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Functional Regulatory T Cells Are Collected in Stem Cell Autografts by Mobilization with High-Dose Cyclophosphamide and Granulocyte Colony-Stimulating Factor

Maud Condomines,*†‡ Philippe Quittet,§ Zhao-Yang Lu,¶ Laure Nadal,* Pascal Latry,§ Ernesto Lopez,§ Marion Baudard,§ Guilhem Requirand,* Christophe Duperray,*† Jean-François Schved,†¶ Jean-François Rossi,*¶ Karin Tarte,§ and Bernard Klein‡*†‡¶

High-dose cyclophosphamide (Cy) and G-CSF are widely used to mobilize hematopoietic stem cells for treating patients with high-dose chemotherapy and autologous stem cell transplantation (ASCT). Because lymphocyte count in the graft collected after Cy-G-CSF treatment is an independent survival factor after ASCT for patients with multiple myeloma, our purpose was to study how Cy-G-CSF treatment affects the phenotype and function of T cells in patients with multiple myeloma. Cy induced a 3-fold decrease of T cell counts with a slow and partial T cell recovery of one-third at the time of hemopoietic stem cell collection. Cy-G-CSF treatment did not affect the relative ratios of central memory, effector memory, and late effector CD4+ or CD8+ T cells, but a decrease in the percentage of naïve CD4+ cells was observed. The percentages of CD25+ cells increased 2- to 3-fold in CD4+ and CD8+ T cells, the former including both activated CD25low and CD25high cells. CD4+CD25high cells were regulatory T cells (Treg) that expressed high levels of FOXP3, CTLA-4, and GITR and displayed in vitro suppressive properties. The recovery of Treg absolute counts after Cy-G-CSF treatment was higher than the recovery of other lymphocyte subpopulations. In conclusion, Cy-G-CSF treatment induces a severe T cell count decrease without deleting Treg, which are potent inhibitors of antitumor response. The present data encourage novel therapeutic strategies to improve T cell recovery following ASCT while limiting Treg expansion. The Journal of Immunology, 2006, 176: 6631–6639.

High-dose chemotherapy (HDC)3 associated with autologous stem cell transplantation (ASCT) improves the rate of complete remission and overall survival in patients with multiple myeloma (MM) and non-Hodgkin lymphoma at relapse (1, 2). Many studies have focused on the efficient mobilization of hematopoietic stem cells (HSC) by administration of hemopoietic growth factors with or without high-dose cyclophosphamide (Cy) (3–8). Cy induces profound aplasia with the production of endogenous mediators and growth factors that favor hemopoietic recovery. These endogenous mediators and the administrated recombinant G-CSF stimulate HSC proliferation and their mobilization into the peripheral blood. This mobilization is attributable to the activation of bone marrow neutrophils by hemopoietic growth factors, in particular G-CSF, resulting in the production of enzymes that cleave adhesion molecules and chemokines and in the release of HSC from the bone marrow environment (9, 10). Because T lymphocytes are major actors in the graft-vs-host reaction in allogenic HSC transplantation, the characteristics of T lymphocytes collected by leukapheresis after HSC mobilization with G-CSF have been analyzed. Some studies indicate that G-CSF doubles the circulating T lymphocyte rate without major changes in the phenotype of T cells (11, 12). Several other studies (13–15) demonstrate that G-CSF might affect the cytotoxic T cell activity by inducing human lymphocytes to preferentially release type 2 cytokines (IL-4, IL-5, and IL-10) rather than type 1 (IFN-γ, TNF-α, and IL-2) upon activation. However, the phenotype and function of T lymphocytes mobilized with Cy and G-CSF have not been studied yet. This is important because Cy is a strong immunosuppressor. In mice, the Cy-induced immunosuppression is followed by a rapid T cell repopulation, likely associated with the production of endogenous T cell growth factors (16). In addition, a selective toxicity of Cy on regulatory T cells (Treg) has been demonstrated (17–19). Treg are CD4+CD25+ T cells that control key aspects of tolerance to self-Ags by suppressing activation of naïve T cells, and they are crucial in the prevention of autoimmune diseases (20, 21). Treg express a specific transcription factor, FOXP3, which controls both their development and function (22). They also constitutively express some molecules associated with T cell activation including glucocorticoid-induced TNF receptor (GITR) and CTLA-4 (23, 24). Because a part of tumor-associated Ags is derived from self-Ags, Treg may be partially responsible for the lack of antitumor immune responses. In animal models, Treg expansion in the lymphoid organs of tumor-bearing animals is correlated with tumor volume, and the removal of Treg enhances antitumor immune responses. In humans, the proportion of Treg is increased in cancer patients. Thus, the depletion of Treg could be useful to promote an antitumor T cell response (20, 21).

