of G-CSF. These data indicate that the co-stimulatory signals induced by CD28/B7 molecules were downregulated in G-CSF-primed bone marrow grafts.

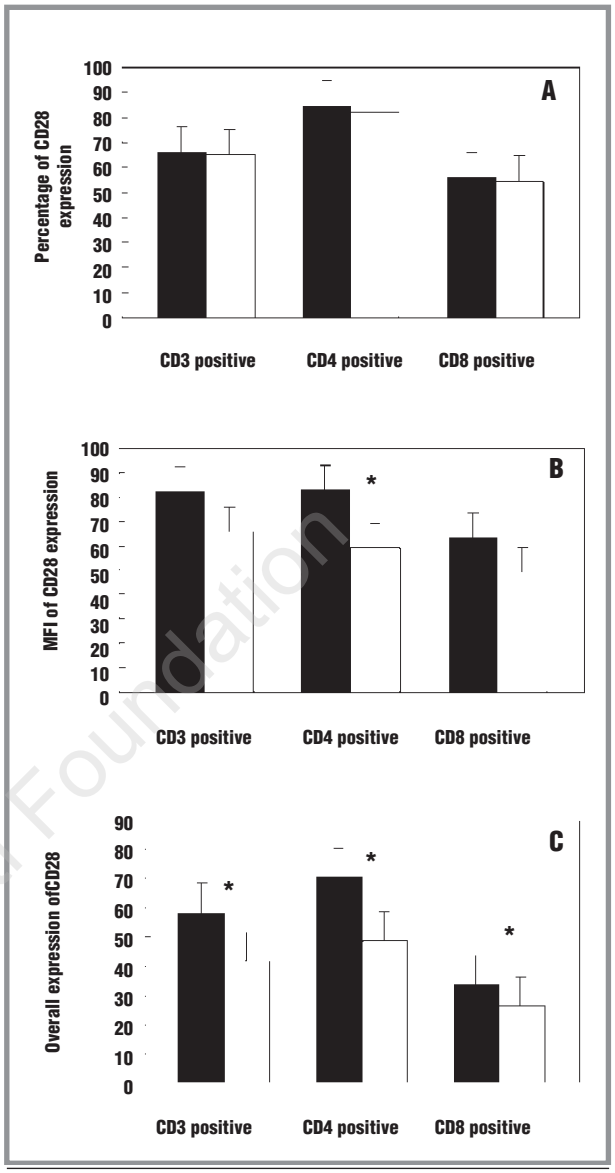

Figure 4. Expression of CD28 co-stimulatory molecules on T-cell subgroups ($CD3^+$,$CD4^+$,$CD8^+$ cells). Percentage (4A), MFI (4B) and overall expression (4C) on T cells of donor bone marrow before (dark boxes) and after (light boxes) treatment with rhG-CSF (5 μg/kg/day). *p <0.05

Discussion

In accordance with Pan's experiment,[7] G-CSF not only reduces the T-cell alloreactivity of murine bone marrow, but may also induce a shift from a type 1 (IFN-γ predominant) toward a type 2 (IL-4 predominant) cytokine profile by increasing IL-4 production and inhibiting IFN-γ secretion. Our data confirmed that G-CSF affects the potential of bone marrow lymphocytes to produce IFN-γ. The amount of IFN-γ secreted by bone marrow T cells was decreased significantly, whereas IL-4 secretion increased. Therefore, T lymphocytes were polarized from a Th1 to Th2 phenotype by G-CSF treatment. The decreased proliferative ability of primed bone marrow T cells suggest that *in vivo* administration of G-CSF may induce hyporesponsive-

