Annals of Hematology (2019) 98:1457–1466
https://doi.org/10.1007/s00277-019-03657-3

ORIGINAL ARTICLE

Evaluation of regulatory T cells (Tregs) alterations in patients with multiple myeloma treated with bortezomib or lenalidomide plus dexamethasone: correlations with treatment outcome

Christina Hadjiaggelidou[1] · Evdokia Mandala[2] · Evangelos Terpos[3] · Efthalia Yiannaki[1] · Dimitra Markala[1] · Theodora Triantafyllou[1] · Athanasios Papatheodorou[3] · Vassiliki Gkastari[1] · Evgenia Verrou[1] · Asimina Papanikolaou[4] · Pavlina Konstantinidou[1] · Eirini Katodritou[1]

Received: 3 December 2018 / Accepted: 4 March 2019 / Published online: 20 March 2019

Abstract

The exact role of regulatory T cells (Tregs) in multiple myeloma (MM) has not been yet determined. Data regarding alterations of Tregs during therapy with novel agents (NA), i.e., bortezomib and lenalidomide are conflicted and limited. We evaluated prospectively alterations of Tregs and searched for correlations with disease characteristics, response, and outcome in 29 patients with active MM treated with either bortezomib-dexamethasone (BD; 11 patients) or lenalidomide-dexamethasone (LenDex, 18 patients). Additionally, we recorded changes of lymphocytes subsets and cytokines related to Tregs function and MM biology, i.e., interleukin (IL) 6, 2, 17, and TGF-β. Compared with controls, patients had significantly higher median levels of Tregs%, IL-6, and IL-17 ($p < 0.001$). Median CD4 T and B cells frequencies were significantly lower, whereas CD8 T and natural killers were increased compared to controls. In BD group, no significant alterations of Tregs% were observed. Patients treated with LenDex, displayed a significant reduction of Tregs% ($p < 0.001$) especially those who achieved at least very good partial response (≥vgPR) ($p = 0.04$). Lymphocyte subsets or cytokines did not significantly change during therapy. In summary, Tregs% are higher in patients with active MM compared with controls, and they significantly decrease after treatment with LenDex but not with BD; After therapy with LenDex, Tregs reduction between baseline and major response correlated with achievement of ≥vgPR suggesting a possible predictive role, that may contribute to therapeutic strategy.

Keywords Multiple myeloma · Tregs · Lenalidomide · Bortezomib

Introduction

Multiple myeloma (MM) is a mature, peripheral B cell malignancy which is characterized by uncontrolled proliferation of plasma cells and bone marrow plasmacytosis in association with excess production of monoclonal protein. It accounts for ≈ 10% of hematological malignancies and has an overall annual incidence of 4.4 cases per 100.000 population [1]. During the last decades, the survival of MM patients has substantially improved because of the wide use of autologous peripheral stem cell transplantation (ASCT) and the introduction of first-generation novel agents (NA), i.e., thalidomide, lenalidomide, and bortezomib [1]. However, despite survival improvements, there is still a long way to go to reach curability of MM. The major pathogenic mechanisms of MM include primary and secondary translocations, defects of cyclins and cell cycle regulators, dysregulation of signal transduction pathways, and interactions between stromal and malignant plasma cells [2]. In addition, MM is accompanied by defects in the immune system which concern abnormalities in number and function of B cells and are responsible for frequent and recurrent

✉ Christina Hadjiaggelidou
chadjiaggelidou@yahoo.com

[1] Department of Hematology, Theagenion Cancer Hospital, Thessaloniki, Greece

[2] Fourth Department of Internal Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece

[3] Department of Clinical Therapeutics, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

[4] Hematopathology Department, Evangelismos General Hospital, Athens, Greece

infectious complications [3]. Additional abnormalities in T, natural killer (NK), NK-like cells (NKL), and dendritic populations are identified which are related with reduced immune surveillance within the context of the disease [3, 4]. During the past decade, regulatory T cells (Tregs) have attracted great attention due to their ability to deteriorate immune responses against a wide panel of neoplasms, including MM[5]. Regulatory T cells represent a critical subset of CD4 T cells, characterized by CD4 + CD25 + Forkhead box P3+ (FoxP3+) phenotype, able to control peripheral tolerance as well as response to foreign and tumor antigens; Tregs are subdivided in two main subpopulations: naive Tregs (nTregs) generated in thymus and induced Tregs (iTregs), acquiring regulatory properties either via IL-10 and/or TGF-β [5–7]. The intracellular Forkhead box protein 3 (FoxP3) was identified as a crucial transcription marker that characterizes both nTregs and iTregs; FoxP3 is required for the suppressive activity of Tregs and mediates both their function and differentiation [8, 9]. While Tregs are critical for the peripheral maintenance of potential autoreactive T cells, they can also be detrimental by suppressing effective anti-tumor responses [5]. Most of the studies have shown that Tregs are elevated in various types of cancer (e.g., lung, ovarian, liver, pancreatic, breast cancers, melanoma), including hematological malignancies (e.g., lymphoproliferative disorders, Hodgkin disease) and can eliminate protective antitumor immunity, contributing in tumors' progression [10, 11]. In MM, data concerning Tregs' number and function are conflicting [7]. Furthermore, data regarding Tregs' alterations during therapy with NA are limited and controversial.

Several cytokines, including interleukins 2, 6, 17 (IL-2, IL-6, IL-17), and TGF-β are related with Tregs function and myeloma biology [12, 13]. In particular, IL-2 reflects CD25 alpha chain receptor and is essential for maintaining Tregs activity in vivo and for the induction of iTregs. It also promotes FoxP3 expression in Tregs, both in vivo and in vitro [5, 6]. IL-6 is vital for the differentiation of plasmablastic cells into mature plasma cells and the proliferation of malignant plasma cells. IL-6 serum levels are elevated in patients with MM and correlate with poor prognosis and high tumor mass [5, 6]; TGF-β inhibits normal B cell development and immunoglobulin production and promotes Tregs proliferation [5, 6]. Interleukin-17 is produced by Th17 cells which are related with Tregs in a reciprocal way [14] and differentiate from naive CD4 cells in the presence of IL-6 with or without TGF-β [15, 16].

