AHOT Acta Haematologica Oncologica Turcica

Acta Haematol Oncol Turc 2025;58(3):162-168

Original Article

DOI: 10.4274/ahot.galenos.2025.2025-4-5

Investigation of CD4$^+$CD25$^+$FoxP3$^+$PD-1$^+$ Regulatory T-cells and PD-1, PD-L1 mRNA Expression in the Bone Marrow in Newly Diagnosed Multiple Myeloma Patients

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ABSTRACT

Aim: Multiple myeloma (MM) is a cancer that plasma cells. Increased regulatory T-cells (Tregs) have been associated with poor clinical outcomes in both newly diagnosed and treated MM patients. Treg cells are a T-cell subgroup that suppresses immune response. Programmed cell death protein-1 (PD-1) signaling pathway plays an essential role in Treg function and development. In literature, there is insufficient knowledge about the distribution of Treg cells in the bone marrow microenvironment despite the presence of these clinical studies in MM. In this study, we aimed to investigate PD-1$^+$ Treg cell distribution and PD-1/PD-ligand 1 (PD-L1) gene expression levels in MM patients' bone marrow.

Methods: Twenty newly diagnosed patients with MM and 9 idiopathic thrombocytopenic purpura patients were included in the study. Cluster of differentiation 4 (CD4), CD25, FoxP3 and PD-1 (CD279) expressions were evaluated by flow cytometry, and messenger ribonucleic acid (mRNA) expression of PD-1 and PD-L1 genes were determined using the quantitative polymerase chain reaction method.

Results: In this study, no significant difference was found among CD4$^+$CD25$^+$FoxP3$^+$PD-1$^+$ Treg cell levels, PD-1, and PD-L1 mRNA expression levels in the patient and control groups. There was no significant difference in Treg cell levels or in PD-1 and PD-L1 mRNA expression levels with respect to patient revised international staging system. There was no significant correlation between the expression of Treg cells, PD-1 mRNA, and PD-L1 mRNA levels ($p>0.05$). A strong correlation was found between PD-1 mRNA and PD-L1 mRNA levels ($p<0.0001$, $r=0.827$).

Conclusion: CD4$^+$CD25$^+$FoxP3$^+$PD-1$^+$ Treg cell and PD-1, PD-L1 mRNA expression levels were found to be high in the patient group, although not significantly. We found a positive correlation between PD-1 and PD-L1 mRNA expression, supporting that PD-1/PD-L1 may be a prognostic biomarker.

Keywords: Biomarkers, clinical hematology, multiple myeloma, targeted therapy

Introduction

Multiple myeloma (MM) is the second most common widespread hematologic malignancy. There is a need for the development of new treatments for MM [1]. Single-agent activity of programmed cell death protein-1 (PD-1) and PD-ligand 1 (PD-L1) blockade is limited in MM patients. Strategies to expand MM-specific T-cells and combination therapies that involve T-cell activation through PD-1/PD-L1 blockade are still being investigated [2]. The PD-1 pathway stimulates helper T-cell populations toward regulatory T-cell (Treg) development. Treg cells express many checkpoint molecules and thus are a target for direct immune checkpoint inhibitors [3]. The number of clinical trials using inhibitors of PD-1 and its ligand PD-L1 is rapidly increasing in MM [4].

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Received: 14.04.2025 **Accepted:** 20.08.2025 **Epub:** 09.09.2025 **Publication Date:** 12.12.2025

Cite this article as: Ekinci D, Özkan A, Salim O, et al. Investigation of CD4$^+$CD25$^+$FoxP3$^+$PD-1$^+$ regulatory T-cells and PD-1, PD-L1 mRNA expression in the bone marrow in newly diagnosed multiple myeloma patients. Acta Haematol Oncol Turc. 2025;58(3):162-168

Tregs are a special T-cell subset that suppress the immune response, thereby maintaining self-tolerance and homeostasis. It has been shown that Tregs can inhibit cytokine production and T-cell proliferation and play a crucial role in preventing autoimmunity [5]. Since Sakaguchi's discovery in 1995 that the interleukin-2 (IL-2) receptor α chain [cluster of differentiation 25 (CD25)] is a phenotypic marker for $CD4^+$ Tregs, studies on Tregs have rapidly increased. Treg cells are primarily a subset of T cells that exhibit the FoxP3 "forkhead winged helix $P3^+$" $CD4^+$, $CD25^+$ phenotype. The primary function of these cells is not to generate an immune response to the individual's own self-antigens and to take part in maintaining and sustaining immune homeostasis [6-8].