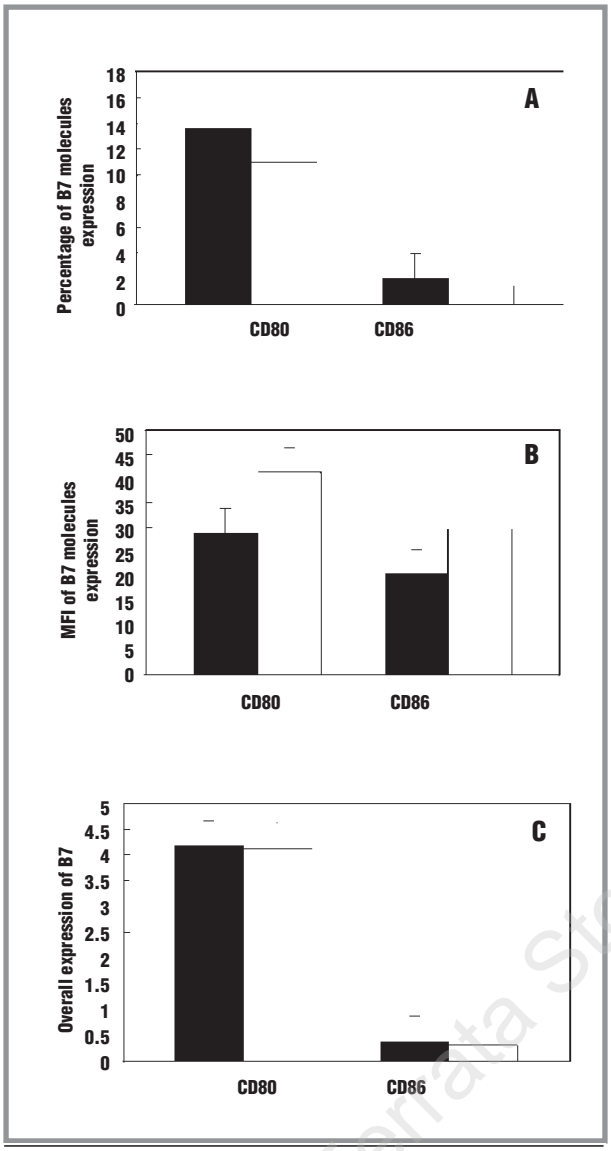

Figure 5. Alteration of B7(CD80/CD86) co-stimulatory molecules expressed on CD19⁺ cells. Percentage (5A), MFI (5B) and overall expression (5C) on CD19⁺ cells of donor bone marrow before (dark boxes) and after (light boxes) treatment with rhG-CSF (5 μg/kg/day).

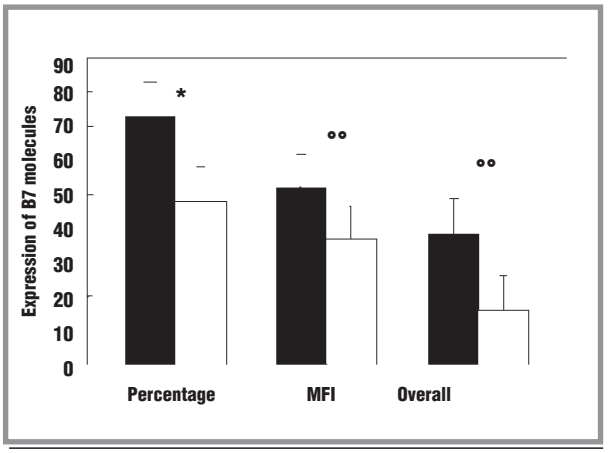

Figure 6. Alteration of B7-2(CD86) co-stimulatory molecules expressed on CD14+ cells of donor bone marrow before (dark boxes) and after (light boxes) treatment with rhG-CSF (5 μg/kg/day). *$p < 0.05$, °$p < 0.001$

ness of T cells both in G-CSF mobilized peripheral blood and bone marrow, simultaneously.[7,15] This study provides a detailed phenotypic profiling of the bone marrow grafts and demonstrates that the absolute number of monocytes actually doubled. It is believed that suppressor monocytes play an important role in peripheral T-cell hyporesponsiveness,[6,16] therefore, it could be expected that monocytes may act similarly on bone marrow T cells.