The primary purpose of this study was to evaluate Tregs alterations during treatment with lenalidomide or bortezomib and to correlate them with response and survival parameters as well as with disease characteristics. Secondarily, we sought out possible changes in the levels of lymphocyte subsets (CD4 T, CD8 T, NK, NKL, CD19 B cells) and the aforementioned cytokines (IL-2, IL-6, IL-17, TGF-β) and searched for correlations with Tregs alterations, disease characteristics, and response parameters. To our knowledge, the current study is the largest study examining Tregs alterations in MM patients treated with NA.

Patients and methods

Patients

A total of 29 patients with symptomatic MM at early lines (diagnosis or 1st relapse) were included in the study. Twenty healthy volunteers were used as controls. The study was prospective, and all patients signed an informed consent. Patients eligible for the study should be above the age of 18 and were expected to survive for more than 3 months. Exclusion criteria were (1) denial of participation, (2) antimyeloma treatment during the last trimester, (3) allo- or auto- stem cell transplantation during the last semester before induction with NA, (4) concomitant autoimmune disease and/or immunodeficiency disorders, (5) active neoplastic malignant disease, except for basal cell carcinoma and in situ cancer of the uterus, (6) any kind of immunosuppressive or immunomodulatory therapy, and (7) current active infectious disease. All patients were treated with NA, i.e., either bortezomib-dexamethasone (BD) (group A) or lenalidomide-dexamethasone (LenDex) (group B), according to our centers' standard practice. The study was performed in accordance with the Declaration of Helsinki with approval from the Institutional Review Board of Aristotle University of Thessaloniki.

Methods

The detection of Tregs and lymphocyte populations was performed in peripheral blood samples using polyparametric flow cytometry analysis and the appropriate isotypic controls. The analysis was implemented with CXP Beckman Coulter and FC Express 4 software on a FC500 flow cytometer (Beckman Coulter).

With regard to Tregs detection, we used directly conjugated monoclonal antibodies (mAbs) against CD4-phycoerythrin-Texas red-x (CD4-ECD), CD25-phycoerthryrin-cyanin 5 (CD25-PC5), CD127-fluorescein isothiocyanate (CD127-FITC), and against the nuclear factor FoxP3-phycoerythrin (FoxP3-PE), (Beckman Coulter, eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Using a sequential gating strategy, Tregs were identified as CD4 + $CD25^{high}CD127^{neg/low}$ FoxP3+ cells quantified from CD4 + T cells and expressed as a percentage of the whole lymphocyte population (Fig. 1). Surface antigens CD4, CD25, and CD127 were stained with the appropriate monoclonal antibodies, and the cells were fixed and

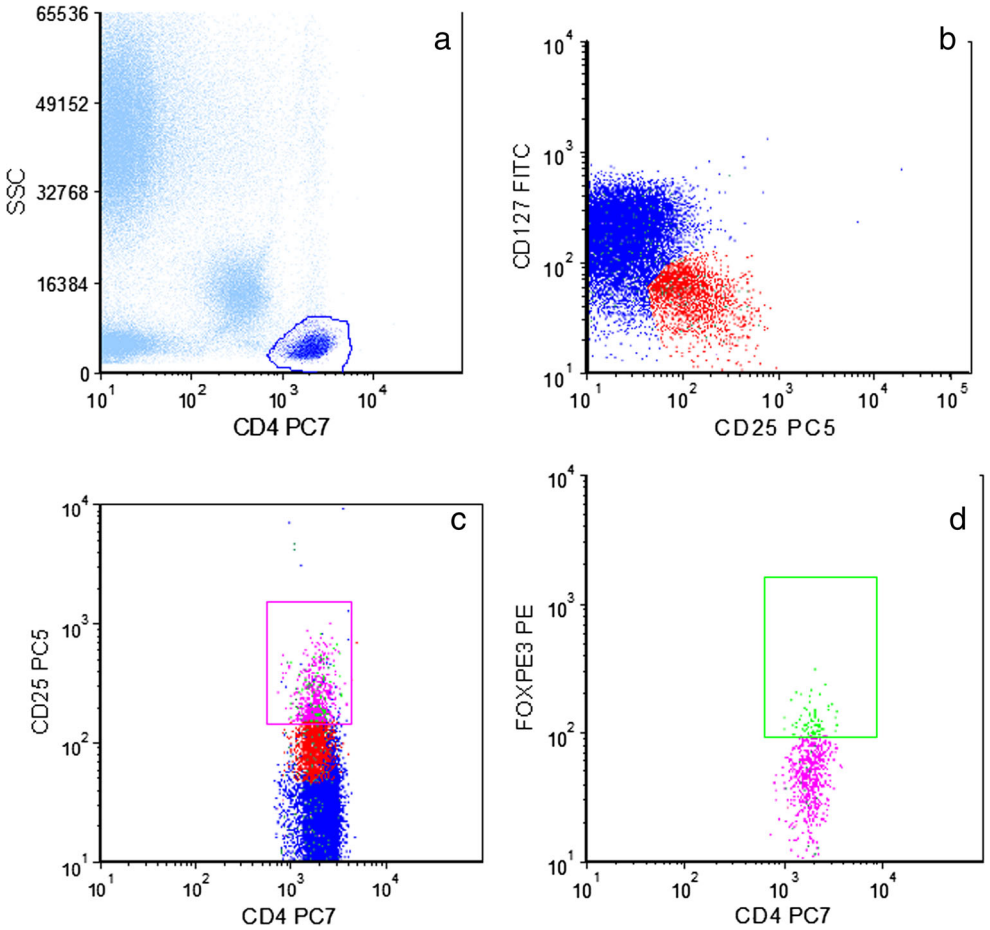

Fig. 1 **a** CD4 T cells were initially separated from the whole lymphocyte population (blue color). **b** CD25 + CD127$^{neg/low}$ cells were then discriminated from CD4 T cells (red color), **c** CD4 + CD25^{high}CD127$^{neg/low}$ cells were gated from CD4+ CD25 + CD127$^{neg/low}$ cells (violet color). **d** Tregs identified as CD4 + CD25^{high}CD127$^{neg/low}$ FoxP3+ cells were finally isolated from CD4 + CD25^{high}CD127$^{neg/low}$ cells (light green color)