A recent study has emphasized that the number of infused autologous lymphocytes collected by Cy and hemopoietic growth factor mobilization was an independent factor for overall survival
and time to progression post HDC and ASCT, in patients with MM (25). Given the lack of data regarding the phenotype and function of T lymphocytes in the HSC graft, the aim of our study was to characterize the behavior of T cell subpopulations after Cy-G-CSF treatment and, in particular, that of Treg. Human CD4+ or CD8+ T cells can be classified into naive T cells, central memory T cells, effector memory T cells, and late effector T cells (26, 27). During an immune reaction against a pathogen, naive T cells are primed by Ag-loaded dendritic cells (DCs) in lymphoid organs, proliferate widely, and differentiate into effector cells, which produce cytokines and are able to kill pathogens. After the acute activation phase, a fraction of the activated T lymphocytes persists as central memory T cells, able to induce a quick and strong immune response upon re-encountering Ag. Naive T cells express CCR7 and two lymph node homing receptors, CD62L, which enables T cells to adhere to endothelial cells, and CCR7, which is a receptor for the CCL19 and CCL21 lymph node chemokines. Central memory T cells are CD45RA- and express the chemokine receptors CCR7 and CD62L. Effector memory T cells are CD45RA- and lack CCR7 and CD62L, but express receptors for inflammatory chemokines (CCR1, CCR3, and CCR5). Late effector T cells are CD45RA+CCR7- with a low expression of CD62L. In healthy individuals, there are very few circulating late effector CD45RA+CCR7-CD4+ T cells compared with the high proportion of late effector CD8+ T cells (28).

We investigated in this study the phenotype and function of T lymphocytes present in the peripheral blood after high-dose Cy-induced aplasia and G-CSF mobilization in 14 patients with MM.

**Materials and Methods**

**Patients and collection of peripheral blood samples**

Fourteen patients with MM (median age, 59 years) who underwent ASCT were included in this study, according to the French ethical laws. The series comprised nine male patients and five female patients. According to the Durie-Salmon classification, 10 patients had stage IIIA disease, two patients had stage IIIB, whereas one patient presented with a plasma cell myeloma of IgA type. The median age of these patients was 59 years (range, 41–75 years). The disease status of each patient before Cy administration was determined by a slow and partial recovery in all 14 patients (Fig. 1B), with a one-fifth partial recovery on the first leukapheresis procedure (median day 10; range, 9–12). PBMCs were obtained by density centrifugation using Ficoll-Hypaque (Cambrex BioScience). After written informed consent, 500 × 10^9 fresh cells from leukemia were used for Treg sorting and functional assays.

**Flow cytometry analysis**

The phenotype of T cells was evaluated with the following mAbs: PE-conjugated anti-CD3, anti-CD4, anti-CD8β, anti-CD25, and anti-pentameric FITC-conjugated anti-CD25, anti-CD4 and anti-CD8 (Beckman Coulter). CD3, CD25, CD8β (BD Systems), CD45RA (BD Systems) and CD62L (BD Biosciences Pharmingen). The phenotype of NK cells was evaluated with a PE-conjugated anti-CD56 antibody (Beckman Coulter). Corresponding isotype-matched murine Abs, recognizing no human Ag, were used as negative controls. Briefly, appropriate amounts of Abs were added to 0.5 × 10^9 cells followed by a 30-min incubation at 4°C. RBCs were then lysed, cells were washed, and 3 × 10^5 PE-conjugated anti-CD45RA and anti-CD62L, or 5 × 10^5 PE-conjugated anti-CD4, anti-CD8 or anti-CD25 cells, were added, and CD45RA+, CD62L-, and CD62L+ T cells were sorted using a FACSAria cell sorter (BD Biosciences). The purity of the T cell subpopulations was >90%.

