

Increased plasma immune cytokines throughout the high-dose melphalan-induced lymphodepletion in patients with multiple myeloma. A window for adoptive immunotherapy.

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Abstract

High-dose melphalan (HDM) followed by autologous stem cell transplantation (ASCT) is a standard treatment for patients with multiple myeloma (MM). However, lymphocyte reconstitution is impaired after HDM. Recent work has suggested that the lymphopenia period occurring after various immunosuppressive or chemotherapy treatments may provide an interesting opportunity for adoptive anti-tumor immunotherapy. The objective of this study is to determine an immunotherapy window after HDM and ASCT evaluating T-cell lymphopenia and measuring circulating immune cytokine concentrations in patients with MM. The counts of T-cell subpopulations reached a nadir at day 8 post-ASCT (day 10 post HDM) and recovered by day 30. IL-6, IL-7 and IL-15 plasma levels increased on a median day 8 post-ASCT, respectively 35-fold, 8-fold and 10-fold compared to pre-HDM levels ($P \leq .05$). The increases in IL-7 and IL-15 levels were inversely correlated to the absolute lymphocyte count, unlike monocyte or myeloid counts. Furthermore, we have shown that CD3 T cells present in the ASC graft are activated, died rapidly when they are cultured without cytokine *in vitro* and that addition of IL-7 or IL-15 could induce their survival and proliferation. In conclusion, the early lymphodepletion period, occurring 4 to 11 days post HDM+ASCT, is associated with an increase of circulating immune cytokines and could be an optimal window to enhance the survival and proliferation of polyclonal T cells present in the ASC autograft and also of specific anti-myeloma T cells previously expanded *in vitro*.

Introduction

High-dose melphalan (HDM) followed by autologous hematopoietic stem cell transplantation (ASCT) has improved the rate of complete remission and overall survival of patients with multiple myeloma (MM) (1) and is now a recognized treatment for this pathology. However, ASCT current procedures allow hematopoiesis reconstitution but do not support efficient immune reconstitution, leaving patients more susceptible to infections. HDM induces severe and persistent immunosuppression characterized by a delayed recovery of CD4 T cells that remain below normal counts for months to years after ASCT (2, 3), a restricted T-cell repertoire (4) and impaired T-cell functions including an increased susceptibility to apoptosis (5), a reduced proliferation intensity upon stimulation with mitogens or defined antigens and a default in Th1 cytokine production that lasts at least one year post ASCT in patients with MM (6, 7). The B cell immune response is also altered after ASCT since levels of plasma antibodies after one recall vaccination are below those found in healthy donors (3). Whereas hematopoietic stem cells (HSC) may differentiate into *de novo* naïve T cells, the thymopoiesis in 50-60 years adults is very low and the recovery of T cells post-ASCT is mainly due to expansion of HDM-resistant patient's T cells and/or of lymphocytes that are present in the leukapheresis product (4, 8). In support with this statement, Porrata *et al* demonstrated that the dose of infused lymphocytes contained in the autograft is directly correlated to the number of circulating lymphocytes recovered 15 days after ASCT and both counts were prognosis factors, with an improved survival in patients having a high lymphocyte count (9, 10). Furthermore, we have shown that leukapheresis products mobilized by cyclophosphamide and G-CSF contained an increased proportion of functional regulatory T cells that could slow down the effector immune cell recovery

(11). Thus, there is a need to improve the immune reconstitution post-ASCT while stimulating an anti-tumor immune response. On the other hand, a chemotherapy induced-lymphopenia is required to obtain clinical efficacy of adoptive anti-tumor T cell transfer in patients with metastatic melanoma (12). Infused anti-tumor T cells take advantage of the emptiness and the high homeostatic proliferation following lymphopenia to expand massively *in vivo* and reach the tumor sites (13). Gattinoni *et al* showed in mice models that lymphopenia occurring after a 5 Gy-irradiation leaves unconsumed IL-7 and IL-15 which increase Th1 cytokine production and anti-tumor cytotoxicity capacity of adoptively transferred antigen-specific CD8 T cells (14). The same group recently reported that plasma concentrations of these homeostatic cytokines were increased after irradiation followed by ASCT in mice and that the graft supported the *in vivo* expansion and increased functionality of infused anti-tumor T cells which eradicated established tumors (15). A recent clinical study achieved in MM patients showed that the early administration – at day 12 post-ASCT – of antigen-primed *in vitro* amplified T cells resulted in a dramatic cellular and humoral immune recovery while a late administration – at day 100 post-ASCT – had no effect (16). Therefore, we hypothesized that HDM followed by ASCT results in an increased availability of homeostatic cytokines which may be favourable to an adoptive immunotherapy. To explore this hypothesis and to define a therapeutic window for adoptive T-cell therapy, we have analyzed the recovery of lymphocyte subpopulations, measured the plasma level of immune cytokines after HDM in patients with MM and evaluated the proliferative capacity of T cells contained in the graft. We show that circulating IL-6, IL-7 and IL-15 mean levels increase 35, 8, and 10 times, respectively at day 8 after ASCT and that IL-2 level was below detection limit. We have also shown that T cells contained in the graft are activated, rapidly

died in culture *in vitro* without cytokines, but that addition of IL-7 or IL-15 could rescue them from apoptosis. Altogether, these data indicate that the optimal window for grafting T cells and improve their survival and expansion *in vivo* should be at day 8 post-ASCT, *i.e.* day 10 post-HDM.

