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SHORT COMMUNICATION

Increase of $CD4^+CD25^+FoxP3^+$Regulatory T Cells in Myeloma

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SUMMARY

Background: **In the tumor microenvironment, regulatory T cells (Treg cells), a small subset of $CD4^+$ T cells, can interrupt antitumor immune reactions by interacting with various other immune cells. The present study aimed to determine the expression level of Treg cells in myeloma compared with that in normal control.**
Methods: **Peripheral blood samples from 90 healthy adults and marrow aspiration samples from 13 patients newly diagnosed with myeloma were collected. Eight-color flow cytometry was performed using a Duraclone IM Treg tube kit. A gating strategy was established based on immunophenotypic characteristics of Treg cells identified as $CD3^+CD4^+CD25^+FoxP3^+CD39^+Helios^+$ cells.**
Results: **A 2.5-fold increase in Treg cells, based on the $CD4^+CD25^+FoxP3^+$ combination, was confirmed in the initial myeloma diagnosis compared with the normal control. Additionally, increased Treg cells had a weak positive correlation with urine M-protein levels ($r = 0.619$, $p < 0.05$).**
Conclusions: **Thus, an increase in Treg cells appears to potentially predict a worse complication of myeloma based on microenvironmental-related biomarkers.**
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KEYWORDS

flow cytometry, myeloma, regulatory T cells, renal impairment

INTRODUCTION

Regulatory T cells (Treg cells) are a small subset comprising 2 - 4% of $CD4^+$ T cells characterized by a $CD3^+CD4^+CD25^{high}CD127^{low}Foxp3^+$ immunophenotype [1,2]. Normally, they play a role in maintaining immune homeostasis by suppressing T cell proliferation and cytokine production [3,4]. In tumors, the number of Treg cells increases, altering the anti-tumor immunity [5]. Thus, a targeted therapy utilizing the immunosuppressive activity of Treg cells might be an option in addition to conventional anticancer treatments [6].
Recent clinical trials for plasma cell myeloma have used a combination regimen of three drugs including proteasome inhibitors and immunomodulatory drugs to improve clinical outcomes as an induction therapy [7].

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Lenalidomide included in the VRD regimen (bortezomib, lenalidomide, and dexamethasone) can induce an immune modulatory effect with an anti-tumor response by expanding antigen-specific $CD8^+$ T cells. In addition, an increase of $CD14^+CD15^+$ myeloid-derived suppressor cells and a reduction of Treg cells have been reported after lenalidomide treatment [8,9].

The present study aimed to compare the expression levels of Treg cells in healthy individuals and patients newly diagnosed with plasma cell myeloma and analyze their correlation with tumor burden.

MATERIALS AND METHODS

The present study included 13 patients with newly diagnosed plasma cell myeloma and 90 healthy individuals as normal controls. Normal controls (n = 90) were adults (18 - 65 years) who had not been diagnosed with a hematologic disorder. Their average age was 45.8 years with a male-to-female ratio of 1:1. Thirteen patients (seven males and six females) were newly diagnosed with plasma cell myeloma based on results of bone marrow examination and related laboratory tests performed at Chungbuk National University Hospital from June 2023 to April 2024. The average age of the patients was 69.5 years. As for M-protein type, out of a total of 13 patients, five had IgG-Kappa type, two had IgA-Kappa, two had IgM-Kappa, one had IgG-Lambda and three had light chain type (one Kappa and two Lam ba). Serum M-protein averaged 19.5% or 2.4 mg/dL and urine M-protein averaged 28.3% or 78.1 mg/dL. The mean serum-free light chain (FLC) ratio (involved to uninvolved) was 527.7. In bone marrow aspirated samples, the percentage of plasma cells in total nucleated cells averaged 44.6%.