**Quantification of FOXP3, CTLA-4, and GITR with real-time RT-PCR**

Total RNA was prepared from sorted CD4+ subpopulations using the RNeasy Kit (Qiagen). cDNA was prepared from 1 μg of RNA using the Superscript II reverse transcriptase (Invitrogen Life Technologies) and oligo(dT)12–18 (Amersham Pharmacia Biotech) as primer. The relative levels of FOXP3, CTLA-4, or GITR mRNA in each subset were determined by real-time PCR on an ABI Prism 7000 Sequence Detector (Applied Biosystems) using the Assays-on-Demand products (Hs00203958_m1 for FOXP3 detection, Hs01175480_m1 for CTLA-4 detection, and Hs01183461_m1 for GITR detection) and the TaqMan Universal Master Mix. Quantitative PCR analysis was completed using ABI Prism 7000 SDS Software. Threshold cycle (Ct) values were measured for β2-microglobulin (β2m) and the genes of interest during the log phase of the cycle. The expression levels of genes of interest were normalized to that of β2m for each sample (ΔΔCt = Ct gene of interest, Ct β2m) and compared with the values obtained for a CD4+CD25+ positive control isolated from a healthy donor, using the following formula: 100/2^ΔΔCt, where ΔΔCt = ΔCt unknown – ΔCt positive control.

**Treg suppression assay**

Mature DCs were generated as detailed previously (29). Briefly, 8 × 10^6 G-CSF-mobilized leukapheresis cells were plated in 2 ml of X-VIVO15 medium (BioWhittaker) per well in six-well flat-bottom plates (Nunc). Nonadherent cells were discarded by gentle rinsing after a 2-h incubation at 37°C in 5% CO2. Adherent cells were cultured in X-VIVO15 medium with 2% human albumin, 100 ng/ml GM-CSF (Leukine; Berlex), and 25 ng/ml IL-4 (Genelix) for 5 days. DCs were then induced to mature for 24 h with 20 ng/ml TNFα (Genelix) and 100 ng/ml PGE2 (PROSTIN E2; Pharmacia) and finally frozen in X-VIVO15-50% albumin-10% DMSO medium.

In each well, 10^4 CD4+CD25- T cells, CD4+CD25+ T cells, or CD4+CD25+CD8α+ T cells were seeded into 96-well U-bottom culture plates in a 200-μl final volume of RPMI 1640 (Invitrogen Life Technologies) supplemented with 5% human serum with or without 10^5 allogeneic mature DCs. Every 2 × 10^4 CD4+CD25+ T cells or 10^5 CD4+CD25+CD8α+ T cells were added to wells containing DC-stimulated CD4+CD25- T cells. At the end of a 7-day culture, cells were pulsed with tritiated thymidine (Amersham Pharmacia Biotech) for 16 h, and tritiated thymidine incorporation was determined as reported previously (30).