permeabilized with formaldehyde and saponine buffers. Subsequently, the nuclear factor FoxP3 was labeled with the appropriate monoclonal antibody. Our management process was initially to quantify CD4+ lymphocytes from the whole lymphocyte population and then gate on CD25^{high}CD127$^{neg/low}$ cells, from which we finally selected and analyzed FoxP3+ cells [17, 18]. According to the aforementioned method, we were able to isolate a population of CD4 + CD25^{high}CD127$^{neg/low}$FoxP3+ regulatory cells (Fig. 1). Concerning process, 100 μl peripheral blood containing 5-10 × 10^3/μL white blood cells were incubated in duplicates for 10 min at room temperature in the dark with 5 μL of the monoclonal antibodies CD4, CD25, and CD127 and 100 μL paraformaldehyde (Reagent A, Invitrogen). Then, we added 5 ml of phosphate-buffered saline (PBS, GIBCO-Invitrogen), centrifuged for 5 min at 540 g and removed the supernatant. The cell pellet was resuspended in 100 μL saponine solution (Reagent B, Invitrogen) and was incubated at room temperature in the dark with 5 μl monoclonal antibody FoxP3-PE for 30 min. After the incubation, we added 5 ml PBS and centrifuged for 5 min at 540 g. The cell pellet was again resuspended in 1 ml PBS, and the duplicates were merged in a single flow cytometry tube which was immediately analyzed. During the procedure, 5 × 10^5 white blood cells were collected.

The identification of the rest of lymphocyte populations (T4, T8, B, NK, and NKL cells) was also performed in peripheral blood samples using directly fluorescent conjugated monoclonal antibodies (Beckman Coulter) against CD3, CD4, CD8, CD16/CD56, CD19, and CD45 antigens (CD3-FITC, CD4-PE, CD8-PE, CD16 + CD56-PE, CD19-PE, CD14-PE, CD45-PC5, CD45-FITC) using five sets of flow cytometry tubes with the following combinations; CD3-FITC/CD4-PE/CD45-PC5, CD3-FITC/CD8-PE/CD45-PC5, CD3-FITC/CD19-PE/CD45-PC5, CD3-FITC/CD16 + 56-PE/CD45-PC5, and CD45-FITC/CD14-PE. T4 and T8 lymphocytes were identified as CD3 + CD4+ και CD3 + CD8+ cells respectively, B lymphocytes as CD3-CD19+, natural killer cells (NK) as CD3-CD16 + CD56+ and natural killer-like cells (NKL) as CD3 + CD16 + CD56+. In particular, 100 μL peripheral blood containing 5-10 × 10^3 WBC/μl was incubated for 10 min in the dark at room temperature with the appropriate monoclonal antibodies, according to the manufacturer's instructions. Red blood cells were lysed by adding 2 ml of lysing solution Versalyse (Beckman Coulter) and incubated in the dark for 10 min. The stained leucocytes were analyzed

by flow cytometry based on forward (FSc) and side (SSc) scatter and on the intense expression of CD45. Lymphocyte subpopulations were expressed both in percentage and absolute values according to the flow cytometer's software (dual platform).

With regard to the measurement of IL-2, IL-6, IL-17, and TGF-β, we used serum which was separated within 4 h and stored at − 70 °C until the day of the procedure. Cytokines were measured using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

The statistical analysis was performed with the common equations. Mann-Whitney *U* test, Pearson's x^2 test, and one-way ANOVA were used for the determination of significant differences and correlations of patients' clinical or laboratory characteristics. Wilcoxon Rank Sum test was used for the evaluation of the statistical changes of the studied parameters, and Cox regression analysis was used to correlate Tregs' alterations with progression free survival (PFS); $p < 0.05$ was considered statistically significant. Data processing and analysis were performed with the software package SPSS v16. Response was estimated according to Uniform Response Criteria [19]. All parameters were estimated before therapy initiation (baseline) and at major response to treatment. Major response was defined as response remaining stable for two consecutive cycles of therapy; PFS was defined as the time from starting therapy for MM until the time of disease progression or death whichever comes first.

Results

Patients characteristics are depicted in Table 1. Patients in both groups were well balanced; marginal differences were observed in hemoglobin, platelets, and stage according to the International Staging System (ISS) (Table 1). Overall, the median number of previous treatment lines was 1 (0–2). Eleven patients were treated with BD (group A), and 18 patients received LenDex (group B); bortezomib was administered in 6–8 cycles every 21 days at the dose of 1.3 mg/m twice weekly (days 1, 4, 8, and 11) in combination with 20–40 mg dexamethasone (days 1–2, 4–5, 8–9, 11–12). Lenalidomide was administered at a dose of 25 mg for 21 consecutive days of a 28-day cycle in combination with 20–40 mg dexamethasone weekly until disease progression or unacceptable toxicity.