Peripheral blood and bone marrow aspirate samples were collected from healthy donors and patients with myeloma into EDTA tubes. Treg cells were counted using a DuraClone IM Treg Tube (Beckman Coulter, Inc., Fullerton, CA, USA), according to the manufacturer's instructions [10]. Cells were analyzed using a Navios flow cytometer (Beckman Coulter, Inc.). Eight-color flow cytometric immunophenotyping was used to detect Treg cell populations with the following monoclonal antibodies: CD45RA (clone 2H4LDH11LDB9)-FITC; CD25 (clone B1.49.9)-PE; CD39 (clone BA54)-PC5.5; CD4 (clone SFCI12T4D11)-PC7, FoxP3 (clone 259D)-A647, CD3 (clone UCHT-1)-AA750, Helios (clone 22F6)-PBE; and CD45(clone J33)-KrO. Data were analyzed using Kaluza analysis software (Beckman Coulter, Inc.). A gating strategy was established based on immunophenotypic characteristics of Treg cells, i.e., $CD3^+CD4^+CD25^+FoxP3^+CD39^+Helios^+$. First, lymphocytes were gated in the $CD45^{bright}/SSC^{low}$ histogram. The $CD3^+CD4^+$ population was then selected. Afterward, the positive region of each marker was quantified by gating (Figure 1). Specimens from healthy individuals were used as residual peripheral blood samples after routine test for experimental setup, while bone marrow samples from patients with myeloma were obtained from Korea Biobank Network. This study was approved by the Institutional Review Board of Chungbuk National University Hospital (CBNUH 2023-04-007-01, CBNUH 2022-07-017-003).

Mann-Whitney U test was used for continuous variables between myeloma patients and normal healthy controls. Spearman's correlation method was used for association analysis between two continuous variables. All statistical analyses were performed using SPSS version 27.0 (IBM SPSS Statistics for Windows; IBM Corp., Armonk, NY, USA). $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Since the healthy individuals in this study were significantly younger than the patients with myeloma, the changes in Treg cells according to age were examined using the results from the healthy individuals. To assess any age-related difference, 10-year interval age groups were set as follows: 20s (n = 8), 30s (n = 7), 40s (n = 26), 50s (n = 32) and 50s (n = 7). Upon comparing the proportion of the $CD3^+CD4^+CD25^+FoxP3$ Treg cells across these groups, a tendency was observed for the values to decrease with age particularly after 40s, although there was no statistically significant difference between the groups.

Additionally, before comparing the proportion of Treg cells, an assessment was conducted to evaluate the impact of sample difference between the control and patient groups. There were differences in samples used from normal controls and myeloma patients when measuring Treg cells. Peripheral blood samples were used from normal controls, whereas bone marrow aspirate samples were collected from myeloma patients. Because there might be differences between the two sampling methods, peripheral blood and bone marrow samples were measured simultaneously for three myeloma patients. The percentage of lymphocytes and the proportion of $CD3^+CD4^+$ T cells differed between bone marrow samples and peripheral blood, while the proportion of regulatory T cells within the total $CD3^+CD4^+$ T cells was similar in both samples. A total of 18 test results were obtained for a total of six markers: $CD4^+CD25^+$, $CD4+FoxP3^+$, $CD4+Helios^+$, $CD4+CD39^+$, $CD25^+FoxP3^+$ and $FoxP3+Helios^+$. These markers showed a positive correlation with an R-squared value of 0.815 indicating agreement (Figure 2).

Although there was no difference in peripheral blood white blood cell counts between normal controls and patients, the percentage of lymphocytes was decreased in patients with multiple myeloma. In addition, hemoglobin and platelet levels were lower compared with the normal controls (Table 1). The proportion of Treg cells using each sample from the normal controls and patients, following the gating strategy described earlier, is

Table 1. Baseline characteristics of normal control and patients with myeloma.

Laboratory Parameters	Normal control (n = 90)			Myeloma patients (n = 13)			
	Mean	SD	Range	Mean	SD	Range	p-value
Age (years)	45.8	10.9	20 - 65	69.5	7.7	50 - 77	< 0.001
Complete blood count							
Hemoglobin (g/dL)	14.6	1.1	12.7 - 16.9	9.8	2.4	5.9 - 14.8	< 0.001
White blood cells (x 10^3/mL)	6.1	1.2	3.2 - 9.8	5.7	3.0	2.1 - 12.1	0.328
Lymphocyte (%)	43.0	12.7	16.1 - 77.6	29.2	9.2	18.0 - 45.0	< 0.001
Platelet count (x 10^3/mL)	250	48.6	159 - 379	172	56.1	53 - 241	< 0.001
Bone marrow plasma cell (%)	-	-	-	44.6	29.2	3.5 - 94.0	
Serum M protein (%)	-	-	-	25.9	19.5	0 - 58.2	
Serum M protein (mg/dL)	-	-	-	2.6	2.4	0 - 7.2	
Serum free light chain ratio (Involved to uninvolved)	-	-	-	527.7	1330.3	0.9 - 4,907.7	
Urine M protein (%)	-	-	-	28.3	144.0	0 - 76.2	
Urine M protein (mg/dL)	-	-	-	78.1	144.0	0 - 453.0	
Beta-2 globulin (mg/L)	-	-	-	5.8	4.2	2.0 - 17.9	