**Results**

**Mobilization of T and NK lymphocytes**

T cell and NK cell subpopulation profiles were studied for 14 patients. Mean white blood cell (WBC) count dropped on day 6 following Cy administration and then increased due to G-CSF in all 14 patients (Fig. 1A). The mean WBC count was 10 × 10^9/L on the day of the first leukapheresis procedure (median day 10; range, 9–12). G-CSF injections were stopped after HSC collection. Cy induced a 7-fold reduction of the CD3+ cell count on day 6 followed by a slow and partial recovery in all 14 patients (Fig. 1B). The mean CD3+ cell count was 1.22 × 10^9/L before Cy-G-CSF treatment and dropped to 0.18 × 10^9/L 6 days after Cy injection, corresponding to the WBC count nadir. It then increased slightly to a mean count of 0.36 × 10^9/L on the first leukapheresis procedure day. The relative ratios of CD4+ and CD8α+ cells within CD3+ cells were not significantly modified throughout the Cy-G-CSF-induced mobilization period (results not shown). NK cell counts paralleled the decrease of CD3+ cells with a one-fifth partial recovery on the first leukapheresis procedure day (Fig. 1C). Thus, Cy administration induced a profound decrease of CD3 and NK cell counts.
Mobilization of naive, central memory, effector memory, and late effector T cells

We studied the subpopulations of naive CD4 $^+$ T cells (CD45RA $^+$, CCR7 $^+$), central memory CD4 $^+$ T cells (CD45RA $^-$, CCR7 $^+$), effector memory CD4 $^+$ T cells (CD45RA $^-$, CCR7 $^-$), and late effector CD4 $^+$ T cells (CD45RA $^-$CCR7 $^-$) throughout the Cy-G-CSF mobilization period in 10 patients with MM. Given the large amount of data, only the mean percentages of CD4 $^+$ or CD8 $^+$ subsets are shown in Fig. 2, A and B. Before Cy-G-CSF treatment, the mean percentages of the various subpopulations of CD4 $^+$ cells were as follows: naive CD4 $^+$ cells, 28% (range, 14–45%); central memory CD4 $^+$ cells, 47% (range, 35.7–65%); effector memory CD4 $^+$ cells, 21% (range, 12.7–31%); and late effector CD4 $^+$ cells, 5% (range, 0.1–16.3%) (Fig. 2A). This profile of CD4 $^+$ cell subpopulation distribution did not differ from those previously reported for a healthy donor population (respectively, 28, 58, 12, and 2%) (31). We found that Cy-G-CSF treatment induced a slight but significant ($p < 0.05$) decrease in naive CD4 $^+$ cells relative rate (from 28 to 19%), whereas the percentages of the three subpopulations of memory CD4 $^+$ cell (central memory, effector memory, and late effector) were not significantly affected (Fig. 2A). Before Cy-G-CSF treatment, CD8 $^+$ T cells comprised 25% (range, 12.3–49%) of naive cells, 14% (range, 6.7–27.5%) of central memory cells, 23% (range, 5.7–40%) of effector memory cells, and 38% (range, 18.6–64%) of late effector cells. The proportions of CD8 $^+$ subsets did not change significantly throughout Cy-G-CSF-induced HSC mobilization (Fig. 2B) and did not differ from those

**FIGURE 1.** Leukocyte and CD3 $^+$ cell counts before and throughout Cy-G-CSF mobilization. The panel shows WBC counts (A), CD3 $^+$ cell counts (B), or NK cell (CD3 $^-$CD56 $^+$) counts (C) measured on the day before Cy administration (day 0), on day 6 after Cy administration (leukocyte nadir), and on the day of the first leukapheresis procedure (median day 10; range, 9–12) for the 14 patients with MM, treated with Cy and G-CSF. Data of individual patients are represented with specific symbol. Black horizontal lines denote mean values at each time point. *, Mean value is significantly different ($p < 0.05$) from that before Cy administration, using the Student $t$ test for pairs. ***, Indicates that the mean value is significantly different ($p < 0.05$) from that before Cy administration and from the nadir value at day 6, using the Student $t$ test for pairs.