Materials and Methods

Patients and collection of peripheral blood samples

Twelve patients with MM (median age: 62 years) who underwent ASCT were included in this study, according to the French ethical laws and after patient's written consent. The series comprised eight male and four female patients. One patient had kappa free light chains MM, one IgA κ MM, one IgA λ MM, three IgG λ MM and six IgG κ MM. Autograft conditioning regimen consisted of 200 mg/m² of melphalan for two days. G-CSF was given from day+5 until hematological engraftment (absolute neutrophil count \geq 500/mm³ for 3 consecutive days). Blood samples were collected after written informed consent, on the day of melphalan administration (day -2), on the day of the autograft (day 0), at day 3 or 4 and 10 or 11 for a first series of 6 patients, or every 2-3 days for 17 days post ASCT for a second series of 6 patients and around day 30. Absolute whole blood cell, monocyte and lymphocyte counts were determined using an ABX PENTRA 60 automaton (HORIBAABX, Montpellier, France). Plasma was frozen at -20°C until use.

Pre-mobilization peripheral blood cells and 100 \times 10⁶ cells from the leukapheresis products of five other patients with MM were collected for functional assays. The mobilization procedure consisted of a single 4-g/m² cyclophosphamide infusion followed by daily subcutaneous injections of 10 μ g/kg/day of G-CSF, until completion of HSC collection (\geq 8 \times 10⁶ CD34⁺ cells/kg) by leukapheresis. Peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation using Lymphocyte Separation Medium (Lonza, Walkersville, MD).

Flow cytometry analysis

The phenotype of T cells was evaluated with the following monoclonal antibodies (MoAbs): phycoerythrin (PE)-conjugated anti-CD3, anti-CD4, anti-CD8, anti-V α 24,

and anti-p α TCR, fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (Beckman Coulter, Villepinte, France). Corresponding isotype-matched murine Abs, recognizing no human antigen, were used as negative controls. Briefly, appropriate amounts of MoAbs were added to 5×10^5 cells followed by a 30-min incubation at 4°C. Red cells were then lysed, cells were washed and 10^4 events in the lymphocyte gate were acquired on a FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA). Analyses were performed with the CellQuest software. Treg were determined as described (11).

Assessment of plasma immune cytokines

Plasma IL-2 and IL-6 amounts were measured by quantitative enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, Minnesota). Plasma IL-7 and IL-15 levels were measured by high sensitivity (≥ 0.1 pg/mL) Quantikine and QuantiGlo ELISA kits (R&D Systems), according to the manufacturer's instructions. A standard curve was prepared for each plate, plotting OD versus different concentrations of recombinant human appropriate cytokine. All standards and samples were tested in duplicate.

In vitro T-cell proliferation assay

Patients' pre-mobilization and post-mobilization PBMC were thawed and seeded at 10^6 cells/ml in 24-well culture plates containing RPMI1640 (Invitrogen, Carlsbad, CA) supplemented with 5% human serum. In some culture groups, 500 U/ml of IL-2 (Chiron, St Louis, MO), 25 ng/ml of IL-15 (R&D) or 25 ng/ml IL-7 (R&D) were added with or without 1 μ g/ml of anti-CD3 MoAb (OKT3, Ortho Biotech Raritan, NJ). Cells were harvested six days later, counted, and cell cycle of CD3 cells determined with PI-labeling.

Statistical analysis

Comparisons of lymphocyte counts and cytokine plasma concentrations were performed using the non-parametric Mann-Whitney test for pairs. A Pearson test was used to set up correlations.

Results

Circulating lymphocyte counts after HDM and ASCT

As shown in Fig. 1A, the mean absolute count of lymphocytes measured in 6 patients dropped to a nadir (14 ALC/mm³) at day 8 post-ASCT, *i.e.* day 10 post-HDM, and resumed to pre-HDM values at days 14-15. Six lymphocyte subpopulations - CD3, CD4, CD8, NK-T, Treg and $\gamma\delta$ T cells - were evaluated in a distinct series of 6 patients at the 4 following points: (the number of blood harvesting was reduced due to ethical limitation) before HDM, at day 3 or 4, day 10 or 11, and between day 26 and day 38. The decrease and expansion of the counts of the subpopulations paralleled that of the ALC (Fig. 1B). CD4 counts remained below 250 / μ L in all six patients and there was a significant decrease in the CD4:CD8 median ratio (from 1.2 before HDM to 0.3 at day 26-38 post ASCT, $P \leq .05$).