Table 2. Comparison of regulatory T cells between 90 normal control and 13 patients with myeloma.

Marker		Normal control (n = 90)			Myeloma patients (n = 13)			Myeloma: Normal	p-value
		Mean	SD	95% CI	Mean	SD	95% CI	Ratio	
Lymphocyte	%	43.0	12.7	40.3 - 45.6	15.0	9.4	6.3 - 20.6	0.5	< 0.001
	/µL	40,787	13,455	37,975 - 43,612	36,445	23,334	23,760 - 49,130		0.180
$CD4^+$ of lymphocytes	%	31.6	6.1	30.3 - 32.8	23.0	7.4	18.5 - 27.5	0.7	< 0.001
	/µL	12,858	4,721	11,865 - 13,842	8,229	3,949	6,082 - 10,376		< 0.001
$CD4^+CD25^+$	%	5.5	2.6	4.9 - 6.0	7.8	3.3	5.8 - 9.8	1.4	0.015
	/µL	689	369	599 - 740	525	259	384 - 667		0.193
$CD4^+CD39^+$	%	3.7	2.1	3.3 - 4.2	7.5	4.0	5.1 - 10.0	2.0	< 0.001
	/µL	442	491	388 - 513	580	536	289 - 873		0.484
$CD4^+FoxP3^+$	%	3.8	2.1	3.4 - 4.2	7.2	2.6	5.6 - 8.8	1.9	< 0.001
	/µL	491	323	425 - 552	562	373	360 - 765		0.575
$CD4^+Helios^+$	%	7.9	3.5	7.2 - 8.6	8.5	3.3	6.6 - 10.5	1.1	0.666
	/µL	1,061	678	908 - 1,157	672	525	387 - 958		0.022
$CD4^+CD25^+FoxP3^+$	%	1.8	0.9	1.6 - 2.0	4.5	2.0	3.3 - 5.7	2.5	< 0.001
	/µL	240	149	203 - 264	339	465	220 - 460		0.182
$CD4^+FoxP3^+Helios^+$	%	2.7	1.5	2.4 - 3.0	6.1	2.9	4.3 - 7.6	2.3	< 0.001
	/µL	345	240	294 - 395	465	327	280 - 644		0.295

illustrated in Figure 1. The results of four single markers ($CD25^+$, $CD39^+$, $FoxP3^+$, and $Helios^+$) and two combination markers ($CD4^+CD25^+$ and $CD4^+$ $CD25^+Fox$ $P3^+$) were obtained. The percentage of $CD3^+CD4^+$ T cells was also reduced in patients with multiple myeloma, whereas the proportion of Treg cells increased (Table 2). On average, the quantitative value of each marker demonstrated a 1.9-fold increase. When various Treg cell-related antibody combinations were used, an increase in Treg cells was observed in myeloma patients