There are two main views regarding the mechanism of action of Tregs. It is believed that in order for Tregs to exhibit their immune suppressive function, they either make cell-to-cell contact or affect distanT-cells by secreting cytokines such as IL-10 or transforming growth factor. The T-cell-inhibiting effects of PD-1 and CTLA-4 are well-known [9]. In cell-to-cell contact, Tregs have been shown to kill antigen-presenting cells or responding T-cells by using granzyme and perforin facilitated by CD39, CD73, and lymphocyte-activation gene-3. However, mechanisms of Treg action also involve cytokines such as transforming growth factor-β, IL-35, IL-10, and galectin-1, mediated by soluble factors or IL-2 deficiency [10-12].

The lack of studies on Treg cells and PD-1/PD-L1 messenger ribonucleic acid (mRNA) expression in the bone marrow microenvironment of MM patients has led us to conduct this study. In this study, $CD4^+CD25^+FoxP3^+PD\text{-}1^+$ Treg cells and PD-1 and PD-L1 mRNA expression levels were determined in the bone marrow of newly diagnosed MM and idiopathic thrombocytopenic purpura (ITP) patients who applied to the Akdeniz University Hospital Adult Hematology Clinic. We believe that this and similar studies will contribute to identifying therapeutic targets in patients by measuring both PD-1 and PD-L1 mRNA expression levels and $PD\text{-}1^+$ Treg cells (%) in the bone marrow microenvironment.

Methods

Ethical approval was obtained from the Faculty of Medicine at Akdeniz University (decision no: 664, date: 14.12.2016,). The study was conducted at the Hematology Outpatient 'Clinic of Akdeniz University Faculty of Medicine' Hospital, with the necessary permissions obtained from the hospital administration, and in accordance with research and publication ethics. Following approval from the ethics committee, 37 patients who had presented with suspected MM or ITP and who had undergone bone marrow aspiration and bone marrow biopsy (BMA/BMB) at the the hematology outpatient clinic within one year were included. One patient who had undergone BMA/BMB for suspected ITP was later diagnosed with myelodysplastic syndrome, and seven patients who had undergone BMA/BMB for suspected MM were excluded from the analysis. They did not meet the criteria for MM diagnosis after BMA/BMB. The final analysis included 20 newly diagnosed MM patients and 9 ITP patients. ITP patients have a bone marrow cell distribution like healthy individuals, on whom a BMB can be performed. Since we could not perform this procedure on a healthy individual, a patient group with similar bone marrow cell distribution was chosen as the control. We believe that our study is the first to provide a new perspective on this subject, as no previous study has used ITP as a control group.

Patient age and gender were recorded. Data on hemoglobin (g/dL), leukocyte (number/mm^3), neutrophil (number/mm^3), lymphocyte (number/mm^3), platelet (number/mm^3), lactate dehydrogenase, total protein, albumin, creatinine, calcium, beta-2 microglobulin, immunoglobulin G (IgG), IgA, IgM, free kappa, and free lambda at the time of MM diagnosis were collected. Additionally, cytogenetic analysis data, including the MM FISH panel [t (14;16), del (17p), t (11;14), t (4;14), and del (13q)], were recorded. MM patients included in the study were staged according to the revised international staging system (R-ISS) and ISS based on the National Comprehensive Cancer Network guidelines and were also classified into myeloma subgroups (e.g., IgA Lambda, IgG Kappa) [13]. This cross-sectional case-control study was conducted in accordance with the Helsinki Declaration. Ethical approval was obtained from the Faculty of Medicine at Akdeniz University for this study (decision no: 664, date: 14.12.2016). Written consent was obtained from all patients.

