

Mobilization with cyclophosphamide reduces the number of lymphocyte subpopulations in the leukapheresis product and delays their reconstitution after autologous hematopoietic stem cell transplantation in patients with multiple myeloma

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Background. Autologous hematopoietic stem cell transplantation is considered the standard of care for younger patients with multiple myeloma. Several mobilization regimens are currently used, most commonly growth factors alone or in combination with chemotherapy. The aim of our study was to investigate the differences in lymphocyte subpopulation counts between three different mobilization regimens on collection day, in the leukapheresis product and on day 15 after autologous hematopoietic stem cell transplantation.

Patients and methods. In total 48 patients were prospectively enrolled in three different mobilization regimens; (i) filgrastim (20), (ii) pegfilgrastim (19) and (iii) cyclophosphamide + filgrastim (9). Lymphocytes, CD16+/56+ natural killer and CD4+/CD25^{high} T regulatory cells were determined by flow cytometry.

Results. We found a statistically significant difference between the mobilization regimens. Cyclophosphamide reduced lymphocyte and natural killer (NK) cell counts on collection day (lymphocytes $1.08 \times 10^9/L$; NK cells $0.07 \times 10^9/L$) compared to filgrastim (lymphocytes $3.08 \times 10^9/L$; NK cells $0.52 \times 10^9/L$) and pegfilgrastim (lymphocytes $3 \times 10^9/L$; NK cells $0.42 \times 10^9/L$). As a consequence lymphocyte and NK cell counts were also lower in the leukapheresis products following cyclophosphamide mobilization regimen (lymphocytes $50.1 \times 10^9/L$; NK cells $4.18 \times 10^9/L$) compared to filgrastim (lymphocytes $112 \times 10^9/L$; NK cells $17.5 \times 10^9/L$) and pegfilgrastim (lymphocytes $112 \times 10^9/L$; NK cells $14.3 \times 10^9/L$). In all mobilization regimens T regulatory cells increased 2-fold on collection day, regarding the base line value before mobilization. There was no difference in T regulatory cell counts between the regimens.

Conclusions. Mobilization with cyclophosphamide reduces the number of mobilized and collected lymphocytes and NK cells as compared to mobilization with growth factors only and results in their delayed reconstitution following autologous hematopoietic stem cell transplantation. We found no difference between filgrastim and pegfilgrastim mobilization.

Key words: mobilization; myeloma; cyclophosphamide; NK cell; stem cell transplantation

Introduction

Novel agents have significantly increased the response rate and overall survival (OS) of patients

with multiple myeloma (MM), but high-dose chemotherapy and autologous hematopoietic stem cell transplantation (AHST) are still considered the standard of care for younger patients.¹⁻⁵ Several

TABLE 1. Patient characteristics

Characteristics	Total number of patients	G-CSF mobilization	Pegfilgrastim mobilization	Cyclophosphamide mobilization
Numbers	48	20	19	9
Sex, M/F	25 / 23	11 / 9	9 / 10	5 / 4
Median age (Range) years	61 (35–71)	60 (35–69)	64 (51–71)	59 (42–63)
ISS stage 1/2/3	13/17/18	3/8/9	7/7/5	3/2/4
Median cycles of chemotherapy	4	4	4	4
Patients with prior radiotherapy	11	3	8	0
Response VGPR/PR/SD		11/9/0	10/8/1	3/5/1
Poor mobilizers	3	1	2	0

F = female; G-CSF = filgrastim; ISS = international staging system; M = male; PR = partial response; SD = stable disease; VPGR = very good partial response

mobilization regimens are currently used, most commonly growth factors alone or in combination with chemotherapy. Considering growth factors, the most commonly used are filgrastim (G-CSF) and the long acting pegfilgrastim.⁶⁻⁸