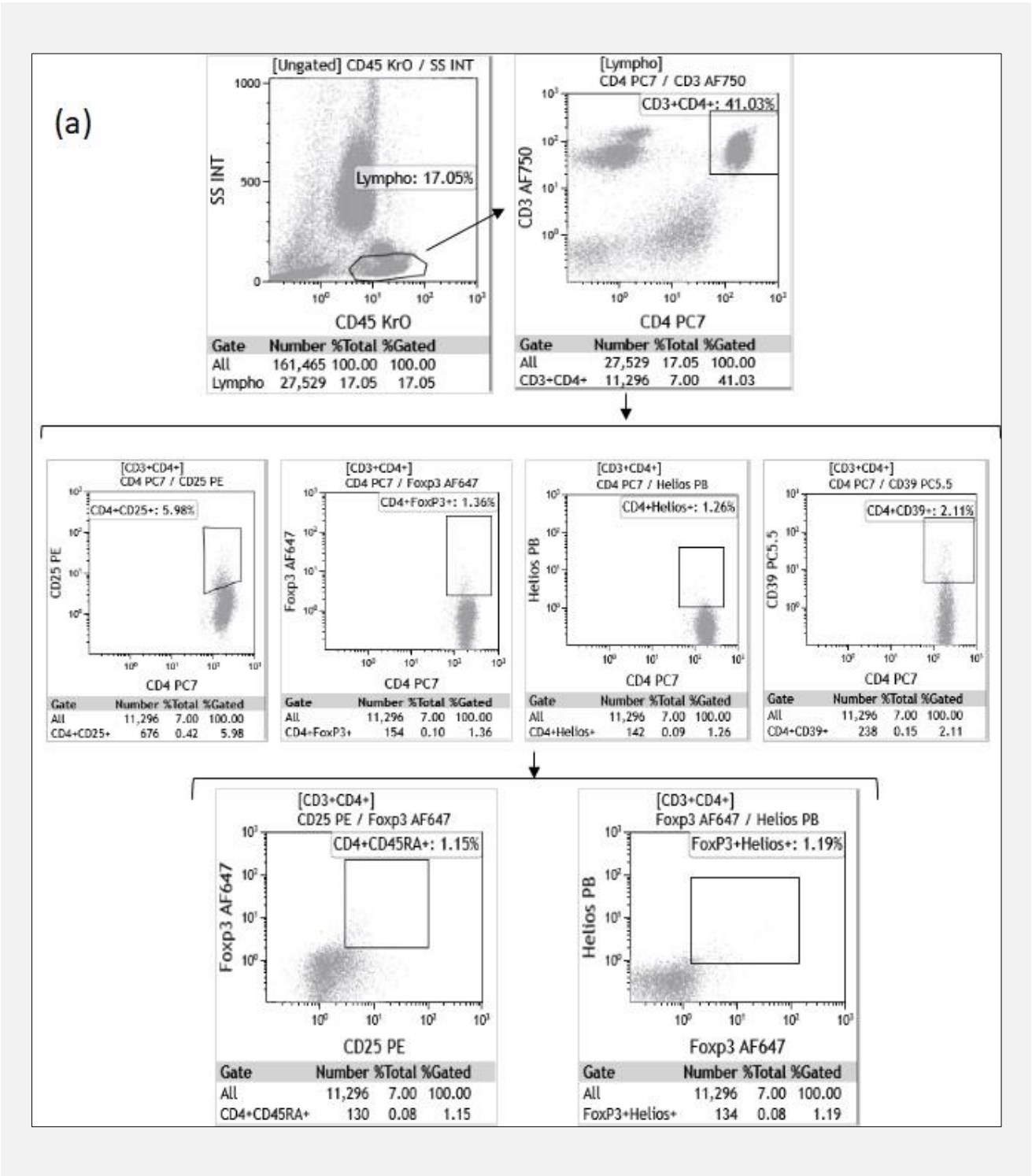

Figure 1a. An example of gating strategy for regulatory T cells in healthy normal control.

Lymphocytes were gated on the $CD45^{bright}SSC^{low}$ histogram, and then the $CD3^+CD4^+$ T cells was defined. Each or combination of immunophenotypic markers (i.e., $FoxP3^+/CD25^+$) were used for measuring regulatory T cells. The proportion of $CD25^+/CD25^+FoxP3^+$ Treg cells of $CD3^+CD4^+$ T cells was statistically higher than that of the healthy individuals.