At baseline, patients had significantly higher median Tregs% compared to controls ($p < 0.001$) (Fig. 2); median CD4 T and B lymphocyte percentage was significantly lower whereas CD8 T and NK cells percentage was higher compared to controls; CD4/CD8 ratio was lower in patients vs. controls. Evaluation of Tregs as well as the rest of lymphocyte subset frequencies measured at baseline and at major response is shown in Table 2. In group A, no significant change of Tregs% was observed during treatment (Fig. 3a). At variance, in group B, there was a significant reduction of Tregs% ($p < 0.001$) (Fig. 3b). In addition, in patients who achieved at least very good partial response (≥vgPR) median Tregs% at response, was lower compared to others (0.01% vs. 0.03%; $p = 0.04$) (Fig. 4). No change on the median percentage of lymphocyte subsets was observed during treatment. However, in patients who achieved ≥vgPR, a significant increase in CD4/CD8 ratio between baseline and response was observed ($p = 0.04$), mainly induced by a decrease of CD8 T cells ($p = 0.04$).

Table 1 Patients' characteristics

Variable	All patients (*N* = 29)	Group A, BD (*N* = 11)	Group B, LenDex (*N* = 18)	*p* value
Age	61 (39–77)	61(43–76)	63 (39–77)	NS
Gender (M/F)	M = 15, F = 14	M = 6, F = 5	M = 9, F = 9	NS
Hb (g/dl)	10.4 (6.6–15.3)	11.1 (8.9–14.6)	12.7 (6.6–15.3)	0.044
PLT (× 10^3/μL)	216 (140–372)	198 (140–286)	260 (153–372)	0.016
LDH (U/L)	188 (88–294)	210 (91–294)	177 (88–236)	NS
Alb (g/dl)	3.8 (1.7–5.1)	3.7 (3–5.1)	3.8 (1.7–5.1)	NS
β2M (mg/L)	3.3 (1.89–24.5)	4 (1.89–24.5)	3.3 (2–17.9)	NS
Myeloma type	IGG = 16, IGA = 8 LC = 2, NS = 3	IGG = 7, IGA = 3, LC = 1	IGG = 9, IGA = 5, LC = 1, NS = 3	NS
ISS	ISS3 = 16, ISS2 = 6, ISS3 = 7	ISS1 = 3, ISS2 = 4, ISS3 = 4	ISS1 = 13, ISS2 = 2, ISS3 = 3	0.04
ORR	22/29	9/11	13/18	NS
≥vgPR	13/29	3/11	10/18	NS

N number, M/F male/female, *Hb* hemoglobulin, *PLT* platelets, *LDH* lactate dehydrogenase, *Alb* albumin, *β2M* beta 2 microglobulin, *Ig* immunoglobulin, *LC* light chain, *NS* non-secretory, *ISS* international staging system, *ORR* overall respose rate, *vgPR* very good partial response, NS non-significant

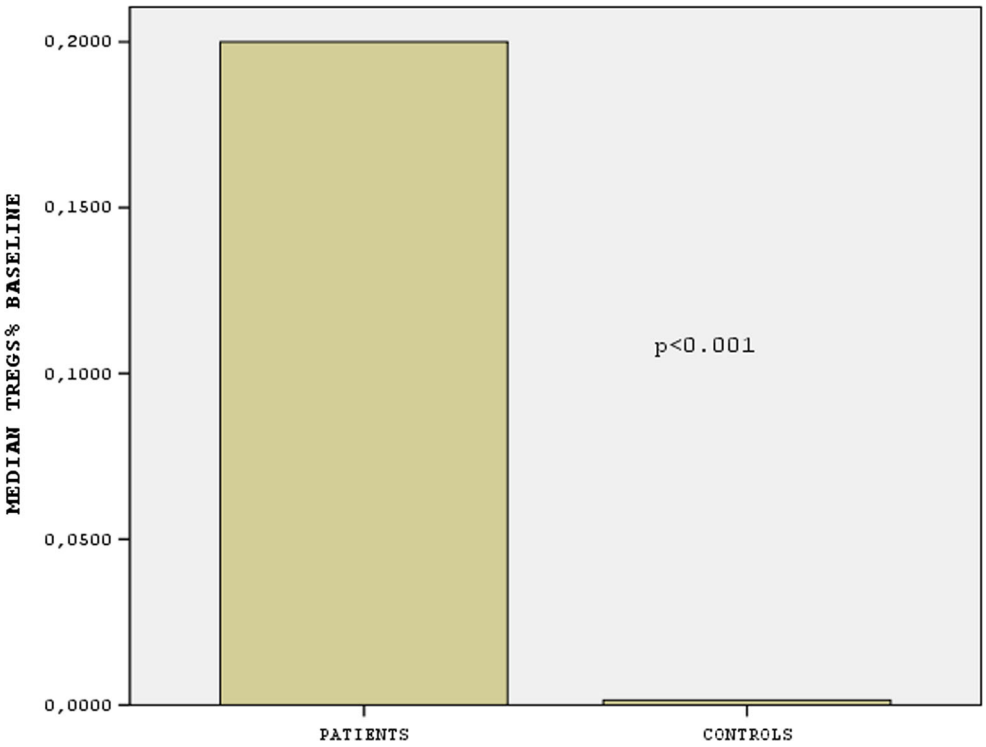

Fig. 2 Median Tregs% are higher compared to controls. Tregs, regulatory T cells

Regarding comparisons of the studied cytokine levels between patients and controls, patients had higher median levels of IL-6 and IL-17 (3.9 pg/mL vs. 2.0 pg/mL and 5.3 pg/mL vs. 3.87 pg/mL, respectively; $p < 0.05$ for both cytokines). The median levels of cytokines at baseline and major response did not show any statistical difference between groups A and B, except for TGF-β at baseline (Table 3). Median cytokine levels did not change significantly after therapy. There were no significant correlations between major disease characteristics as shown in Table 1 and Tregs frequencies in either group of patients.