Immune cytokines post-HDM

Circulating levels of IL-2, IL-6, IL-7 and IL-15 were measured using ELISA. No IL-2 could be detected (Elisa sensitivity ≥ 7 pg/mL) in 5 patients throughout the 30-day follow-up. An increase in the plasma levels of the other 3 cytokines occurred after HDM for all the eleven patients tested (Fig. 1A and Fig. 2A-C). Due to ethical limitations in the number of allowed blood harvesting, we first determined in a series of 5 patients the plasma cytokine levels at day -2, day 0, day 3 or 4, day 10 or 11 and around one month post-ASCT. Then, in a second series of 6 patients, plasma samples were harvested every 2-3 days after day 4 in order to better determine the day of maximal concentration for each cytokine. Cytokine levels in these 2 patients' series are shown in Fig. 2. IL-6 peaked at day 6-8 in agreement with our previous data (17) (Fig. 2Aii). As shown in Fig. 1A, the mean maximal IL-6 concentration at day 8 was 7.9 pg/mL (range, 4.7-13.4), *i.e.* 35 fold higher than that measured before

HDM ($P \leq .05$). Mean IL-7 concentration peaked also at day 8 with an 8-fold increase (12.9 pg/ml, range, 8–22, $P \leq .05$) compared to pre-HDM levels (Fig. 1 A). The day of IL-15 maximal concentration was more variable, ranging from day 8 to day 12-13, depending on the patients (Fig. 2Aii). At day 8, the mean IL-15 level (36 pg/ml, range, 23.6–55.6 pg/mL) was increased 10 fold compared to pre-HDM levels ($P \leq .05$) (Fig. 1A).

Correlation between plasma cytokine concentrations and absolute lymphocyte counts.

The mean concentrations of plasma IL-6, IL-7 and IL-15 were each inversely correlated to the mean absolute lymphocyte counts (ALC) ($P = .026$, $P = .014$ and $P = .002$, respectively, Fig. 1A). These mean cytokine levels were not correlated to the whole leukocyte counts (WLC) or the absolute monocyte counts (data not shown). When patients' individual values were considered, IL-7 and IL-15 levels were inversely correlated to ALC in 4 out of 6 patients but IL-6 was not. Also, absolute monocyte counts were not correlated to cytokine levels except for one patient with a significant inverse correlation between IL-7 or IL-15 levels and monocyte counts. WLC were inversely correlated to IL-6 levels in two patients, to IL-7 levels in two patients and to IL-15 levels in 3 patients (data not shown).

Response of leukapheresis T cells to cytokines

We then investigated the proliferation and growth potential of T cells present in the autograft mobilized by cyclophosphamide and G-CSF of 5 patients. We have previously shown that mobilized CD3 T cells contained 2-fold more activated cells (CD25⁺ cells) than those before mobilization (11). The mobilized activated CD3⁺ cells were non-cycling in vivo. The majority of CD3 cells (98.6%) were in the G0/G1 phase and only 1.2% of CD3 cells were in the S phase (Fig. 3A). We looked for

whether immune cytokines could induce proliferation and growth of the mobilized T cells in 6-day cultures *in vitro* (Fig. 3A and Fig. 3B). Without adding cytokines, 80% of mobilized T cells died within 6-day cultures compared to 50% of pre-mobilization T cells (Fig. 3B). Addition of IL-2, IL-7 or IL-15 prevented T cell death and increased cell cycling (Fig. 3A and 3B). The IL-2-, IL-7- or IL-15-induced proliferation was higher ($P = .043$) in T cells harvested before than after mobilization. Addition of anti-CD3 MoAb (OKT3) and each of the 3 cytokines resulted in a large growth and proliferation of T cells harvested before or after mobilization in short-term cultures.

Discussion

T-cell functions are impaired after HDM and ASCT in patients with MM despite a recovery of normal numbers of T lymphocytes (5-7). We confirm here that all T-cell subpopulations were depleted by HDM infusion and recovered within one month after ASCT, while CD4 T cell counts remain low. CD4 T cell counts were already below normal before HDM in 3/6 patients, likely due to the lymphopenia induced by high dose cyclophosphamide (4 g/m^2) used for hematopoietic progenitor mobilization as reported (11).

This study confirms previous results showing elevated IL-6 plasma levels post-ASCT (17, 18). This increase can be explained by the HDM-induced stress and also by the deletion of cells able to consume IL-6. We show here for the first time that IL-7 and IL-15 plasma levels increase and peak at a median day 8 after HDM and ASCT in patients with MM, supporting results found in mice by Restifo and coworkers (15). One likely mechanism is the deletion of IL-7- and IL-15-consuming cells, in particular lymphocytes since the cytokine concentrations are inversely correlated to ALC and the maximal increase in plasma cytokine level is observed at the time of the lymphocyte nadir. These results challenge recent observations published by van Rhee and coworkers who did not detect increased serum IL-15 concentrations post-ASCT in 2 patients with MM (19). This discrepancy could be due to a different sensitivity of the ELISA used. It has been documented that IL-7 and IL-15 serum levels are increased in children receiving allogeneic HSC transplant, with peaks occurring within the first 14 days after HSC transplantation (20, 21). High IL-15 serum level even predicts the occurrence of acute graft-versus-host disease (21). Since conditioning regimens in these studies were different from HDM, the lymphopenia is

the common feature explaining the increase in circulating cytokine, by defect in consumption, whatever the drugs utilized.