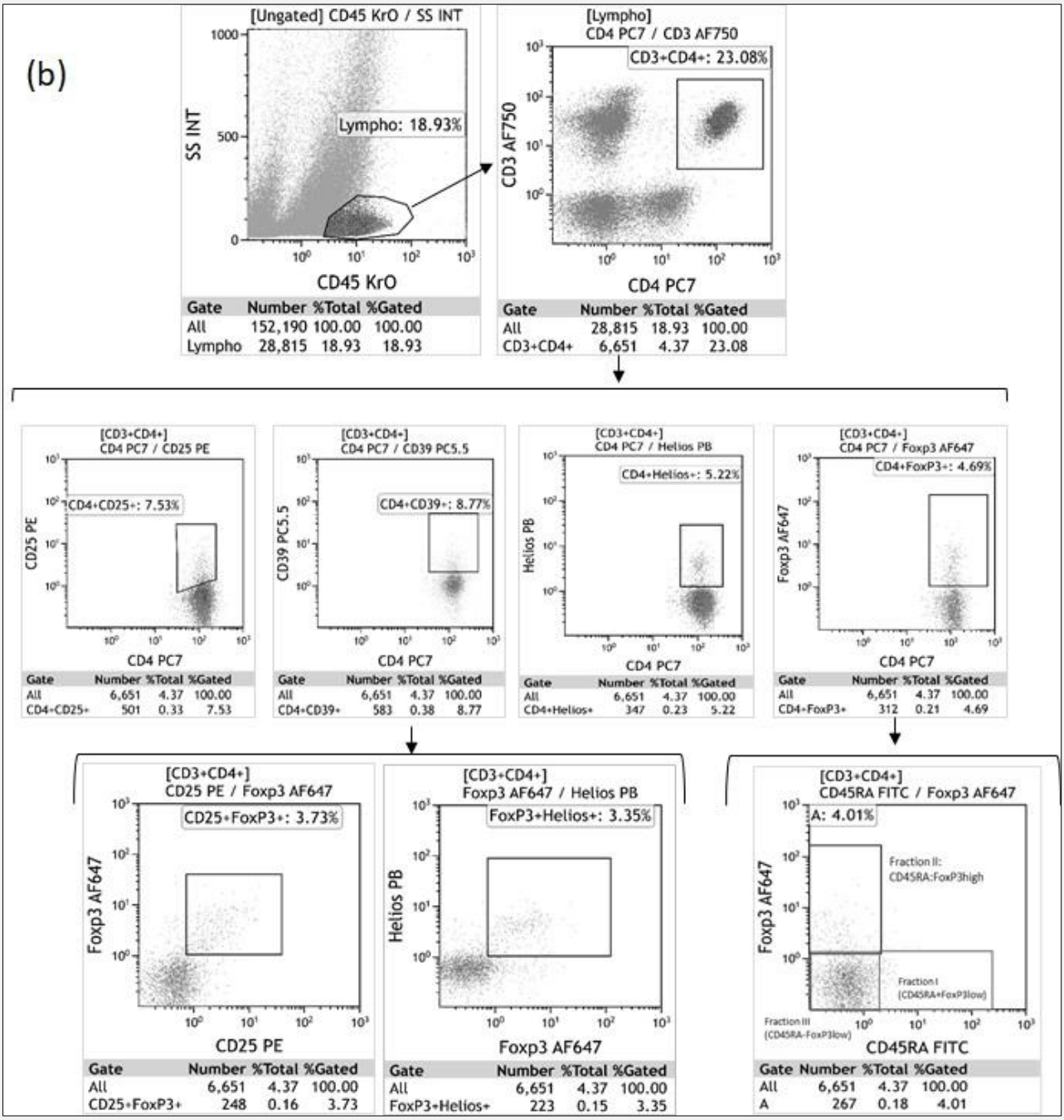

Figure 1b. An example of gating strategy for regulatory T cells in patient with myeloma.

Lymphocytes were gated on the $CD45^{bright}SSC^{low}$ histogram, and then the $CD3^{+}CD4^{+}$ T cells was defined. Each or combination of immunophenotypic markers (i.e., $FoxP3^{+}/CD25^{+}$) were used for measuring regulatory T cells. The proportion of $CD25^{+}/CD25^{+}FoxP3^{+}$ Treg cells of $CD3^{+}CD4^{+}$ T cells was statistically higher than that of the healthy individuals.