Mononuclear Cell Separation

Samples from patients whose diagnosis was confirmed by flow cytometry and bone marrow smear examination were evaluated. During BMA, and biopsy, performed aseptically from the right iliac crest of patients with MM and ITP, 3 mL of bone marrow aspirate was taken. The aspirate was collected in a tube containing sodium heparin and another tube containing ethylenediamine tetraacetic acid in addition to routine procedures. After 3.5 mL of ficoll histopaque-1077 (Sigma-Aldrich) was added to the Falcon tubes, bone marrow diluted with PBS was slowly added at a ratio of 1:2. The sample was centrifuged for 30 minutes at 2000 rpm (800 g) and 20 °C, using a Beckman-Coulter centrifuge. Mononuclear cells were taken with a pipette. PBS was added and centrifuged. The cells obtained were diluted with Becton Dickinson (BD) Pharmingen Stain Buffer (FBS) to 10^6 cells per mL.

Flow Cytometry

Cell surface staining: Appropriate amounts of surface antibodies (CD4 and CD25 (20 µl), CD279 (PD-1) (5 µl), CD45 (3 µl)) and isotypes were pipetted into 12x75mm tubes. Allophycocyanin-H7-CD45 [catalog number (cat. no.) 560178], phycoerythrin -CD25 (cat. no. 555432), fluorescein isothiocyanate-CD4 (cat. no. 555346), Brilliant Violet 421-PD-1 (cat. no. 562516) - 100 µl of cells were added to each tube, vortexed, and incubated for 20 minutes in the dark at room temperature. Two mL wash buffer (FBS) was added. The sample was centrifuged at 250 x g for 10 minutes the supernatant was removed. To fix the cells, the cell pellet remaining in the tube was gently

resuspended and 2 mL 1x human FoxP3 buffer A was added on top. Two mL BD FBS, was added to the tubes, and the pellet was gently resuspended.

Intracellular staining: 0.5 mL 1x working solution human FoxP3 buffer C was added to permeabilize the cells. It was vortexed and incubated for 30 minutes at room temperature in the dark. FoxP3 antibody (20 µL) was added to the tubes and incubated for 30 minutes at room temperature in the dark. Alexa Fluor 647-FoxP3 (cat. no. 560045). The washing process was repeated with BD FBS, and after the supernatant was removed, the cell pellet remaining in the tubes was diluted with 400 µL wash buffer and analyzed as soon as possible. Treg cells were identified by flow cytometry as $CD4^+$, $CD25^+$, $FoxP3^+$, $PD\text{-}1^+$ based on their phenotypic properties [14]. During the reading, a minimum of 15,000 to 25,000 CD4 positive lymphocytes were counted. Samples were evaluated by flow cytometry (FACSCanto 2, CA) and analyzed using FACS Diva software [15].

Real-Time Polymerase Chain Reaction

Bone marrow samples were pelleted by centrifugation. Erythrocytes were removed using lysis buffer solution, and total RNA was obtained from the remaining nucleated cells by the silica column method (NucleoSpin® RNA Blood (MN-740200.50, Germany). RNA quality and quantity were measured spectrophotometrically with a Nanodrop (Nano). RNA samples were stored at -20 °C for cDNA synthesis. Before starting cDNA synthesis, all RNA samples were adjusted to 100 ng/µL. The EasyScript Plus TM cDNA Synthesis Kit, (ABM-G236, Canada) was used for cDNA synthesis. Before starting cDNA synthesis, all RNA samples were adjusted to 100 ng/µL. The EasyScript Plus TM cDNA Synthesis Kit, (ABM-G236, Canada) was used for cDNA synthesis. Total RNA samples that we kept at -20 °C were thawed at room temperature. Samples were İncubated at 25 °C for 10 minutes, at 40 °C for 50 minutes, and at 85 °C for 5 minutes in a thermal cycler, respectively, in accordance with the kit procedure. The melting curve was analyzed using LightCycler® 480 Software release 1.5.0. The cycle values of the target and reference genes were normalized using the REST© program, with the RPL13A gene used as a reference. PCR for all patients and controls was repeated twice. The primers were synthesized by Sentegen Biotechnology (Türkiye). The primers used for the PD-1 gene were FW: CCCTGGTGGTTGGTGTCGT and R: GCCTGGCTCCTATTGTCCCTC; while those for the PD-L1 (CD274) gene were FW: GGTGCCGACTACAAGCGAAT and R: ATGGTCACTGCTTGTCCAGATG [16].