Discussion

Immune dysfunction is an important feature of MM and has been associated with reduced survival [3]. Studies have shown that Tregs implicated in immune surveillance are expanded in tumors and correlate with worse prognosis [10, 11]. Data regarding alterations of Tregs in patients with MM are controversial [7]. In the current study, we have demonstrated that myeloma patients had higher Tregs frequencies compared to HV. Similarly, Feyler et al. [20], as well as Beyler et al. [21] reported

Table 2 Median Tregs% and lymphocyte subsets% at baseline and at response

Variable %	All patients	Group A (BD)	Group B (LenDex)	*p*
Tregs Base	0.138 (0.01–5.7)	0.03 (0.0–1.20)	0.2 (0.02–5.7)	NS
Tregs Res		0.06 (0.0–0.32)	0.02 (0.0–0.12)	NS
NK Base	19.1 (2.8–31.3)	18.6 (2.8–25.5)	22 (10.5–31.3)	NS
NK Res		15.5 (8.1–19.3)	17 (5.7–28)	NS
CD4 Base	38 (14.3–70)	35.2 (14.3–70)	34 (23.7–54.2)	NS
CD4 Res		40.4 (35.5–61.3)	34 (15–48)	NS
CD8 Base	33.5 (12–55)	18.9 (12–38.5)	31.8 (24–55)	NS
CD8 Res		33.5 (14.1–43.8)	32.6 (21.8–45)	NS
NKL Base	6 (0.8–17.7)	4.7 (0.8–7.8)	5.5 (1.1–17.7)	NS
NKL% Res		3.2 (1.07–8.48)	4.5 (1–19.5)	NS
CD19 Base	6.7 (0.5–24.2)	8.3 (1.5–24.2)	4.3 (0.5–17)	0.02
CD19 Res		2.2 (0.0–10.8)	2.3 (0.6–20.5)	NS

N number, *Base* baseline, *Res* response, *Tregs* T-regulatory cells, *CD* classification determinant, *NK* natural killers, *NKL* natural killers like, *NS* non-significant

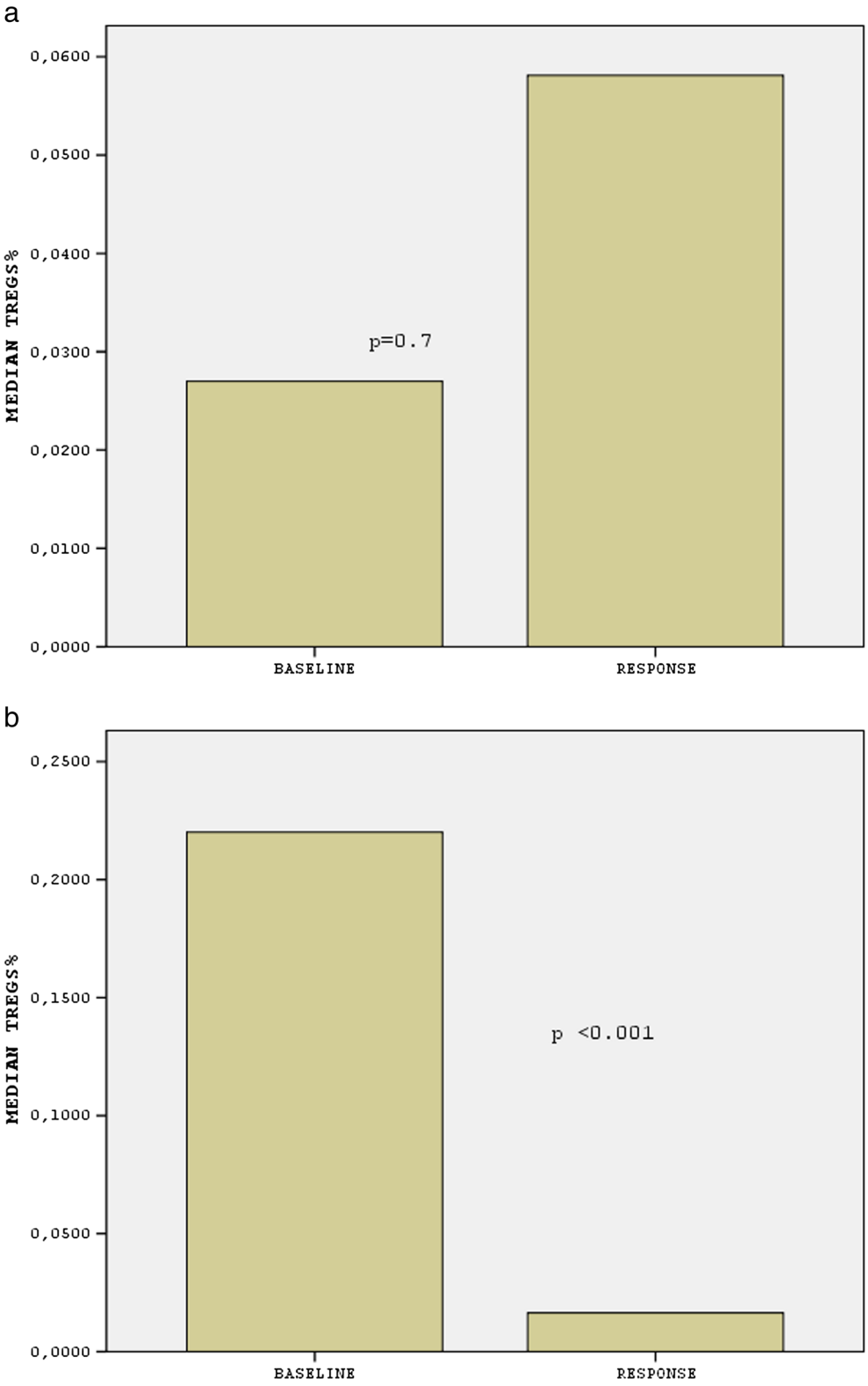

Fig. 3 **a** Tregs alterations before and after treatment with BD. Tregs regulatory T cells, BD bortezomib/dexamethasone. **b** Tregs alterations before and after treatment with Len/Dex. Tregs, regulatory T cells; Len/Dex, lenalidomide/dexamethasone

increased frequencies of peripheral blood Tregs in MM patients. Contrasting to the previous studies, Prabhala et al. [22] and Gupta et al. [23] reported reduced Tregs' numbers in MM patients. These conflicting results may be due to different gating strategies applied throughout the aforementioned studies. Feyler et al. identified Tregs as CD4 + CD25 + FoxP3+ cells and Beyler et al. took into consideration the intensity of