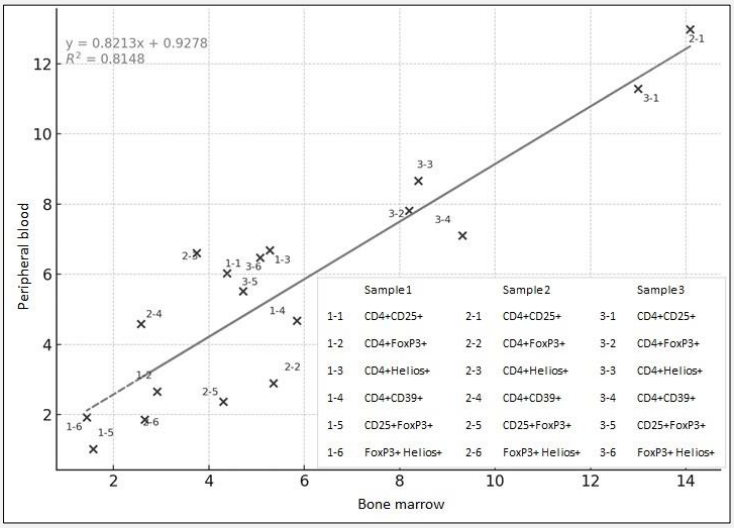

Figure 2. Linear correlation between the proportion of Treg cells/total lymphocytes in BM samples and peripheral blood by flow cytometry.

All 6 markers showed strong positive correlation.

compared with the normal controls in all analyses except for those involving Helios. Among these, the $CD4^+CD25^+FoxP3^+$ combination showed the greatest difference in proportion and exhibited the smallest standard deviation. In addition, when applying CD45RA/FoxP3 gating after CD4+ selection, effector T cells (Fraction II: CD45RA-FoxP3high) were detected and it did not differ significantly from CD4+FoxP3+ population ($p = 0.79$) (Figure 1b).

In addition, the correlation between the expression of Treg cells and myeloma tumor burden-related markers were analyzed. Bone marrow plasma cell percentage, serum M-protein level (% or mg/dL), serum ratio, urine M-protein level (% or mg/dL), and β-2 microglobulin (mg/L) were selected as tumor burden markers for myeloma. Among these, $CD4^+CD25^+$ Treg cells showed a positive correlation with urine M-protein quantitative value (urine M-protein in %: $r = 0.619$, $p < 0.05$; urine M-protein in mg/dL: $r = 0.569$, $p < 0.05$).

DISCUSSION

In previous studies, various antibodies such as CD25, CD39, FoxP3, and Helios were used to quantify Treg cells. When added to $CD3^+CD4^+$ antibodies, CD25, CD39, and FoxP3 were individually observed to be statistically different between the normal controls and patients with myeloma. Among them, $CD4^+CD25^+FoxP3^+$ Treg cells showed a small standard deviation with a non-overlapping 95% CI. Therefore, it was considered that this combination of antibodies was the most appropriate to show the difference in the expression of Treg cells in the normal controls and the patients with myeloma.

Notably, among several immunophenotypical markers representing Treg cell differentiation, FoxP3 is an antigen commonly expressed. Treg cells can be classified into natural Treg cells (nTreg) and induced-to-adjust Treg cells (iTregs) based on their production and biological characteristics. CD25 is also expressed in nTregs cells that develop in the thymus. $CD25^+$ Treg cells are known to have a higher affinity for apoptosis and self-antigen with a different transcriptome than $FoxP3^+$ Treg cells [6]. Therefore, $CD25^+FoxP3^+$ Treg cells seem to more accurately represent Treg cells. On the other hand, $Helios^+$ have recently been mentioned as markers indicating activated Treg cells [11,12]; however, further verification using a larger number of samples is necessary in the future.

In the present study, a 2.5-fold increase in Treg cells, based on the $CD4^+CD25^+FoxP3^+$ combination, was confirmed in initial myeloma diagnosis compared with normal controls. To date, various pathological characteristics, serological test values, cytogenetics, and genetic factors have been used as prognostic indicators for predicting survival in hematologic neoplasms. Changes in the function of various immune cells as part of the tumor microenvironment are also likely to have similar clinical significance. Increased Treg cells are known to interfere with antitumor immunity and induce adverse outcomes, making them a factor to consider in risk strat-

ification analysis [5].