The combination of chemotherapy and growth factors is commonly used for mobilization because of the higher yield of stem cells and fewer collection procedures.^{6,9} Usually, chemotherapy regimens include cyclophosphamide at different doses. Intermediate dose cyclophosphamide (3 - 4 g/m²) significantly increases the proportion of patients achieving the target dose of collected stem cells as compared to low dose cyclophosphamide (1.5 g/m²) with a significantly faster neutrophil and platelet engraftment, probably due to a higher dose of infused stem cells.¹⁰

Cyclophosphamide induces bone marrow aplasia followed by delayed reconstitution of lymphocytes resulting in partial recovery on collection day.¹¹ As a consequence lower doses of lymphocytes are collected and reinfused. There is accumulating evidence that the dose of infused lymphocytes and their recovery on day 15 and 30 after AHSCT are independent prognostic factors for overall survival in patients with non-Hodgkin lymphomas and myeloma.¹²⁻¹⁵ Among lymphocyte subpopulations the CD16+/56+ natural killer (NK) cells are particularly strong independent predictors of survival.¹²

T regulatory (Treg) cells express CD4+CD25^{high} and are crucial in the tolerance to self-antigens. Since tumor antigens are derived from self-antigens, Treg cells may decrease the antimyeloma effect. Treg cells directly inhibit NK cell effector function and Treg depletion exacerbates NK cell proliferation and cytotoxicity in in-vitro essays.^{16,17}

In MM the induction of immunosuppression by Treg cells is believed to be associated with myelomagenesis and the progression of MM.^{18,19} The effect of cyclophosphamide on Treg cells depends on the dose of cyclophosphamide and the addition of G-CSF. Low doses of cyclophosphamide have a specific toxicity to Treg cells, thereby decreasing their numbers and potentially increasing an immune response against myeloma cells.²⁰ On the opposite, high doses of cyclophosphamide in combination with G-CSF increase the number of Treg cells by 2-3 fold.¹¹ The aforementioned data support the role of immunosurveillance on the progression of MM and the concept of an autologous graft-versus-myeloma effect in AHSCT.

The aim of this study was to investigate the influence of three different mobilization regimens (i) filgrastim, (ii) pegfilgrastim and (iii) cyclophosphamide + filgrastim on the lymphocyte subpopulations during the mobilization procedure, in the leukapheresis product and after AHSCT. To the best of our knowledge, ours is the first study to compare the effects of three different mobilization regimens on lymphocyte subpopulations in newly diagnosed patients with MM after novel agent induction.

Patients and methods

In all, 48 patients with newly diagnosed symptomatic MM following induction treatment with bortezomib and dexamethasone were enrolled into this prospective single center study to receive stem cell mobilization with three different regimens; (i) filgrastim, (ii) pegfilgrastim and (iii) cyclophosphamide + filgrastim in a 2 : 2 : 1 ratio. Patients

were enrolled by two of the authors (MS, BS) to achieve same baseline characteristics according to age, number of treatment cycles, sex, ISS stage and response to induction treatment (Table 1). Only newly diagnosed MM patients that received induction treatment with 3 - 6 cycles of bortezomib (1.3 mg/m² sc. on days 1, 4, 8 and 11) and dexamethasone (40 mg iv. on days 1–4 of cycle 1 and on days 1, 4, 8 and 11 of the following cycles) were enrolled. Response before enrollment was assessed according to IMWG criteria.²¹ Since a bone marrow biopsy is considered mandatory for defining complete response (CR), none of the patients in our study was considered in CR before mobilization, as a bone marrow biopsy was not mandatory at the time of enrollment. Patients with treatment refractory MM were excluded. This study was approved by the Local Ethics Committee (Number 149/04/11) and all patients gave their informed consent according with the Declaration of Helsinki.

Peripheral blood stem cell mobilization

Filgrastim mobilization: Patients received subcutaneous filgrastim 10 mcg/kg (rounded up to 300 mcg or 480 mcg vial size or combination thereof) daily for five days. Peripheral circulating CD34+ cells monitoring was commenced on day five and apheresis was commenced at CD34+ levels greater than 20 × 10⁶ /L. Additional filgrastim was allowed on day 6.

