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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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High-dose melphalan (HDM) followed by autologous stem cell transplantation (ASCT) is an standard treatment for patients with multiple myeloma. 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 antitumor immunotherapy. The objective of this study was to determine an immunotherapy window after HDM and ASCT, evaluating T cell lymphopenia, and measuring circulating immune cytokine concentrations in patients with multiple myeloma. 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 with pre-HDM levels ($p \leq 0.05$). 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, die 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 4–11 d post-HDM and 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. The Journal of Immunology, 2010, 184: 1079–1084.

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 on stimulation with mitogens or defined Ags and a default in Th1 cytokine production that lasts at least 1 y post-ASCT in patients with MM (6, 7). The B cell immune response is also altered after ASCT, because levels of plasma Abs after one recall vaccination are below those found in healthy donors (3). Whereas hematopoietic stem cells (HSCs) may differentiate into de novo naïve T cells, the thymopoiesis in adults 50–60 y old is very low (8) and does not occur before 2 mo post-ASCT (9, 10). The early recovery of T cells post-ASCT is mainly due to expansion of patient’s HDM-resistant T cells and/or of lymphocytes that are present in the leukapheresis product (4, 11, 12). In support of 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 d after ASCT and both counts were prognosis factors, with an improved survival in patients having a high lymphocyte count (13, 14). 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 immune effector cell recovery (15). Thus, there is a need to improve the immune reconstitution post-ASCT while stimulating an antitumor immune response. However, a chemotherapy-induced lymphopenia is required to obtain clinical efficacy of adoptive antitumor T cell transfer in patients with metastatic melanoma (16). Infused antitumor T cells take advantage of the emptiness and the high homeostatic proliferation after lymphopenia to expand massively in vivo and reach the tumor sites (17). Gattinoni et al. (18) 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 antitumor cytolyis capacity of adoptively transferred Ag-specific CD8 T cells. 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 antitumor T cells that eradicated established tumors (19). A recent clinical study achieved in MM patients showed that the early administration, at day 12 post-ASCT, of Ag-primed in vitro amplified T cells resulted in a dramatic cellular and humoral immune recovery, whereas a late administration, at day 100 post-ASCT, had no effect (20). Therefore, we hypothesized that HDM, followed by ASCT, results in an increased availability of homeostatic cytokines that may be favorable 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 the 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, namely, day 10 post-HDM.

Materials and Methods
Patients and collection of peripheral blood samples
Twelve patients with MM (median age: 62 y) who underwent ASCT were included in this study, according to the French ethical laws and after the patient’s written consent. The series comprised eight male and four female patients. One patient had κ-free L chains MM, one IgAκ MM, one IgAλ MM, three IgGκ MM, and six IgGλ MM. Autograft conditioning regimen consisted of 200 mg/m² melphalan for 2 days. G-CSF was given from day +5 until HSC collection (day 14 or 15, and around 1 mo after ASCT (day 32–34). The series comprised eight male and four female patients. PBMCs were obtained by density centrifugation of 200 mg/m² melphalan for 2 days. G-CSF was given from day +5 until HSC collection (day 14 or 15, and around 1 mo after ASCT (day 32–34). The series comprised eight male and four female patients. PBMCs were obtained by density centrifugation with propidium iodide-labeling.

Flow cytometry analysis
The phenotype of T cells was evaluated with the following mAbs: PE-conjugated anti-CD3, anti-CD4, anti-CD8, anti-Vα24, and anti-pan TCR, FITC-conjugated anti-CD25 (Beckman Coulter, Villepinte, France). Corresponding isotype-matched murine Abs, recognizing no human Ag, were used as negative controls. Briefly, appropriate amounts of mAbs were added to 5 × 10⁶ cells, followed by a 30-min incubation at 4°C. Red cells were then lysed, cells were washed, and 10⁶ events in the lymphocyte gate were acquired on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Analyses were performed with the CellQuest software. Regulatory T cells (Treg) were determined as described (15).

Assessment of plasma immune cytokines
Plasma IL-2 and IL-6 amounts were measured by quantitative ELISA (R&D Systems, Minneapolis, MN). 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’ premobilization and postmobilization PBMCs were thawed and seeded at 10⁶ cells/ml in 24-well culture plates containing RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% human serum. In some culture groups, 500 U/ml IL-2 (Chiron, St. Louis, MO), 25 ng/ml IL-15 (R&D Systems), or 25 ng/ml IL-7 (R&D Systems) were added with or without 1 μg/ml anti-CD3 mAb (OKT3, Ortho Biotech, Raritan, NJ). Cells were harvested 6 d later, counted, and cell cycle of CD3 cells determined with propidium iodide-labeling.

Statistical analysis
Comparisons of lymphocyte counts and cytokine plasma concentrations were performed using the nonparametric Mann-Whitney U 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 lymphocyte count (ALC) measured in six patients dropped to a nadir (14 ALC/mm³) at day 8 post-ASCT, namely, day 10 post-HDM, and resumed to pre-

![FIGURE 1](http://www.jimmunol.org/)
HDM values at days 14–15. Six lymphocyte subpopulations: CD3, CD4, CD8, NK-T, Treg, and γδ T cells, were evaluated in a distinct series of six patients at the four following points: (the number of blood harvesting was reduced because of 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 were below 250/μl 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, \( p \leq 0.05 \)).