The immune modulatory effect of G-CSF on peripheral T cells is thought to be mediated exclusively through other effector cells.[6,11,12,16] We confirmed that G-CSF treatment preferentially increased DC2 numbers, resulting in a lower DC1/DC2 ratio. Russoan *et al.* recently demonstrated that polarization of the T-cell response into Th1 or Th2 depends upon the type of APC in humans: CD11c⁺ DC1 induce Th1 responses, whereas, interleukin-3 receptor α positive (IL-3α⁺) DC2 induce Th2 reponses.[17] It is conceivable that G-CSF might decrease the availability of DC1 in bone marrow grafts, leading to the relatively higher number of DC2 and Th2 polarization in the donors. In several experimental models, donor Th2 cells have a decreased potential to induce GVHD.[7,15,18] It is also possible that donor DC2 in bone marrow contribute to the pathogenesis of GVHD after transfer to the recipient through indirect presentation of host antigen to donor T cells and the induction of a Th2 response. Interestingly, umbilical cord blood, another source of allogeneic stem cells for transplantation associated with a relatively low incidence of acute GVHD, contains DC2 but not DC1.[19]

Some researchers have proposed that B7-1 and B7-2 molecules expressed on APC bind to CD28 and cytotoxic T lymphocyte antigen-4 (CTLA-4), thereby providing a co-stimulus for T-cell activation and colony expansion.[20,21] Experimental and clinical data suggest that CD28/B7 co-stimulatory molecules may play an important role in regulating T-cell function and the development of GVHD after allogeneic marrow transplantation in human and mice.[22,23] In this study we observed that CD28 expression on T cells, and B7 expression on B cells and monocytes were downregulated. Therefore, the unexpectedly low proliferative ability of bone marrow T cells may be a consequence of the larger number of CD14⁺/B7^{lo} cells, CD19⁺/B7^{lo} cells and the low expression

of CD28 on T cells, suppressing CD28/B7-mediated signal transduction.

The *double-negative* T cells ($CD3^+CD4^-CD8^-$) are believed to have immunosuppressive activity and play a regulatory role in the development of the GVHD reaction through inhibition of alloreactivity.[24-26] We found that the G-CSF-primed bone marrow grafts and normal bone marrow grafts contained comparable numbers of double-negative T cells. Other T-suppressor cells, such as $CD8^+CD28^-$ cells, have been described.[27,28] In our study, the number of $CD8^+CD28^-$ cells in bone marrow grafts was similar after *in vivo* administration of G-CSF, indicating that there was not the same preferential G-CSF-induced increase of $CD3^+CD4^-$ $CD8^-$ and $CD8^+CD28^-$ T-suppressor cells in bone marrow as in G-CSF-mobilized peripheral blood grafts.[6] We have found that there were larger quantities of $CD8^+CD28^-$ cells in peripheral blood after G-CSF mobilization than prior to this treatment (*data not published*).

The $CD4^+CD25^+$ thymic-derived regulatory T cells are critical to the maintenance of self-tolerance, prevention of autoimmunity and control of GVHD. Recent studies showed that the role played by $CD4^+CD25^+$ regulatory T cells in GVHD is different in mice and human.[29,30]

To explain this discrepancy, Stanzani *et al.* pointed out that circulating human interleukin-2 receptor α-chain (IL-2Rα, CD25)-expressing T cells represent a heterogeneous population, containing not only regulatory but also activated effector T cells.[30] No definite conclusion can be drawn from our study given the similar numbers of $CD4^+CD25^+$ T cells in bone marrow grafts before and after G-CSF treatment. The effect of $CD4^+CD25^+$ regulatory T cells in hematopoietic stem cell transplantation remains to clarified.

In conclusion, our data suggest that *in vivo* administration of G-CSF might alter the composition of bone marrow grafts, polarize Th1 to Th2, and induce hyporesponsiveness of T cells. Downregulation of CD28/B7 co-stimulatory signals and the preferential increase of monocytes and DC2 may contribute to the hyporesponsiveness of T cells. The potential correlations between our data on G-CSF-primed bone marrow grafts and the occurrence of acute and chronic GVHD, relapse, or other transplantation-related complications are presently being investigated in our institute.

HXJ: conception and design, revising the article critically and final approval of the version to be published; CYJ: analysis and interpretation of data, drafting the article and final approval of the version to be published; ZXY: analysis and interpretation of data, drafting the article and final approval of the version to be published. The authors reported no potential conflicts of interest.

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