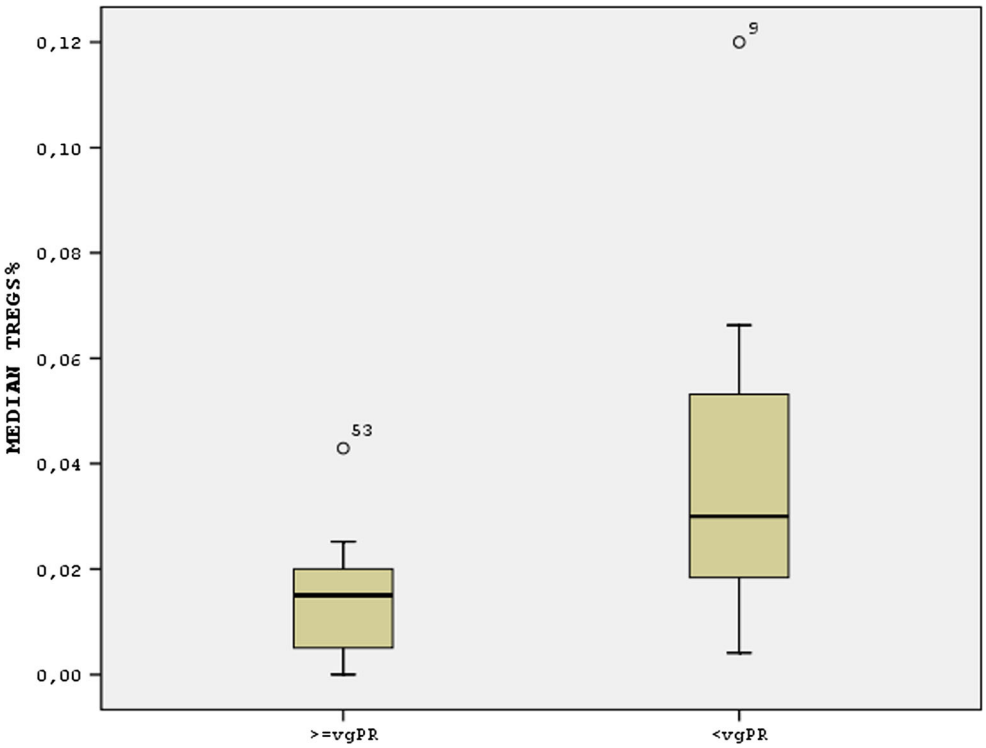

Fig. 4 Len/Dex patients;Tregs alterations at major response. Tregs, regulatory T cells; Len/Dex, lenalidomide/dexamethasone; vgPR, very good partial response

CD25 expression and identified Tregs as CD4 + CD25^{high}FoxP3+ cells [20, 21]. On the other hand, Prabhala et al. identified Tregs as CD4 + FoxP3+, and Gupta et al. characterized Tregs with the inclusion of CD127 in gating [22, 23]. In our study, we identified Tregs as CD4 + CD25^{high}CD127$^{low/dim}$FoxP3+ cells based on literature data according to which high expression of CD25 combined with low or negative expression of CD127 on CD4 + FoxP3+ cells characterize regulatory cells with mainly immunosuppressive properties and consist 1–2% of the total number of Tregs [17, 18].

As far as Tregs alterations during therapy with lenalidomide are concerned, all 18 patients in group B who were treated with LenDex combination displayed significant reduction of Tregs frequencies which were more profound in the 13 patients which achieved ≥vgPR, suggesting a possible relation of immune surveillance with quality of response. Data concerning Tregs alterations during or after therapy with immunomodulatory drugs like lenalidomide are opposing. Galustian et al. [24] reported that the expansion of CD4 + CD25 + ^{high}CTLA4 + Foxp3+ Tregs which were isolated from peripheral blood mononuclear cells (PBMCs) treated with IL-2 were decreased after incubation with lenalidomide, and this was mainly attributed to the suppression of transcription factors FoxP3 and OX40 (CD134). According to Galustian et al., lenalidomide may enhance anti-tumor immunity by inhibiting the suppressive effects of regulatory cells [24]. On the contrary, Muthu Raja et al. reported that

Table 3 Median cytokines at baseline and response

Variable	All patients	Group A (BD)	Group (LenDex)	*p*
IL-2 (pg/ml) Base	7.7 (5.5–18.7)	7.9 (5.5–9.8)	7.9 (6.08–18.7)	NS
IL-2 (pg/ml) Res		8.1 (6.5–34.8)	8.07 (5.6–11)	NS
IL-6 (pg/ml) Base	3.1 (1.6–103.7)	5.4 (1.7–33.9)	3.8 (1.6–103.7)	NS
IL-6 (pg/ml) Res		8.5 (2.2–30.4)	4.4 (1.8–29.06)	NS
IL-17 (pg/ml) Base	5.3 (3.8–9.5)	5.4 (3.8–7.6)	5.3 (4.16–9.5)	NS
IL-17 (pg/ml) Res		4.6 (3.7–6.1)	6.00 (2.5–10.7)	NS
TGF-β (ng/ml) Base	19.2 (0.26–35.9)	14.8 (8.9–30.5)	22.8 (0.26–35.9)	0.046
TGF-β (ng/ml) Res		22.5 (14.3–33.07)	19.3 (0.7–32.5)	NS

Base baseline, *Res* response, *IL-2* Interleukin-2, *IL-6* Interleukin-6, *IL-17* Interleukin-17, *TGF-β* transforming growth factor-β, *NS* non-significant

lenalidomide in combination with dexamethasone, not only failed to inhibit the proliferation of Tregs, but it increased Tregs numbers in 15 patients with newly diagnosed myeloma, and this could negatively influence the anti-tumor immune response, according to authors speculations [25]. The opposite results demonstrated in our study compared to the study of Muthu Raja could be attributed to the difference of the quality of response to therapy. More specifically, in Muthu Raja's et al. study, only three patients achieved ≥vgPR; furthermore, eight patients with minimal response were included in the group of responding patients. In our study, complete response and very good partial response was achieved in 13 patients, and this was correlated with significant decrease in Tregs frequencies.