**Mobilization of naive, central memory, effector memory, and late effector T cells**

We studied the subpopulations of naive CD4 $^+$ T cells (CD45RA $^+$, CCR7 $^+$), central memory CD4 $^+$ T cells (CD45RA $^-$, CCR7 $^+$), effector memory CD4 $^+$ T cells (CD45RA $^-$, CCR7 $^-$), and late effector CD4 $^+$ T cells (CD45RA $^-$CCR7 $^-$) throughout the Cy-G-CSF mobilization period in 10 patients with MM. Given the large amount of data, only the mean percentages of CD4 $^+$ or CD8 $^+$ subsets are shown in Fig. 2, A and B. Before Cy-G-CSF treatment, the mean percentages of the various subpopulations of CD4 $^+$ cells were as follows: naive CD4 $^+$ cells, 28% (range, 14–45%); central memory CD4 $^+$ cells, 47% (range, 35.7–65%); effector memory CD4 $^+$ cells, 21% (range, 12.7–31%); and late effector CD4 $^+$ cells, 5% (range, 0.1–16.3%) (Fig. 2A). This profile of CD4 $^+$ cell subpopulation distribution did not differ from those previously reported for a healthy donor population (respectively, 28, 58, 12, and 2%) (31). We found that Cy-G-CSF treatment induced a slight but significant ($p < 0.05$) decrease in naive CD4 $^+$ cells relative rate (from 28 to 19%), whereas the percentages of the three subpopulations of memory CD4 $^+$ cell (central memory, effector memory, and late effector) were not significantly affected (Fig. 2A). Before Cy-G-CSF treatment, CD8 $^+$ T cells comprised 25% (range, 12.3–49%) of naive cells, 14% (range, 6.7–27.5%) of central memory cells, 23% (range, 5.7–40%) of effector memory cells, and 38% (range, 18.6–64%) of late effector cells. The proportions of CD8 $^+$ subsets did not change significantly throughout Cy-G-CSF-induced HSC mobilization (Fig. 2B) and did not differ from those

**FIGURE 2.** Subpopulations of naive and memory CD4 $^+$ or CD8 $^+$ T cells before and throughout Cy-G-CSF mobilization. Results are the mean values of naive (CD45RA $^+$, CCR7 $^+$), central memory (CD45RA $^-$, CCR7 $^+$), effector (CD45RA $^-$, CCR7 $^-$) CD4 $^+$ (A) or CD8 $^+$ (B) T cells determined at the time points described in Fig. 1, for 10 patients with MM. *, Mean value is significantly different ($p < 0.05$) from that before Cy administration, using the Student $t$ test for pairs. Because SDs could be high due to interpatient variability, they were not shown.
patients are shown in Fig. 3B. A mean percentage of 46% of CD4+ T cells and of 13% of CD8− T cells expressed CD25 on the day of the first leukapheresis procedure (median day 10, after Cy administration, range 9–12 days) (Fig. 3B). These mean percentages were significantly increased 2.0- and 2.9-fold respectively, compared with those observed before Cy-G-CSF treatment (p ≤ 0.01). In humans, CD4+ CD25low cells contain both Treg that express high levels of CD25 (CD4+CD25high) and activated nonregulatory CD4+ CD25low cells (33). These two populations could be found before but also after Cy-G-CSF treatment, suggesting that Treg are still present after Cy administration (Fig. 4A). The mean percentages of both CD4+ CD25high and CD4+ CD25low subsets were increased 2-fold (p ≤ 0.01) at the time of HSC collection compared with those observed before Cy-G-CSF treatment (Fig. 4, B and D). The mean CD4+ CD25high and CD4+ CD25low cell counts were decreased 5.6- and 3.6-fold, respectively (p ≤ 0.01), on day 6 after Cy administration. At the time of HSC collection, the mean CD4+ CD25high and CD4+ CD25low cell counts did not significantly differ from those prior Cy-G-CSF treatments, contrary to CD3+ cell or NK cell counts, which remained at least 3-fold decreased (Fig. 1, B–C). Because these data are intriguing given the reported toxicity of Cy to Treg (17, 18, 34), the phenotype and function of CD4+ CD25high cells in the leukapheresis product were studied in detail. CD4+ CD25high, CD4+ CD25low, and CD4+ CD25− cells contained in the leukapheresis products from six patients with MM were purified using a FACS Aria cell sorter (Fig. 5A). FOXP3, CTLA-4, and GITR gene expressions were assayed using real-time RT-PCR. FOXP3 gene expression in CD4+ CD25high cells was 60- to 200-fold higher than in CD4+ CD25− (p < 0.001) cells, and 6- to 20-fold higher than in CD4+ CD25low cells (p < 0.01) (Fig. 5B). CD4+ CD25high, CD4+ CD25low, and CD4+ CD25− cells contained in the peripheral blood before mobilization from four patients with MM were also purified. A similar pattern of FOXP3 expression was found in the three purified subpopulations (data not shown). CD4+ CD25high cells harvested before (data not shown) and on the day of leukapheresis (Fig. 5, C and D) also overexpressed the CTLA-4 (p = 0.03) and GITR (p = 0.05) genes, other markers associated with Treg function. The expressions of CTLA-4 and GITR were confirmed at the protein level. As shown in Fig. 5E for one representative patient of five patients with MM, CD4+ CD25high cells were strongly labeled with anti-CTLA-4 Ab, unlike CD4+ CD25− cells. CD4+ CD25low cells displayed an intermediate CTLA-4 expression. CD4+ CD25high cells collected on the day of leukapheresis also expressed higher levels of GITR than CD4+ CD25low or CD4+ CD25− cells. A similar high GITR and CTLA-4 protein expression by CD4+ CD25high cells harvested before the mobilization was found for five patients with MM (data not shown).