Pegfilgrastim mobilization: Patients received a fixed subcutaneous dose of 12 mg pegfilgrastim on day one. Peripheral circulating CD34+ cells monitoring was commenced on day five and apheresis was commenced at CD34+ levels greater than 20 × 10⁶/L. No additional growth factors were allowed.

Patients received cyclophosphamide 4 g/m² intravenous with mesna prophylaxis. Subcutaneous filgrastim 10 mcg/kg was started on day 5. Peripheral circulating CD34+ cells monitoring was commenced on day five and apheresis was commenced at CD34+ levels greater than 20 × 10⁶/L. Filgrastim was continued daily until completion of apheresis or for up to day 20.

Blood samples and flow cytometry

Ethylenediaminetetraacetic acid (EDTA) anticoagulated peripheral blood samples were obtained from all patients at three time points; (i) before mobilization, (ii) on collection day and (iii) on day fifteen after AHSCT. A sample of the leukapheresis product was obtained on day one of collection. The

immunolabeling was performed on samples according to the manufacturer's recommendation. Samples were incubated with 10–20 µL of antibody in the dark for 20 minutes. Red blood cells were then lysed. NK cells were enumerated as CD16+/56+ cells and Treg cells were enumerated as CD4+CD25^{high} cells. Samples were analyzed using Immunotech (Beckman-Coulter) antibodies on the Beckman Coulter Cytomics FC500 analyzer.

Stem cell collection target and definition of poor mobilizers

The collection of at least 2 × 10⁶ CD34+ cells/kg recipient body weight was required. Patients with a circulating CD34+ cell count below 20 × 10⁶/L up to six days after mobilization with filgrastim and pegfilgrastim or up to twenty days after cyclophosphamide and filgrastim or patients with a yield of less than 2 × 10⁶ CD34+ cells/kg in three apheresis procedures were considered poor mobilizers and were excluded from further evaluation.²²

High-dose therapy and AHSCT

AHSCT was planned 20–40 days after successful stem cell collection. Patients received melphalan (200 mg/m²) and stem cells were reinfused 24 hours later.^{3,5,23} The mean stem cell dose was 2.6 × 10⁶ CD34+ cells/kg, 2.6 × 10⁶ CD34+ cells/kg and 3.1 × 10⁶ CD34+ cells/kg for patients after filgrastim, pegfilgrastim and cyclophosphamide + filgrastim mobilization respectively. Patients received prophylaxis with levofloxacin and posaconazole, and G-CSF support until leukocyte engraftment at the discretion of the treating physician.

Statistical analysis

The SPSS Statistics 21 (IBM, USA) software package was used for the statistical analysis. One-way ANOVA test was used to calculate differences between cohorts. Additional post-hoc analysis using the Tukey's HSD test was used to validate differences between two cohorts. For difference between only two cohorts the t-test for independent samples was used. Results were considered statistically significant if p < 0.05.

Results

No differences between the three regimens were noted in lymphocyte, NK cell and Treg cell counts