One can ask here whether these concentrations of circulating cytokines are bioactive *in vivo*. The amount of circulating cytokine in the blood is a very minor part of the amount of the cytokine produced in the whole body. This was demonstrated in patients with MM treated with HDM + ASCT and with anti-IL-6 MoAb (17). In the current study, a mean IL-6 concentration of 7.9 pg/ml is detected on day 6-8 post ASCT. Given a blood volume of 5 liters, the mean amount of circulating IL-6 is about 39 ng. In patients with MM treated with HDM + ASCT and with anti-IL-6 MoAb, we have calculated a median daily IL-6 production of 35 μ g/day at day 9 post ASCT (17). Thus, in the case of IL-6, the amount of circulating IL-6 is 1/1000 of the total amount of IL-6 produced per day. These estimations fit well with the increase of CRP production after HDM and ASCT (17) - CRP production is controlled by IL-6 in human *in vivo* (22) – and with an efficacy dose of 5 μ g/kg/day for recombinant IL-6 in humans (23). Given these data for IL-6, one can anticipate that the increased concentrations of circulating IL-7 and IL-15 at day 8 post-ASCT indicates an increase biological activity of these cytokines *in vivo*. What is the usefulness of these observations? Increasing data support the idea that the early period post-lymphodepletion is propitious to promote *in vivo* amplification of adoptively transferred T cells and to enhance their functions. Several studies in mice and humans showed that homeostatic expansion is associated with faster and more efficient immune response and that immunization with tumor antigens during lymphopenia generates CD8 T cells with enhanced anti-tumor capacities (12, 24-27). Also, an infusion of polyclonal costimulated T cells in the early period post-ASCT accelerates the recovery of CD4 and CD8 T cells following chemotherapy-induced

lymphodepletion in patients with lymphoma (28). In patients with MM, CD4 T cell counts were doubled at day 40 in 10 patients with MM by infusing *in vitro* amplified polyclonal T cells 12 days post-ASCT (16). We have shown here that the CD3 cells present in the hematopoietic progenitor graft died within 6 days *in vitro* if they are cultured without cytokines but are able to survive and proliferate vigorously in the presence of IL-15 or IL-7. Such a death of grafted T cells should occur in the current ASCT procedures, since T cells are grafted two days after HDM, at a time when circulating immune cytokine concentration is low. As the lymphocyte count in the graft or after HDM and ASCT is an independent prognostic survival factor (9, 10), an easy improvement will be to graft one part of the leukapheresis ($3 \times 10^6/\text{kg}$ CD34⁺ cells) 2 days post-HDM and another part containing at least $20 \times 10^6/\text{kg}$ CD3 cells when IL-7 and IL-15 plasma concentrations are increased - *i.e.* at day 8 post-ASCT. This will make it possible the grafted T cells to survive and expand, taking advantage of the lymphopenia-associated burst of immune cytokine production.

A second interest will be to use this window of post-HDM+ASCT immune cytokine burst to develop adoptive immunotherapy with anti-myeloma T cells. Indeed, IL-7, produced by stromal cells, is required for homeostatic expansion of naïve and memory CD4 and CD8 T cells and is critical for their survival (29). IL-15 drives antigen-independent homeostatic memory CD8⁺ $\alpha\beta$ T cell proliferation (29, 30). IL-7 and IL-15 are also required for $\gamma\delta$ T cell homeostatic expansion (31). We previously demonstrated that $\gamma 9\delta 2$ T cells exert anti-myeloma specific cytotoxicity, can be expanded 100-fold with IL-2 and biphosphosphonate *ex vivo* (32) and are present in mobilized autografts (11). These $\gamma 9\delta 2$ T cells could be expanded *ex vivo* 2 weeks before HDM and then grafted at day 8 post ASCT. We have checked that they expressed CXCR4 to be able to home into the bone marrow (32). HSC harvests also

contain anti-tumor CD8⁺ T cells, in particular those directed against the HM1.24 antigen (33, 34) or cancer testis antigens (unpublished observations). CD8 T cells recognizing several myeloma antigens as MUC-1 (35), cancer-testis antigens (36-38) or IgG epitopes (39), detected in peripheral blood of patients may also be present in HSC harvests. Once stimulated *ex vivo* with antigen pulsed dendritic cells (40), these anti-myeloma cell CD8⁺ T cells are able to kill myeloma cells (33). These anti-myeloma cell T cells could be expanded *ex vivo* and also *in vivo*, if they are infused at the time of the burst of circulating immune cytokines.