To prove definitively that CD4+ CD25high cells were Treg, we studied their ability to inhibit the activation of autologous CD4+ CD25− cells by allogeneic DCs. CD4+ CD25high cells harvested either before or after Cy administration abrogated activation of CD4+ CD25− cells induced by allogeneic DCs, at 1:1 ratio of CD4+ CD25− cells to Treg (Fig. 6). CD4+ CD25low cells were efficiently stimulated by DCs, contrary to CD4+ CD25high cells, and were unable to inhibit activation of CD4+ CD25− cells (Fig. 6). Altogether, these data clearly demonstrate that circulating CD4+ CD25high cells present during Cy-G-CSF mobilization are functional Treg, as are circulating CD4+ CD25high cells harvested before Cy-G-CSF treatment, whereas the CD4+ CD25low cells are activated non-Treg.

Immune cell composition of leukapheresis products

Table I depicts the cell composition of leukapheresis products of the 14 patients with MM. These products contained median values of 6.2 × 10⁶ CD34+ cells/kg (range, 2.68–9.1), 21.1 × 10⁶ CD3−

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**Figure 3.** Percentages of CD25+ lymphocytes within CD4+ or CD8− T cells. A, Representative dot plots showing CD25 expression in CD4+ and CD8− cells before Cy administration and on the day of the first leukapheresis procedure. Peripheral blood cells were stained with anti-CD25-FITC and anti-CD4-PE or anti-CD8-PE Abs. Control samples were stained with the corresponding IgG2a-FITC, IgG1-FTTC, or IgG2a-PE isotype-matched 25 murine Ab. PBMCs were gated to include only small lymphocytes by forward and side scatter. Numbers on dot plots indicate the percentages of CD25+ cells calculated within CD4+ or CD8− cells. B, Increased proportion of peripheral CD4+ CD25− and CD8− CD25− T cells throughout the mobilization procedure in 14 patients. Results are mean values ± SD of percentages of CD25+ cells within CD4+ or CD8− T cells for 14 patients with MM, determined before (day 0) and after Cy administration, at the time points described in Fig. 1. *p<0.05. **p<0.05. Mean value is significantly different (p < 0.05) from that before Cy administration, using the Student t test for pairs.
cells/kg (range, 13–116), including $1.5 \times 10^6$ CD4$^+$ cells/kg and $6.3 \times 10^6$ CD8$^+$ cells/kg, $3.8 \times 10^6$ NK cells/kg (range, 1.5–46.9) and $0.3 \times 10^6$ γδ T cells/kg (range, 0.1–5.3). They contained nearly $1 \times 10^6$ CD4$^+$ CD25$^{high}$ cells/kg (range, 0.4–5.1) with the phenotype and suppressive properties of Treg. The ratio of CD34$^+$ cells to Treg ranged from 13.4 to 0.5 for a median value of 7.