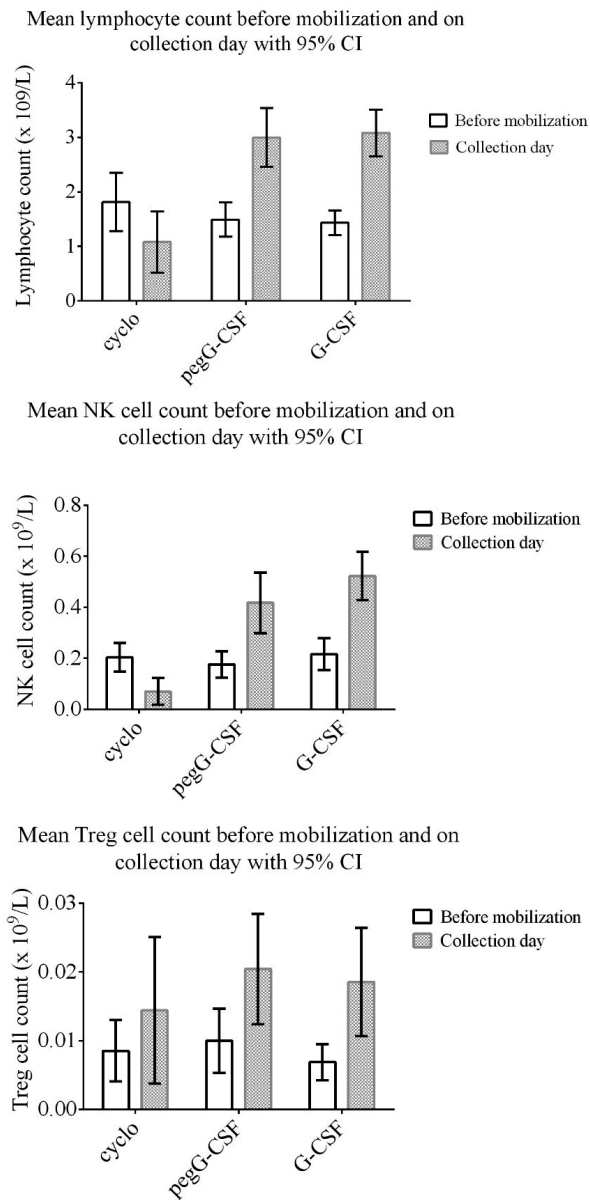


FIGURE 1. The mean lymphocyte, natural killer (NK) cell and Treg cell counts (10⁹/L) before mobilization and on collection day with 95 % confidence interval (CI) for mobilization with cyclophosphamide (cyclo), pegfilgrastim (pegG-CSF) and filgrastim (G-CSF).

before mobilization. Mobilization with cyclophosphamide reduced the lymphocyte and NK cell counts on collection day by 2-fold as compared to baseline value before mobilization. On the contrary mobilization with G-CSF and pegfilgrastim induced a 2- fold increase in lymphocyte and NK cell counts on collection day as compared to baseline value before mobilization (Figure 1). This resulted in different lymphocyte and NK cell counts on collection day between the mobilization regimens (p

< 0.001). In pairwise comparison the differences between mobilization with cyclophosphamide and G-CSF only, and cyclophosphamide and pegfilgrastim only were statistically significant ($p < 0.001$). The differences in lymphocyte and NK cell counts on collection day resulted in a significantly different lymphocyte and NK cell composition of the leukapheresis product (Table 2). All three mobilization regimens increased Treg cell counts on collection day by 2-fold with no differences in absolute values between the three regimens. Comparison between the groups mobilized with G-CSF or pegfilgrastim showed no difference in lymphocyte, NK and Treg cell counts on the collection day, in the leukapheresis product and on day 15 after AHSCT. Because no difference in lymphocyte and NK cell counts during mobilization and in the leukapheresis product was noted between the G-CSF and pegfilgrastim group, and due to the small patient sample, we analyzed lymphocyte counts on day 15 after AHSCT between cyclophosphamide mobilization and growth factors only (G-CSF and pegfilgrastim combined) mobilization. Mobilization with growth factors resulted in a higher lymphocyte count on day 15 post AHSCT compared to cyclophosphamide mobilization ($p < 0.04$).

Three patients failed mobilization with G-CSF or pegfilgrastim and were excluded from further evaluation. All three were salvaged with plerixafor and successfully underwent AHSCT. No patient failed mobilization with cyclophosphamide. All transplanted patients successfully engrafted.

Discussion

The use of cyclophosphamide mobilization in MM patients results in lower mobilization failure, higher stem cell yield and fewer collection procedures but at the cost of higher toxicity.^{6,9,10} Furthermore, cyclophosphamide affects lymphocyte subpopulations possibly affecting the response after AHSCT. In our study, we prospectively analyzed the impact of cyclophosphamide, G-CSF and pegfilgrastim mobilization on lymphocyte, NK and Treg cell counts during mobilization, in the leukapheresis product and after AHSCT in newly diagnosed patients treated with bortezomib and dexamethasone.