Statistical Analysis

The data were analyzed utilizing Statistical Package for the Social Sciences statistics for Windows version 23 (IBM Corp., Armonk, NY, USA). In the within-group analysis, the chi-square test of independence was used to analyze the relationship between categorical variables, and the Mann-Whitney U and Kruskal-Wallis tests were used to compare continuous data. The correlation between continuous variables was evaluated using Spearman's rho correlation. A p value less than 0.05 was considered statistically significant.

Results

There is similarity in all individuals in the study in terms of age and biochemical parameters. This enables us to compare the Treg and PD-1/PD-L1 mRNA values obtained in the patient and control groups. It was observed that the female and male ratios were similar in the control and patient groups, and the study groups were homogeneous in terms of gender (p=0.53). The mean age of all individuals in the study was 60.59±8.62. It was observed that the mean ages were not significantly different between the control and patient groups (p=0.90).

It was observed that the MM subtypes were IgA K (Kappa) at 15%, IgA L (Lambda) at 10%, IgG K at 25%, and IgG L at 35%. The disease stage levels of the patients were stage 1 at 25%, stage 2 at 15%, and stage 3 at 60%. Five patients were R-ISS 1 (25%), seven patients were R-ISS 2 (35%), and eight patients were R-ISS 3 (40%) (Table 1).

In the study, there was no statistically significant difference in the levels of $CD4^+CD25^+FoxP3^+PD\text{-}1^+$ Treg cells (%) (1.4-11.2%, 0.01-3.5%) between the patient and control groups (p=0.095) (Figure 1a). However, the cell percentage was higher in myeloma patients than in our control group. Relative PD-1 mRNA expression levels in the patient and control groups (0.1-6.62, 0.1-2.17) did not show a statistically significant difference (p=0.167) (Figure 1b). There was no statistically significant difference in the relative PD-L1 mRNA expression levels between the patient and control groups (0.13-37.77, 0.014-11.61) (p=0.69) (Figure 1c). The gating strategy for Tregs is shown in Figure 2. There was no statistically significant difference in the levels of $CD4^+CD25^+FoxP3^+PD\text{-}1^+$ Treg cells (%) (p=0.68), PD-1 mRNA expression levels (p=0.59), and PD-L1 mRNA expression levels (p=0.7) between the groups according to R- of the patients. There was no significant correlation between the expression levels of Treg cells and the levels of PD-1 mRNA and PD-L1 mRNA (p>0.05). There was a strong correlation between PD-1 mRNA and PD-L1 mRNA levels in the control and patient groups (p<0.0001, r=0.827).

Discussion

In MM, as with other hematologic cancers, the relationship between Tregs and cancer has been questioned and has become an area of research. In recent years, the increase in clinical trials of PD-1 and PD-L1 inhibitors in hematologic cancers has led to more scrutiny of the relationship between MM and Tregs, and has also necessitated the development of new research strategies and parameters. In 2014, Braga et al. [15] investigated Tregs in the bone marrow of MM patients as potential biomarkers, therapeutic targets, and prognostic indicators in the local immune environment. Compared to controls, FoxP3 expression was found to be six-fold higher in MM patients. In another study conducted by D'Arena et al. [16] in 2016, the percentage and absolute number of circulating Tregs ($CD4^+$ $CD25^+$high CD127-low) of 39 untreated MM patients and 44 monoclonal gammopathy of undetermined significance (MGUS) patients were tested and compared.