**Discussion**

Mobilization regimens have been optimized to collect a maximum of CD34$^+$ HSC to reduce aplasia and the number of leukaphereses. High-dose Cy associated with G-CSF is widely used because it results in a 2- to 3-fold increase in the number
of CD34⁺ HSC collected per leukapheresis procedure compared with G-CSF alone (7, 8). However, very little attention has been given to the impact of Cy-G-CSF combination on the number, phenotype, and function of the T cells that are collected in the leukapheresis product and infused to the patient.

In a cohort of 14 consecutive patients with MM, we show that Cy-G-CSF treatment induced a severe T cell and NK cell counts...
decrease. Circulating CD3+ cell counts were reduced 7-fold on day 6 after Cy administration and slowly and partially recovered, contrary to a rapid recovery of neutrophil and platelet counts. On the contrary, mobilization with G-CSF alone was shown to double circulating CD3+ cell counts (11, 12). Thus, although Cy increases the mobilization and collection of CD34+ cells, it dramatically reduces the number of circulating T cells. The effect of the mobilization regimen on T cell count might be of importance because Porrata et al. (25) have recently shown that absolute lymphocyte count in the autograft collected after Cy and hemopoietic growth factors treatment may affect survival in MM patients after ASCT. Patients receiving a high number of lymphocytes had higher response rates and lower relapse rates, resulting in improved survival. In particular, the autograft absolute lymphocyte count was an independent prognostic factor for overall survival and progression-free survival in MM patients (25). In this study, the lymphocyte population was not characterized and may include T cells, B cells, NK cells or, eventually, nonlymphocyte cells.

T cells comprise several subsets with distinct phenotype and function. Based on CD45RA and CCR7 chemokine receptor expression, we investigated four CD4+ or CD8+ T cell subpopulations: naive T cells (CD45RA+, CCR7+), central memory T cells (CD45RA−, CCR7+), effector memory T cells (CD45RA−, CCR7−), and late effector T cells (CD45RA+, CCR7−). Before Cy-G-CSF treatment, the proportions of the four CD4+ subsets and the four CD8+ subsets in patients with MM were similar to those previously described in healthy individuals. They remained stable throughout the partial T cell recovery after Cy administration, except for a slight decrease in the naive CD4+ subset. Thus, the current data indicate that the Cy-G-CSF mobilization regimen, while inducing a severe reduction in T cell count, did not significantly affect the proportions of memory and naive T cells. This is important considering that the memory T cell repertoire in the autograft is critical to T cell immunity reconstitution after HDC (35).

We then investigated T cell activation. The percentages of CD25+ cells in CD4+ or CD8+ cells increased 2- or 3-fold throughout post-Cy lymphocyte recovery. In healthy individuals or patients with MM, CD4+CD25+ cells include Treg that highly express CD25 (CD4+CD25high cells) and activated nonregulatory CD4+ T cells (CD4+CD25low cells). The current study indicates that the percentages of both circulating subpopulations doubled during Cy-G-CSF mobilization. Concerning CD4+CD25low cells, such a high proportion of activated cells (40% of CD4+ cells) was not reported in studies that explored the phenotype of G-CSF mobilized T cells (12, 14, 36). This may be explained by an in vivo production of a cytokine burst able to activate T cells, which occurs after Cy treatment. In particular, Proietti et al. (16, 37) demonstrated in a murine model that one of these mediators was IFN-α.

We demonstrated that CD4+CD25high cells collected in the leukapheresis product have the phenotype of Treg. They highly express the FOXP3, CTLA-4, and GITR genes and the CTLA-4 and GITR proteins compared with CD4+CD25low or CD4+CD25− cells. FOXP3 is one of the best Treg-specific markers available to date (22). CTLA-4 is usually up-regulated on T cells after activation. It has been shown to be constitutively expressed by Treg and is involved in their suppressive function (24). GITR, a member of the TNF receptor superfamily, is a costimulatory molecule over-expressed on Treg but also found on activated T cells. Activation of GITR may abrogate Treg suppressive function (23, 38).