Our study shows that mobilization with cyclophosphamide resulted in lower absolute lymphocyte and NK cell counts as compared to growth factor only mobilization (Figure 1) and as a conse-

TABLE 2. Mean lymphocyte (Lym), natural killer (NK) and T regulatory (Treg) cell counts with 95 % confidence interval (CI) before mobilization, on collection day, in the leukapheresis product and on day 15 after autologous hematopoietic stem cell transplantation (D+15) for mobilization regimens using cyclophosphamide, filgrastim (G-CSF) and pegfilgrastim

Time point	Collection regimen	Cyclophosphamide		Pegfilgrastim		G-CSF		Sig.
		Mean value	95% CI	Mean value	95% CI	Mean value	95% CI	
Before mobilization	Lym ($\times 10^9/L$)	1.81	1.27–2.34	1.48	1.17–1.80	1.43	1.20–1.66	
	NK ($\times 10^9/L$)	0.20	0.15–0.26	0.18	0.12–0.23	0.22	0.15–0.28	
	Treg ($\times 10^6/L$)	8.53	4.1–12.9	10	5.28–14.6	6.86	4.24–9.48	
Collection day	Lym ($\times 10^9/L$)	1.08	0.52–1.64	3	2.46–3.54	3.08	2.65–3.51	< .001
	NK ($\times 10^9/L$)	0.07	0.01–0.13	0.42	0.3–0.54	0.52	0.43–0.62	< .001
	Treg ($\times 10^6/L$)	14.4	3.73–25.1	20.4	12.4–28.5	18.5	10.6–26.4	.646
Leukapheresis product	Lym ($\times 10^9/L$)	50.1	36–64.2	112	96.6–129	112	94.7–130	< .001
	NK ($\times 10^9/L$)	4.18	0.72–7.64	14.3	11.9–16.6	17.5	12.7–22.4	< .001
	Treg ($\times 10^6/L$)	1030	392–1670	1030	400–1650	714	430–997	0.531
D+15	Lym ($\times 10^9/L$)	0.31	0.14–0.47	0.47	0.38–0.55	0.47	0.36–0.59	0.115
	NK ($\times 10^9/L$)	0.05	0–0.85	0.10	0.04–0.16	0.10	0–0.34	0.357
	Treg ($\times 10^6/L$)	27.8	4.45–51.1	36.6	0–101	2	0–5.74	0.691

Lym = lymphocytes; NK = natural killer cells; Treg = T regulatory cells

quence the leukapheresis products also contained lower counts of lymphocytes and NK cells. It resulted in slower reconstitution of lymphocytes on day 15 after AHSCT in patients mobilized with cyclophosphamide as compared to those mobilized with growth factors only. The number of lymphocytes and NK cells is important because studies have shown that the infused dose of lymphocytes and the early recovery affects survival in patients after AHSCT suggesting a graft-vs-myeloma effect.^{12–15,24} Counts of at least $0.5 \times 10^9/L$ lymphocytes on day 15 after AHSCT predict better OS in patients with MM.¹³ In our group 2 out of 9 patients receiving cyclophosphamide mobilization achieved this cutoff value as opposed to 7 out of 19 and 9 out of 17 patients receiving G-CSF and pegfilgrastim mobilization, respectively. Besides a graft-vs-myeloma effect, the early reconstitution of lymphocytes and NK cells protects against viral infections including cytomegalovirus, influenza virus, HIV-1, and hepatitis C virus thereby decreasing infectious complications after AHSCT and possibly increasing the age limit of patients eligible for AHSCT.^{25,26}

Our findings encourage further research because of the expanding use of lenalidomide consolidation and maintenance after AHSCT. Lenalidomide activity is partly exerted through immunomodulation and activation of NK cells.^{27,28} Therefore per-

sistent lower lymphocyte and NK cell counts after AHSCT may decrease the efficacy of lenalidomide in cyclophosphamide mobilized patients. Trials on lenalidomide maintenance have not focused on the impact of the mobilization regimen or lymphocyte subpopulations on treatment outcome.^{3,29}