In conclusion, this study defines an optimal window for grafting autologous stem cells but also autologous polyclonal T cells, for the *in vivo* expansion of adoptively transferred cytotoxic anti-tumor T cells, such as $\gamma\delta$ T cells or antigen-driven CD8 T cells amplified *in vitro*, and for anti-myeloma vaccination.

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Legends to figures

Figure 1: Lymphocyte counts and cytokine plasma levels before and after HDM and ASCT.

A. Absolute lymphocyte counts (ALC) and cytokine plasma levels.

Data are the mean values \pm SD of absolute lymphocyte counts (ALC), of IL-6, IL-7 and IL-15 plasma concentrations measured in 6 patients before HDM infusion (day -2), on the day of ASCT (day 0) on day 3 or 4, on day 6, on day 8, on day 10 or 11, on day 12 or 13, on day 14 or 15 and around one month after ASCT (day 32-34).

§ and * indicate that the mean ALC or cytokine value is significantly different ($P \leq .05$) from that of day-2, using a Mann-Whitney test for pairs.

B. T-cell subpopulation counts.

Data are the median values of CD3⁺, CD4⁺, CD8⁺ T cell, $\gamma\delta$ T cell, Treg and NK-T cell counts measured in 6 patients before HDM infusion (day -2), on the day of ASCT (day 0) on day 3 or 4, on day 10 or 11 and around one month after ASCT (day 26-39). * Indicates that the mean value is significantly different ($P \leq .05$) from that before HDM infusion, using a Mann-Whitney test for pairs.

Figure 2: Plasma levels of IL-6, IL-7 and IL-15 in 11 patients before and after HDM and ASCT.

Plasma concentrations of IL-6 (A), IL-7 (B) and IL-15 (C) of individual patients are represented with specific symbol. Day 0 is the day of ASCT and day -2 the day of HDM infusion. The Ai, Bi and Ci panels display the cytokine concentrations (pg/mL) in plasma samples from a series of 5 patients. The Aii, Bii and Cii panels display cytokine concentrations in plasma samples of a second series of 6 patients (plasma samples were harvested every 2-3 days after day 4).

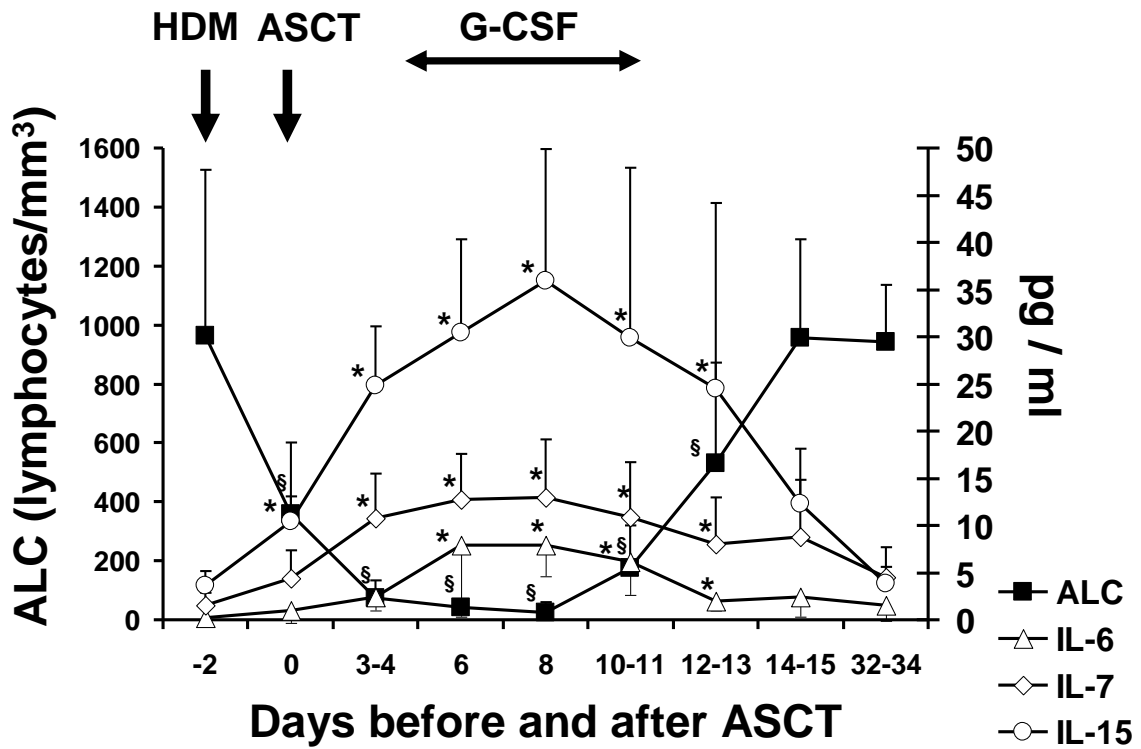
Figure 3: Proliferation and growth of circulating CD3 cells harvested before and after mobilization induced by cytokines with or without OKT3.