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 $7 \text{ pg/ml}$) in five patients throughout the 30-d follow-up. An increase in the plasma levels of the other three cytokines occurred after HDM for all the 11 patients tested (Figs. 1A, 2A–C). Because of the ethical limitations in the number of allowed blood harvesting, we first determined in a series of five patients the plasma cytokine levels at day −2, day 0, day 3 or 4, day 10 or 11, and ~1 mo post-ASCT. Then, in a second series of six patients, plasma samples were harvested every 2–3 d after day 4 to better determine the day of maximal concentration for each cytokine. Cytokine levels in these two patients’ series are shown in Fig. 2. IL-6 peaked at days 6–8 in agreement with our previous data (21) (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), that is, 35-fold higher than that measured before HDM (\( p \leq 0.05 \)). Mean IL-7 concentration also peaked at day 8 with an 8-fold increase (12.9 pg/ml, range, 8–22, \( p \leq 0.05 \)) compared with pre-HDM levels (Fig. 1A). The day of IL-15 maximal concentration was more variable, ranging from day 8 to day 12–13, depending on the patients (Fig. 2Cii). At day 8, the mean IL-15 level (36 pg/ml, range, 23.6–55.6 pg/ml) was increased 10-fold compared with pre-HDM levels (\( p \leq 0.05 \)) (Fig. 1A).

**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 is the day of HDM infusion. Ai, Bi, and Ci display the cytokine concentrations (pg/ml) in plasma samples from a series of five patients. Aii, Bii, and Cii display cytokine concentrations in plasma samples of a second series of six patients (plasma samples were harvested every 2–3 d after day 4).
Correlation between plasma cytokine concentrations and ALCs

The mean concentrations of plasma IL-6, IL-7, and IL-15 were each inversely correlated to the mean ALC (p = 0.026, p = 0.014 and p = 0.002, respectively, Fig. 1A). These mean cytokine levels were not correlated to the whole leukocyte counts 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 four of six 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: whole leukocyte counts 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 compared the proliferation and growth potentials of T cells present in the autograft mobilized by cyclophosphamide and G-CSF with those of circulating T cells harvested before mobilization, to evaluate the impact of the mobilization regimen on T cell function. We have previously shown that mobilized CD3+ T cells contained 2-fold more activated cells (CD25+ cells) than those before mobilization (15). The mobilized activated CD3+ cells were noncycling 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, which was, however, significantly higher than that observed in circulating CD3+ cells before mobilization, presumably because of the cyclophosphamide-induced homeostatic recovery phase (Fig. 3A). We looked for whether immune cytokines could induce proliferation and growth of the mobilized T cells in 6-d cultures in vitro (Fig. 3A, 3B). Without adding cytokines, 80% of mobilized T cells died within 6-d cultures compared with 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, 3B). The IL-2-, IL-7-, or IL-15-induced proliferation was higher (p = 0.043) in T cells harvested before than after mobilization. However, the death rescue by the cytokines was effective with no statistical difference in pre- or postmobilization T cells (Fig. 3B) and the addition of a nonspecific TCR stimulation by an anti-CD3 mAb (OKT3) resulted in a large growth and proliferation of T cells, equal in both groups. Thus, mobilized T cell growth capacity is not impaired, but their survival in medium without cytokines is reduced.

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 in this study that all T cell subpopulations were depleted by HDM infusion and recovered within 1 mo after ASCT, whereas CD4 T cell counts remain low. CD4 T cell counts were already below normal before HDM in three of six patients, likely because of the lymphopenia induced by high-dose cyclophosphamide (4 g/m²) used for hematopoietic progenitor mobilization as reported (15). We have previously shown that for grafts containing a mean of 6.4 × 10⁹/kg CD34+ cells, they contain a mean of 32.7 × 10⁹/kg of CD3 T cells, 7.4 × 10⁹/kg of NK cells, 1 × 10⁹/kg of γδ T cells, and 1.3 × 10⁹/kg of Treg cells (15). Considering a body diffusion volume of ~60 l (unpublished results), this CD3 cell dose, without in vivo expansion, will increase the circulating lymphocyte count of 4 cells/mm³, in an individual of 70 kg. Thus, these T cell numbers may not impact on the early T cell recovery, as suggested by one study on CD34+ cell-selected versus unselected transplantation (10). However, other authors showed that patients receiving CD34+ cell-selected grafts had significantly delayed CD4 and CD8 T cell recoveries by day 100 than patients receiving unselected autografts (i.e., containing passenger myeloid and T cells) (9, 22). Also, the prognostic value of the autograft lymphocyte count (14) suggests that T cells coinfused with the CD34+ cells could undergo a peripheral expansion and play an important role in the early lymphocyte recovery. In the same time, de novo naïve T cells are generated in the thymus and contribute to a complete lymphocyte recovery by 1 or 2 y post-ASCT (8–10). Moreover, CD4 T cell regeneration mainly requires the thymopoiesis and is therefore very slow in adults, whereas some subsets of CD8 T cells expand rapidly by a thymus-independent pathway (11).