We also demonstrated that CD4+CD25high cells collected in the leukapheresis product strongly suppress the proliferation of autologous CD4+CD25− cells induced by allogeneic DCs, unlike CD4+CD25low cells. Thus, CD4+CD25+ cells in the leukapheresis product contain both activated lymphocytes and functional Treg, and the frequency of Treg in CD4+ cells is doubled at the time of HSC collection compared with pre-Cy-GSF treatment.

This observation is noteworthy because low doses of Cy have a specific toxicity to Treg in rodents, leading to Treg apoptosis and loss of function (17–19, 34, 39). In particular, Ghiringhelli et al. (18) demonstrated that a single injection of a low dose of Cy in tumor-bearing rats abrogated the rise of Treg that generally occurred during tumor progression, resulting in the restoration of an antitumor immune response. More recently, Lutsiak et al. (34) demonstrated that in addition to decreasing the Treg cell number, a low dose of Cy inhibits the suppressive capability of Treg in mice. The fact that Treg are still present and fully functional after mobilization with high-dose Cy and G-CSF could be assigned to G-CSF because it increases the number of circulating Treg in healthy individuals (40). Furthermore, Hadaya et al. (41) have demonstrated that G-CSF treatment prevents Cy acceleration of autoimmune diabetes in NOD mice by stimulating the
expansion of regulatory CD4+ CD25+ CD62L-high T cells. It has also been reported in a NOD mouse model that G-CSF injection promotes the splenic accumulation of plasmacytoid tolerogenic DCs, which have the property to expand Treg (42). Our data indicate that the median Treg count in leukaphereses is approximately one-seventh that of CD34+ cells. Because >3 × 10^6/kg CD34+ cells are generally grafted to patients, >4 × 10^6/kg Treg are also grafted. These Treg could contribute to hindering the reconstitution of T cells after HDC. In particular, given the well-documented suppressive activity of Treg on antitumor T cells (43–45), these grafted Treg could affect the emergence of antimyeloma T cells after HDC. In this small series of 14 patients with MM, we found no relationship between the grafted Treg counts and the response rate to HDC, and the follow-up of these patients is as yet too short to evaluate a link between Treg count and event-free or overall survival. Our study points out the interest to monitor grafted T cell subsets and Treg counts on a large series of patients to look for their impact on response, event-free and overall survival rates.

The present data could be useful to improve the treatment of patients with MM using high-dose melphalan supported by ASCST. Indeed high-dose melphalan induces a profound and long-lasting cell immunosuppression (46). Because we show in this study that the grafted T cells comprise activated T cells, one can suggest that these patients could be provided with T cell growth factors to favor T cell recovery and growth. According to the recent data indicating that grafted lymphocyte counts could predict survival in patients with MM (25), this may dramatically improve treatment response. IL-2 would have a limited interest because 1) systemic IL-2 administration induces severe toxicity, 2) IL-2 is necessary to the homeostatic maintenance of the pool of natural Treg (47), and 3) IL-2 is required to expand Treg in vitro and to induce their suppressive properties (48). IL-7 or IL-15 could be more promising in a posttransplant setting. Indeed, these two cytokines have reduced systemic toxicities compared for their ability to affect the number and function of T cells that could be collected and grafted to patients.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


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**Table I. Cell composition of infused leukapheresis products**

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<th>Patient</th>
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<th>CD3+ (×10^6/kg)</th>
<th>CD4+ (×10^6/kg)</th>
<th>CD8+ (×10^6/kg)</th>
<th>NK Cells (×10^6/kg)</th>
<th>γδ T Cells (×10^6/kg)</th>
<th>Treg (×10^6/kg)</th>
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*Data represent amounts of each cell type that are infused per autograft per patient. MNC, Mononuclear cells.