PBMC of five patients with MM were harvested before mobilization by cyclophosphamide and G-CSF infusion (PBMC) and at the time of HSC collection (leukapheresis). PBMC were cultured in RPMI 1640 culture medium and 5% human serum with either IL-2, IL-15 or IL-7 with or without OKT3 (1 µg/mL). Cells were harvested 6 days later, counted, the CD3 were determined by FACS labelling and their cell cycle determined with CD3 and PI labelling. Results are the median percentages of CD3 cells in the S phase (A) and the median increase of the CD3 cell number (B) after 6 days of culture.

* Indicates that the percentage of CD3 in the S phase or the increase in CD3 numbers is significantly different ($P \leq .05$) at the time of HSC collection than those before mobilization using a Wilcoxon test for pairs. ** Indicates that the percentage of CD3 in the S phase or the increase in CD3 numbers from PBMC cultured with cytokines and OKT3 is significantly different ($P \leq .05$) from those of the same PBMC cultured with cytokines alone.

FIGURE 1

A



B

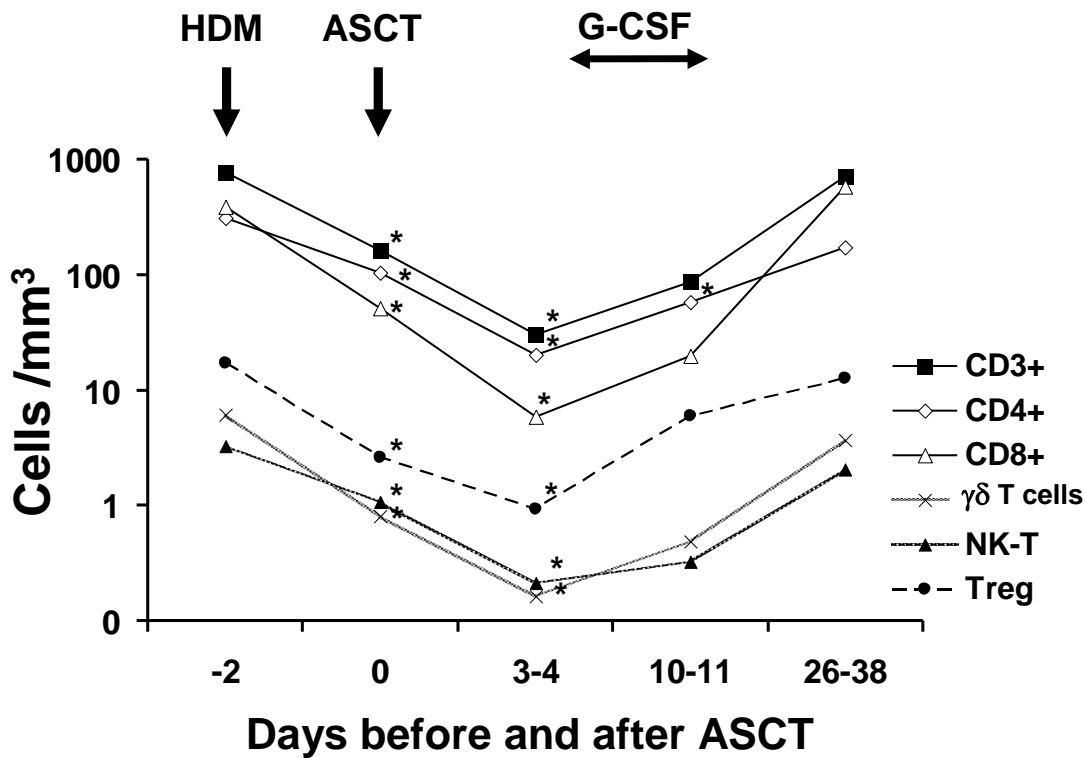
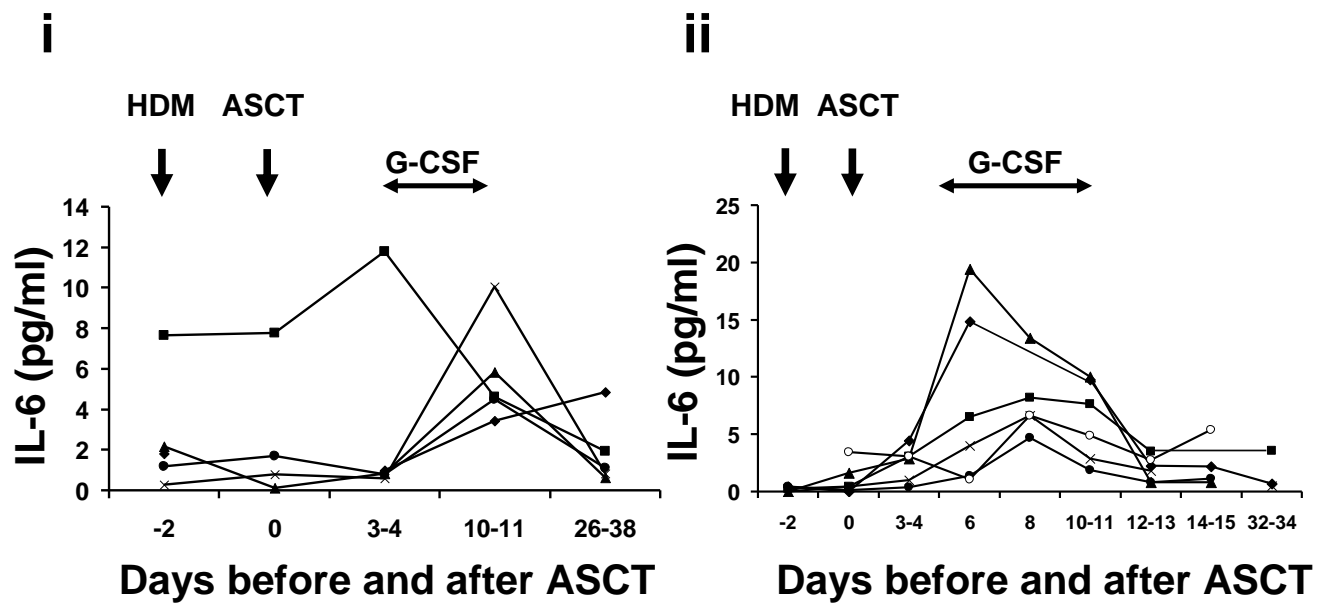