In group A, no significant alterations of Tregs were observed during treatment with BD. In particular, patients who received bortezomib exhibited a non-significant increase in Tregs frequencies. Data concerning Tregs alterations during therapy with bortezomib are very limited. Blanco et al. reported that the addition of bortezomib to CD4 T cells cultures not only does not affect the viability of nTregs, but furthermore it promotes the emergence of a distinct suppressor CD4 T cell population while eliminating the activities of conventional T cells [26]. Interestingly, a few studies have reached the conclusion that high numbers of circulating CD4 + CD25 + FoxP3+ cells are associated with reduced incidence of graft versus host disease (GVHD) after allogeneic stem cell transplantation [27, 28]. According to Blanco et al., resistance of Tregs to the pro-apoptotic effect of bortezomib could be used as a potential therapeutic tool against GVHD [26].

With regard to correlation of Tregs with disease outcome, Giannopoulos et al. have shown that patients treated upfront with thalidomide triplets, who had high Tregs frequencies, displayed significantly reduced overall survival compared with patients with reduced Tregs [29]. Muthu Raja et al. reported that patients with higher levels of Tregs in the peripheral blood had shorter time to progression compared to patients with lower levels [30]. In our study Tregs% reduction between baseline and response marginally correlated with PFS, in patients treated with LenDex in early lines, in the univariate cox regression analysis (data not shown). However, this observation could not be validated in a multivariate cox regression model due to the limited number of patients. Overall, the correlation of Tregs with survival parameters needs further evaluation.

In accordance with published studies [14–16], in the current study, patients with MM had increased levels of IL-17 compared to controls; this could be related to the increased amounts of IL-6 in the bone marrow of myeloma patients with active disease which promote the production of Th17 cells from CD4 naive cells. In patients treated with LenDex, there was a marginal reduction of IL-17 at major response; in combination with the decrease of Tregs frequency in the same group of patients, the reduction of IL-17 probably reflected disease response. Nevertheless, the relationship between Th17 cells and Tregs in MM requires further investigation [14].

Immune impairment in MM concerns both humoral and cellular immune system and consists of decreased levels of uninvolved immunoglobulins and significant reductions in the numbers of CD4 T and CD19 B populations, correlating with increased morbidity and mortality [2–4]. In our study, median frequencies of CD4 T and CD19 B cell counts in myeloma patients were significantly lower compared to controls; in line with previous reports, CD4/CD8 ratio was significantly lower in MM patients compared to HV because of both CD4 T reduction and CD8 T increase, reflecting disease activity; CD19 B, CD4 T, CD8 T, NK cells, and CD4/CD8 ratio did not significantly change after therapy in patients treated with LenDex (data not shown); Contrasting to our findings, Gandhi et al. demonstrated that dexamethasone reduces lenalidomide-induced immunomodulation of T and NK function [31]. This discrepancy between our study and Gandhi's study was observed probably because the inhibitory effect of dexamethasone is considered as dose-dependent. Muthu Raja has demonstrated that CD4 T cells along with Tregs are reduced after treatment with cyclophopshamide, thalidomide, and dexamethasone combination (CDT) in patients that achieved ≥ vgPR, probably because of the cytotoxic effect of cyclophosphamide [30]. Of note, in our study, CD4/CD8 ratio was increased in patients that achieved ≥ vgPR probably as a result of the significant decrease of CD8 T cells.

In conclusion, we have demonstrated that Tregs levels are significantly higher in patients with active MM compared with healthy controls, indicating that there is strong correlation of immune responses and disease biology. Most importantly, Tregs significantly reduced after treatment with LenDex, and their alterations correlated with quality of response underscoring the impact of immune surveillance on treatment outcome; correlation of Tregs alterations with survival parameters needs further evaluation in larger cohorts of MM patients. Bortezomib-based treatment had no impact on Tregs numbers, underscoring the different mode of action of proteasome inhibitors. Finally, no relation between Tregs and disease characteristics or immune response-mediating cytokines was observed, indicating that immune mechanisms underlying MM are still obscure and require further investigation.