Table 1. Experimental results of MM patients and controls

Patient	Gender	Age	ISS	R-ISS	PD-1⁺ Tregs(%)	PD-1 mRNA copy	PD-L1 mRNA copy
1	F	55	I	1	2.3	0.65	1.05
2	M	75	II	2	1.7	0.92	0.91
3	F	55	I	2	1.4	4.83	2.67
4	M	66	III	3	1.8	0.28	0.15
5	M	67	I	1	1.43	2.98	4.27
6	F	73	II	2	1.9	2.31	5.33
7	F	51	III	3	2.9	6.30	5.06
8	F	65	III	3	2.1	0.40	1.31
9	M	53	III	3	1.5	0.21	0.52
10	F	60	III	3	2.8	4.08	5.891
11	M	45	III	3	1.4	0.18	0.20
12	F	50	III	2	11.2	0.42	1.21
13	F	62	I	1	2.7	0.10	0.16
14	M	67	III	2	2.6	0.21	0.28
15	F	58	1	1	1.8	0.53	0.13
16	M	65	III	3	2.5	0.66	1.23
17	F	66	III	3	1.9	1.64	1.91
18	F	57	I	1	2.8	6.10	37.7
19	M	60	III	2	1.8	6.62	13.1
20	F	59	II	2	1.96	3.01	2.21
Control	**Gender**	**Age**					
1	F	61			1.1	0.29	0.01
2	F	55			0.01	1.06	0.88
3	M	81			0.26	0.19	0.31
4	F	64			0.7	0.28	0.73
5	F	58			0.01	0.48	2.46
6	M	40			2.9	0.58	2.86
7	M	61			3.5	0.81	3.84
8	F	68			0.01	2.17	11.6
9	F	60			1.3	0.10	0.42

F: Female, M: Male, R-ISS: Revised International Staging System, PD-1/PDL-1: Programmed cell death protein 1/death ligand 1, mRNA: Messenger ribonucleic acid

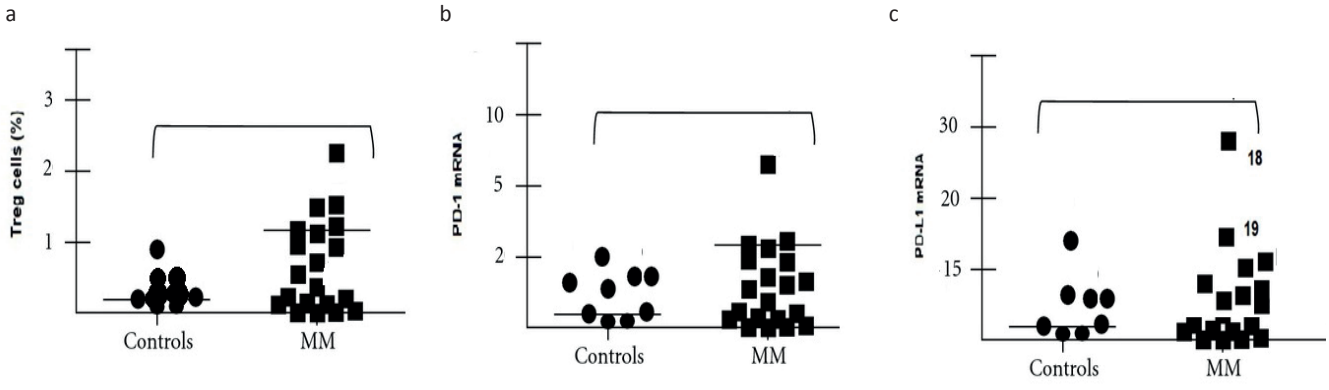
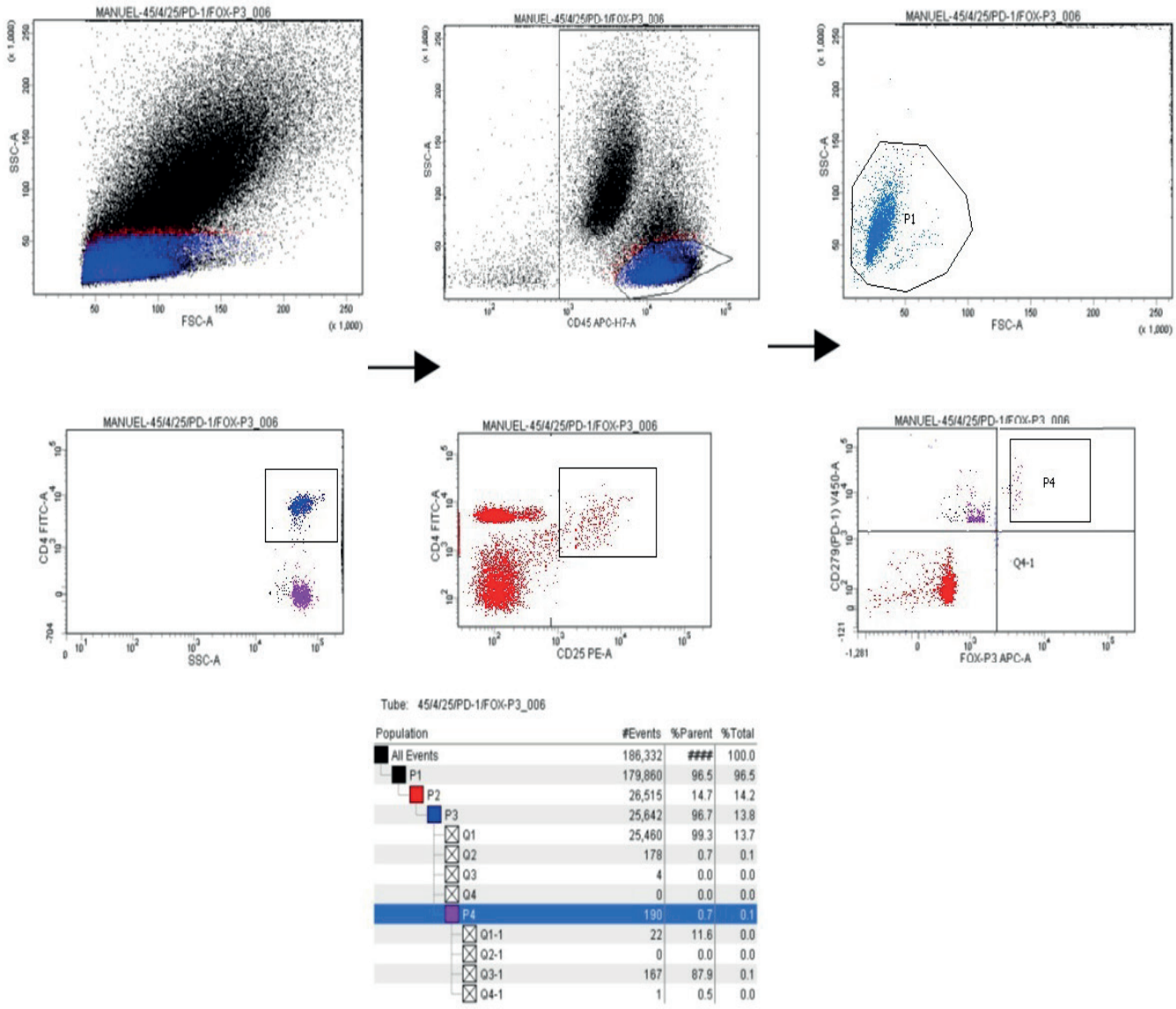