This study confirms previous results showing elevated IL-6 plasma levels post-ASCT (21, 23). This increase can be explained by the HDM-induced stress and also by the deletion of cells able to consume IL-6. We show in this study 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 (19). One likely mechanism is the deletion of IL-7- and IL-15-consuming cells, in particular lymphocytes because

![FIGURE 3.](http://www.jimmunol.org/)
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 two patients with MM (24). 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 d after HSC transplantation (25, 26). High IL-15 serum level even predicts the occurrence of acute graft-versus-host disease (26). Because 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 used.

One can ask at this time 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 and ASCT and with anti-IL-6 mAb (21). 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 l, the mean amount of circulating IL-6 is ~39 ng. In patients with MM treated with HDM and ASCT and with anti-IL-6 mAb, we have calculated a median daily IL-6 production of 35 μg/d at day 9 post-ASCT (21). 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 are in agreement with the increase of C-reactive protein production after HDM and ASCT (21). C-reactive protein production is controlled by IL-6 in human in vivo (27), and with an efficacy dose of 5 μg/kg for recombinant IL-6 in humans (28). 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 increased biological activity of these cytokines in vivo. What is the usefulness of these observations? Increasing data support the idea that the early period postlymphodepletion 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 Ags during lymphopenia generates CD8 T cells with enhanced antitumor capacities (16, 29–32). Recently, two clinical trial studies in metastatic melanoma revealed that lymphodepletion-induced high levels of circulating IL-7 and IL-15 were associated with a longer in vivo persistence of infused autologous antitumor T cells (33, 34). Wallen et al. made an intrapatient comparison of two consecutive infusions of T cell clones, the first without fludarabine and the second with fludarabine as a conditioning regimen. They reported median plasma IL-7 and IL-15 levels of 3 pg/ml and 6 pg/ml, respectively, at the time of the second T cell infusion (34). The persistence of T cells from the second infusion was 2.9-fold longer than that of T cells from the first infusion, underlying the benefit effect of the conditioning regimen. Dudley et al. showed mean plasma IL-7 and IL-15 levels of ~25 pg/ml and 30 pg/ml, respectively, at the time of tumor-infiltrating lymphocyte administration after cyclophosphamide and fludarabine conditioning regimen. These levels increased when the lymphodepletion was augmented by total body irradiation, which resulted in a significantly improved clinical response rate (33). As we observed similar IL-7 and IL-15 plasma concentrations in MM patients, one can assume that these levels will efficiently support the growth of infused T cells after HDC and ASCT. Although a direct correlation between the increased cytokine level and T cell expansion has not been proved in humans, it has been evidenced in preclinical animal models (18, 19).

Also, an infusion of polyclonal costimulated T cells in the early period post-ASCT accelerates the recovery of CD4 and CD8 T cells after chemotherapy-induced lymphodepletion in patients with lymphoma (35). 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 d post-ASCT (20). We have shown in this study that the CD3 cells present in the hematopoietic progenitor graft died within 6 d 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, because T cells are grafted 2 d after HDM, at a time when circulating immune cytokine concentration is low. An easy improvement of the T cell recovery post-ASCT, will be to graft one part of the leukapheresis (3 × 10^6/kg CD34+ cells) 2 d post-HDM and another part containing at least 20 × 10^6/kg CD3 cells when IL-7 and IL-15 plasma concentrations are increased, namely, at day 8 post-ASCT. This will make it possible for 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 and ASCT immune cytokine burst to develop adoptive immunotherapy with antimielyoma T cells. Indeed, IL-7, produced by stromal cells, is required for homeostatic expansion of naive and memory CD4 and CD8 T cells and is critical for their survival (36). IL-15 drives Ag-independent homeostatic memory CD8+ αβ T cell proliferation (36, 37). IL-7 and IL-15 are also required for γδT cell homeostatic expansion (38). We previously demonstrated that γδT2 cells exert antimielyoma-specific cytotoxicity, can be expanded 100-fold with IL-2 and biphosphosphonate ex vivo (39), and are present in mobilized autografts (15). These γδT2 cells could be expanded ex vivo 2 wk 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 (39). HSC harvests also contain antimielyoma CD8+ T cells, in particular those directed against the HM1.24 Ag (Ag, 40, 41) or cancer tests Ags (unpublished observations). CD8 T cells recognizing several myeloma Ags as MUC-1 (42), cancer testis Ags (43–45), or IgG epitopes (46) may also be present in HSC harvests. Once stimulated ex vivo with Ag-pulsed dendritic cells (47), these antimielyoma cell CD8+ T cells are able to kill myeloma cells (40). These antimielyoma 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 antimielyoma T cells, such as γδ T cells or Ag-driven CD8 T cells amplified in vitro, and for antimielyoma vaccination.

Disclosures

The authors have no financial conflicts of interest.

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