FIGURE 2

A



B

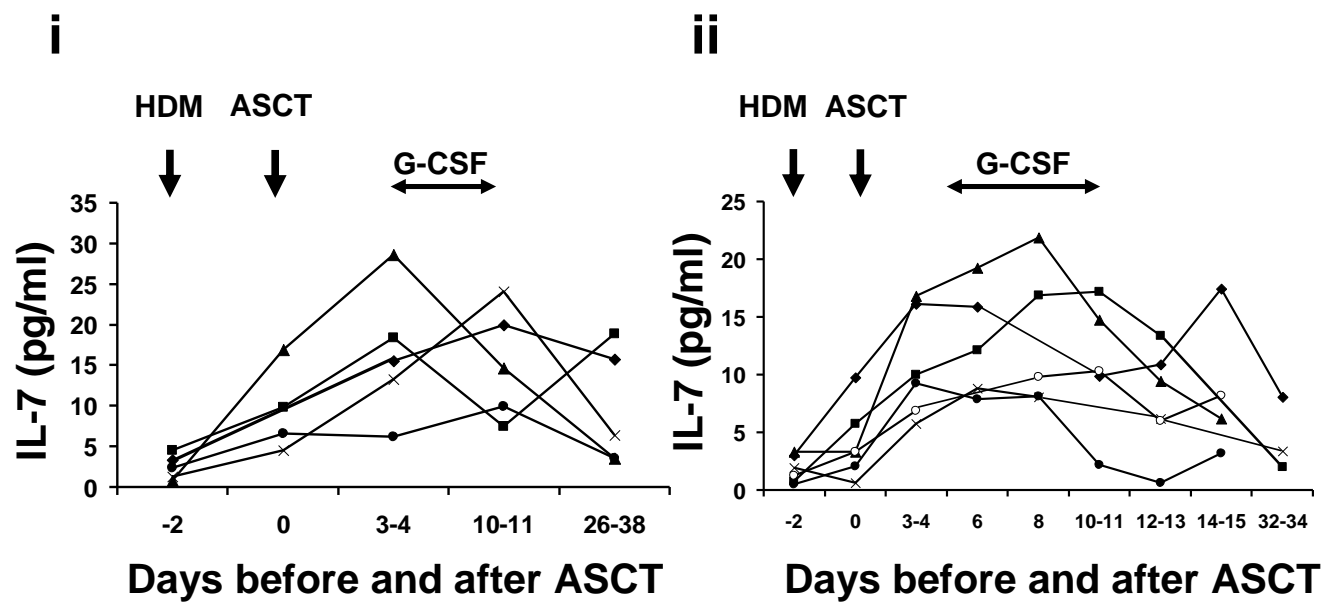


FIGURE 2

C