Treg cells are associated with MM progression, probably through immunosuppression and inhibition of NK cell function.^{16–19} Previous studies show that low doses of cyclophosphamide are highly toxic to Treg cells.²⁰ In contrast, Condomines in a study of 14 patients mobilized with high dose cyclophosphamide found a 2-fold increase in Treg cells, speculating that a cytokine burst following aplasia due to cyclophosphamide is responsible.¹¹ Our data shows a 2-fold increase in Treg cells on collection day compared to baseline value before mobilization in all three mobilization regimens. We speculate that growth factors (G-CSF and pegfilgrastim) are responsible for the increase in Treg cell counts and not the cytokine burst following aplasia due to cyclophosphamide. Evidence from animal models show that G-CSF has a strong effect on promotion of Treg cell numbers and function.^{30,31} A small clinical trial on 29 patients receiving mobilization with high doses of filgrastim (16 mcg/kg) showed that, in addition to stem cells, Treg cell counts increased during mobilization.³²

On day 15 after AHSCT Treg cells are the only subpopulation with higher counts compared to baseline value before mobilization and the value on collection day. Our data shows a 4-fold higher Treg cell count on day 15 after AHSCT as compared to baseline value before mobilization and 2-fold higher as compared to the value on the collection day. We speculate that G-CSF application after AHSCT is the major contributor for the increase of Treg cells on day 15 after AHSCT and not growth factors given during mobilization. The lower value of Treg cells on day 15 after AHSCT in the G-CSF regimen cannot be properly evaluated due to the small patient group.

In our study we investigated two different growth factors, namely G-CSF and the long acting pegfilgrastim. Stem cell mobilization with both growth factors results in comparable toxic profiles and a similar recovery of leukocytes and platelets after AHSCT.⁸ However, results from small trials suggest that pegfilgrastim mobilizes a different CD34+ cell subset due to higher expression levels of various genes indicative of early haematopoiesis.^{33,34} Whether this has an impact on lymphocyte subpopulations is not known from the available literature. Our study shows that both growth factors are comparable regarding the increase in lymphocytes, NK and Treg cells during mobilization and in the leukapheresis product. Counts on day 15 after AHSCT are comparable, except Treg cell counts, which are primarily a result of G-CSF application after AHSCT and not during mobilization (Table 2).

In the current study we enrolled only patients receiving induction with bortezomib and dexamethasone. Triple agent induction including cyclophosphamide or immunomodulatory drugs is currently the standard induction regimen.^{4,35} Weekly applications of cyclophosphamide during induction might decrease Treg cells and improve the immune status of patients before mobilization resulting in a different composition of the leukapheresis product and altered reconstitution after AHSCT. In addition, the optimal dose and interval of cyclophosphamide on Treg cell depression is not known. To answer these questions further investigator driven studies are needed.

Our study has some limitations. Other factors may influence the reconstitution of lymphocyte subpopulations after AHSCT. The transplantation of cells expressing dipeptidyl peptidase-4 (CD26) has been shown to influence reconstitution of lymphocyte subpopulations. Monocytes expressing CD26+ improve the reconstitution of helper,

suppressor and NK lymphocytes and a higher number of transplanted CD26+ lymphocytes accelerates the reconstitution of NK lymphocytes.³⁶ Cotransplantation of mesenchymal stromal cells during AHSCT improves lymphocyte recovery after AHSCT.³⁷

In summary, we found no differences between G-CSF and pegfilgrastim mobilization regarding the increase in lymphocytes, NK and Treg cells during mobilization and in the leukapheresis product. Therefore mobilization with pegfilgrastim is comparable to G-CSF regarding lymphocyte subpopulations and can be used instead for stem cell mobilization. Our results show that mobilization with cyclophosphamide reduces the number of lymphocytes and NK cells on collection day and in the leukapheresis product thereby reducing the absolute number of lymphocytes on day 15 post AHSCT. Hence, cyclophosphamide mobilization might decrease graft-vs-myeloma effect possibly affecting the outcome of AHSCT.

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