Figure 1. (a,b,c). Treg and PD-1 PD-L1 expression
MM: Multiple myeloma, PD: Programmed cell death protein 1, PD-L1: Programmed cell death-ligand 1, mRNA: Messenger ribonucleic acid



Tube: 45/4/25/PD-1/FOX-P3_006

Population	#Events	%Parent	%Total
All Events	186,332	####	100.0
P1	179,860	96.5	96.5
P2	26,515	14.7	14.2
P3	25,642	96.7	13.8
Q1	25,460	99.3	13.7
Q2	178	0.7	0.1
Q3	4	0.0	0.0
Q4	0	0.0	0.0
P4	190	0.7	0.1
Q1-1	22	11.6	0.0
Q2-1	0	0.0	0.0
Q3-1	167	87.9	0.1
Q4-1	1	0.5	0.0

Figure 2. Gating strategy for Tregs
Fox: Forkhead box, PD: Programmed cell death

Twenty healthy individuals were tested as a control group. However, there is a significant disagreement in the literature regarding the numbers and functions of Tregs in MM.

The average percentage of circulating Tregs was found to be 2.1±1.0% (0.75-6.1%) in MM patients, 2.1±0.9% (0.3-4.4%) in MGUS patients, and 1.5±0.4% (0.9-2.1%) in controls [16,17]. Prabhala et al. [18] found lower Treg cell numbers compared to the control group. In contrast, Beyer et al. [19] showed the presence of strong inhibitory function in MGUS and MM with high Treg cell numbers. A recent study reported that regulatory T-cells (Tregs) were more abundant in the bone marrow in patients with MM [20]. Our study supports this result. We found that PD-1+ Treg cell percentage was higher in myeloma patients than in our control group. However, there was no statistically significant difference in the levels of $CD4^+CD25^+FoxP3^+PD\text{-}1^+$ Treg cells (%) (1.4-11.2%, 0.01-3.5%) between the patient and control groups. In another study conducted on peripheral blood lymphocytes in ITP patients, the $CD4^+$ CD25 high $FoxP3^+$ Treg cell rate was found to be 9.69±3.70% [21]. In our study, unlike this study, 0.01±3.5% was measured in lymphocytes obtained from bone marrow, not from peripheral blood, and this difference in percentage occurred because PD-1 expression was also included [21]. The differences in Treg function and numbers in these studies may be due to differences in purification techniques and evaluations. Although peripheral blood is typically used in such studies, we used bone marrow material, which is more difficult to obtain.

Clinical studies have been conducted on PD-1 and PD-L1 inhibitors for hematological malignancies. Badros et al.

[22] published the results of their combination study of pembrolizumab, low-dose dexamethasone, and pomalidomide in relapsed/refractory MM. In this study, PD-L1 expression was evaluated as positive or negative using immunohistochemical staining of bone marrow biopsies in 29 patients, and it was found to be correlated with treatment response. In analyses of pre-treatment bone marrow samples, there is increased expression of PD-L1 in responding patients, independent of PD-1 expression. In a recent study conducted, it was found that low-dose, single-fraction radiotherapy in combination with pembrolizumab was safe and induced an early response. They stated that larger studies were needed to confirm their findings [23].

The number of studies that have investigated the correlation of Treg cell levels with PD-1 mRNA and PD-L1 mRNA levels is limited in the literature. However, in our study, no positive or negative relationship was found between Treg cell expression and PD-L1 and PD-1 mRNA levels. A recent review reported that studies with proteasome inhibitors and monoclonal antibodies are ongoing, and long-term analyses are awaited [24]. Studies like ours contribute to expanding the knowledge base and accelerating clinical studies.

Study Limitations

Limitations of this study include the selection of bone marrow, which is not as easily accessible as peripheral blood, this may have limited both the small patient population and our comparison group.

Conclusion

Although not statistically significant, $CD4^+CD25^+FoxP3^+PD\text{-}1^+$ Treg cell percentages and PD-1, PD-L1 gene expression distributions differed in our patient control groups. We found both higher cell percentages and higher gene expressions in our patient group compared to controls. There was no significant relationship between Treg cell expression and both PD-1 mRNA and PD-L1 mRNA levels. However, a strong correlation was found between PD-1 mRNA and PD-L1 mRNA levels. This strong relationship supports the use of blockade points in the treatment of myeloma patients. In addition, selecting bone marrow, which is difficult to access, as the study material and including ITP patients as the control group increases the specificity of our study and its contribution to science.

In the coming years, our study should be supported by further studies with a larger number of patients to identify who will benefit from treatment strategies targeting Treg cells and the PD-1/PD-L1 pathway.

Ethics

Ethics Committee Approval: Ethical approval was obtained from the Faculty of Medicine at Akdeniz University (decision no: 664, date: 14.12.2016). The study was conducted at the Hematology Outpatient "Clinic of Akdeniz University Faculty of Medicine" Hospital, with the necessary permissions obtained from the hospital administration, and in accordance with research and publication ethics.

Informed Consent: Written consent was obtained from all patients.

Footnotes

Authorship Contributions

Surgical and Medical Practices: O,S,. L.Ü., Concept: D.E., A.Ö., A.T., N.S.E., Design: D.E., A.Ö., N.S.E., L.Ü., Data Collection or Processing: D.E., O.S., A.T., M.U., N.S.E., Analysis or Interpretation: D.E., A.Ö., O.S., A.T., M.U., N.S.E., L.Ü., Literature Search: D.E., A.Ö., N.S.E., L.Ü., Writing: D.E., A.Ö., A.T., N.S.E., L.Ü.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This study was supported by the Akdeniz University Scientific Research Projects Management Unit (Project No: FDK-2017-2382).

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