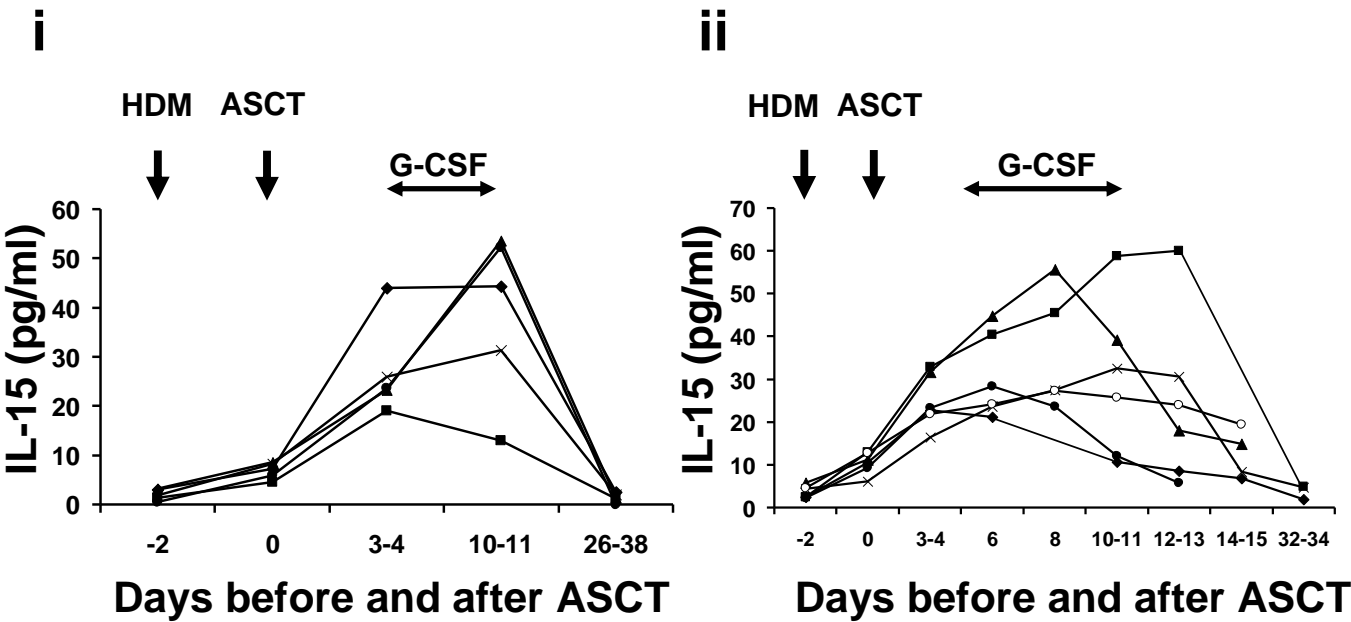
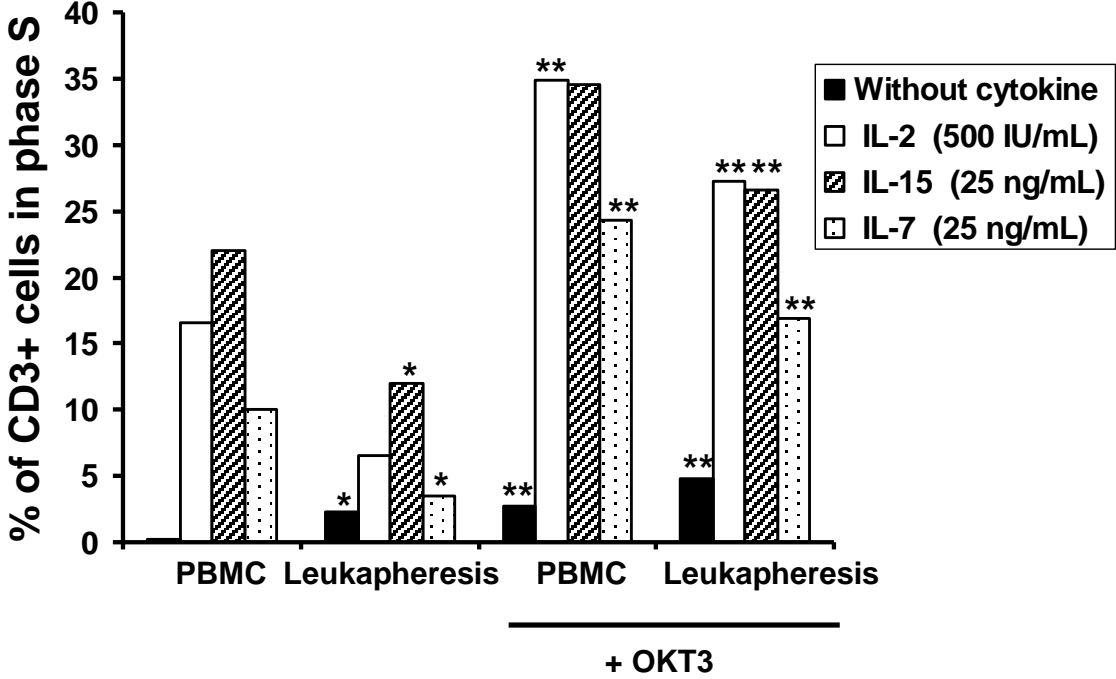


FIGURE 3

A



B