Compliance with ethical standards

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

Informed consent Informed consent was obtained from all patients for being included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Rajkumar SV (2013) Multiple myeloma: 2013 update on diagnosis, risk-stratification, and management. Am J Hematol 88:226–235
2. Morgan GJ, Walker BA, Davies FE (2012) The genetic architecture of multiple myeloma. Nat Rev Cancer 12:335–348
3. Pratt G, Goodyear O, Moss P (2007) Immunodeficiency and immunotherapy in multiple myeloma. Br J Haematol 138:563–579
4. Dosani T, Carlsten M, Maric I, Landgren O (2015) The cellular immune system in myelomagenesis: NK cells and T cells in the development of MM and their uses in immunotherapies. Blood Cancer J 5:e321
5. Fehervari Z, Sakaguchi S (2004) Development and function of CD25+CD4+ regulatory T cells. Curr Opin Immunol 16:203–208
6. Workman CJ, Szymcak-Workman AL, Collison LW, Pilai MR, DAA V (2009) The development and function of regulatory T cells. Cell Mol Life Sci 66:2603–2622
7. Raja Muthu KR, Hajek R. Regulatory cells and multiple myeloma (2012) https://www.intechopen.com /doi.org /https://doi.org/10.5772/31207
8. Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057–1061
9. Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4+CD25 regulatory T cells. Nat Immunol 4:330–336
10. Curiel TJ (2008) Regulatory T cells and treatment of cancer. Curr Opin Immunol 20:241–246
11. Wang HY, Wang RF (2007) Regulatory T cells and cancer. Curr Opin Immunol 19:217–223
12. Merico F, Bergui L, Gregoretti MG, Ghia P, Aimo G, Lindley IJD, Caligaris-Cappio F (1993) Cytokines involved in the progression of multiple myeloma. Clin Exp Immunol 92:27–31
13. La Cava A (2008) Tregs are regulated by cytokines: implications for autoimmunity. Autoimmun Rev 8:83–87
14. Braga WMT, Atanackovic D, Colleoni GWB (2012) The role of regulatory T cells and TH17 cells in multiple myeloma. Clin Dev Immunol 2012:293479
15. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441:235–238
16. Noonan K, Marchionni L, Anderson J, Pardoll D, Roodman GD, Borrello I (2010) A novel role of IL-17-producing lymphocytes in mediating lytic bone disease in multiple myeloma. Blood 116:3554–3563
17. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, Fazekas de St Groth B (2006) Expression of interleukin IL-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 203:1693–1700
18. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4(+) Treg cells. J Exp Med 203:1701–1711
19. Durie BG, Harousseau JL, Miguel JS, Bladé J, Barlogie B, Anderson K, Gertz M, Dimopoulos M, Westin J, Sonneveld P, Ludwig H, Gahrton G, Beksac M, Crowley J, Belch A, Boccadaro M, Cavo M, Turesson I, Joshua D, Vesole D, Kyle R, Alexanian R, Tricot G, Attal M, Merlini G, Powles R, Richardson P, Shimizu K, Tosi P, Morgan G, Rajkumar SV (2006) International uniform criteria for multiple. Leukemia 20:1467–1473
20. Feyler S, von Lilienfeld-Toal M, Jarmin S, Lee Marles L, Rawstron A, Ashcroft AJ, Owen RG, Selby PJ, Cook G (2009) CD4(+)CD25(+)FoxP3(+) regulatory-T cells are increased whilst CD3(+)CD4(−)CD8(−)αβTCR(+) double negative T cells are decreased in the peripheral blood of patients with multiple myeloma which correlates with disease burden. Br J Haematol 144:686–689
21. Beyler M, Kochanek M, Giese T, Endl E, Weihrauch MR, Knolle PA, Classen S, Schultze JL (2006) In vivo peripheral expansion of naive CD4+CD25^{high}FoxP3+ regulatory T cells in patients with multiple myeloma. Blood 107:3940–3949
22. Prabhala RH, Neri P, Bae JE, Tassone P, Shammas MA, Allam CK, Daley JF, Chauhan D, Blanchard E, Thatte HS, Anderson KC, Munshi NC (2006) Dysfunctional T regulatory cells in multiple myeloma. Blood 07:301–304
23. Gupta R, Ganeshan P, Hakim M, Verma R, Sharma A, Kumar L (2011) Significantly reduced regulatory T cell population in patients with untreated multiple myeloma. Leuk Res 35:874–878
24. Galustian C, Meyer B, Labarthe MC, Dredge K, Klaschka D, Henry J, Todryk S, Chen R, Muller G, Stirling D, Schafer P, Bartlett JB, Dalgleish AG (2009) The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. Cancer Immunol Immunother 58:1033–1045
25. Muthu Raja KR, Kovarova L, Hajek R (2012) Induction by lenalidomide and dexamethasone combination increases regulatory cells of patients with previously untreated multiple myeloma. Leuk Lymphoma 53:1406–1408
26. Blanco B, Pérez-Simón JA, Sánchez-Abarca LI, Caballero-Velazquez T, Gutierrez-Cossío S, Hernández-Campo P, Díez-Campelo M, Herrero-Sanchez C, Rodriguez-Serrano C, Santamaría C, Sánchez-Guijo FM, Del Cañizo C, San Miguel JF (2009) Treatment with bortezomib of human CD4+ T cells preserves natural regulatory T cells and allows the emergence of a distinct suppressor T-cell population. Haematologica 94:975–983
27. Atanackovic D, Cao Y, Luetkens T, Panse J, Faltz C, Arfsten J, Bartels K, Wolschke C, Eiermann T, Zander AR, Fehse B, Bokemeyer C, Kroger N (2008) CD4+CD25+FOXP3+ T regulatory cells reconstitute and accumulate in the bone marrow of patients with multiple myeloma following allogeneic stem cell transplantation. Haematologica 93:423–430
28. Rezvani K, Mielke S, Ahmadzadeh M, Kilical Y, Savani BN, Zeilah J, Keyvanfar K, Montero A, Hensel N, Kurlander R, Barrett AJ (2006) High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. Blood 108:1291–1297
29. Giannopoulos K, Kameniska W, Dmoszynska A (2012) The frequency of T regulatory cells modulates the survival of multiple myeloma patients: detailed characterization of immune status in multiple myeloma. Br J Cancer 106:546–552
30. Muthu Raja KR, Kovarova L, Kaisarova P, Bartonova J, Hajek R, Michalek J (2011) Regulatory T cells predicts progression in previously untreated myeloma patients and treatment by

cyclophosphamide, thalidomide plus dexamethasone reduces regulatory T cells. Blood 118:2870

31. Gandhi AK, Kang J, Capone L, Parton A, Wu L, Zhang LH, Mendy D, Lopez-Girona A, Tran T, Sapinoso L, Fang W, Xu S, Hampton G, Bartlett JB, Schafer P (2010) Dexamethasone synergizes with lenalidomide to inhibit multiple myeloma tumor growth, but reduces lenalidomide-induced immunomodulation of T and NK cell function. Curr Cancer Drug Targets 10:155–167