Another new finding of the present study was that increased Treg cells had a weak positive correlation with urine M-protein levels ($r = 0.619$, $p < 0.05$). Various chemical laboratory tests can be used for diagnosis and follow-up for myeloma. Among them, urine M-protein is useful because it can predict myeloma-related initial renal damage and subsequent renal failure [13,14]. Furthermore, serum creatinine and β-2 microglobulin have been suggested as biomarkers of renal impairment [15]. Further analysis revealed that the increase of Treg cells was statistically positively correlated with serum creatinine (Spearman's rank 0.618, $p < 0.05$). Renal dysfunction is associated with tumor burden, and Tregs may serve as an indirect marker of this. It has also been suggested that in immune-mediated renal disease, functional abnormalities of Tregs, which are involved in systemic immune responses, can have a direct impact on the kidneys [16]. Therefore, it is possible that increased Treg cells in myeloma may further worsen renal function. Unfortunately, no correlation was observed between plasma cells in bone marrow and Treg cells. It may be due to site variation, as the differential count of plasma cells in bone marrow in performed by identifying the most ideal zone on the slide. So, quantification of plasma cells in the overall bone marrow may vary depending on the examiner, which could lead to differences in the results. Due to the limited number of samples, further study including functional analysis on the renal impairment prediction model of Treg cells seems warranted.

In conclusion, the present study showed that $CD4^+$ $CD25^+FoxP3^+$ Treg cells in patients with initial myeloma increased by 2.5 times compared with normal controls and that this increase of Treg cells may be associated with renal impairment. In addition, it suggested that among previously reported immunophenotypical markers of Treg cells, $CD25^+FoxP3^+$ more appropriately reflected an activated state. Although the number of samples included in this study was very small, it showed that Treg cells have the potential as a microenvironment-related biomarker for predicting worse complications of myeloma patients.

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Data Availability:

The data may be requested from the corresponding author.

Declaration of Interest:

The authors declare that they have no competing interests.

References:

1. Grant J, Bourcier K, Wallace S, et al. Validated protocol for FoxP3 reveals increased expression in type 1 diabetes patients. Cytometry B Clin Cytom 2009;76:69-78. (PMID: 18690669)

2. Pitoiset F, Barbié M, Monneret G, et al. A standardized flow cytometry procedure for the monitoring of regulatory T cells in clinical trials. Cytometry B Clin Cytom 2018;94:621-6. (PMID: 29316248)

3. Castillo-Aleman YM, Bencomo-Hernandez AA, Ventura-Carmenate Y. CD4+Foxp3+Helios+ regulatory T cells: Role in immunostasis and clinical applications. J Cell Mol Immunol 2022; 1(1):5-7. https://probiologists.com/Uploads/Articles/36_637834347727869463.pdf

4. Liston A, Gray DH. Homeostatic control of regulatory T cell diversity. Nat Rev Immunol 2014;14:154-65. (PMID: 24481337)

5. Curiel TJ. Regulatory T cells and treatment of cancer. Curr Opin Immunol 2008;20:241-6. (PMID: 18508251)

6. Li C, Jiang P, Wei S, Xu X, Wang J. Regulatory T cells in tumor microenvironment: new mechanisms, potential therapeutic strategies and future prospects. Mol Cancer 2020 Jul 17;19(1):116. (PMID: 32680511)

7. Jung SH, Jo JC, Song GY, et al. Frontline therapy for newly diagnosed patients with multiple myeloma. Blood Res 2020;55(S1): S37-S42. (PMID: 32719175)

8. Busch A, Zeh D, Janzen V, et al. Treatment with lenalidomide induces immunoactivating and counter-regulatory immunosuppressive changes in myeloma patients. Clin Exp Immunol 2014; 177:439-53. (PMID: 24712857)

9. Galustian C, Meyer B, Labarthe MC, et al. The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. Cancer Immunol Immunother 2009;58:1033-45. (PMID: 19009291)

10. Kronenberg K, Riquelme P, Hutchinson JA. Standard protocols for immune profiling of peripheral blood leucocyte subsets by flow cytometry using DuraClone IM reagents. 2021. https://pdfs.semanticscholar.org/7846/e3295ee8b3f75c62d7c77aa88cb56234df47.pdf

11. Elkord E, Abd Al Samid M, Chaudhary B. Helios, and not FoxP3, is the marker of activated Tregs expressing GARP/LAP. Oncotarget 2015;6:20026-36. (PMID: 26343373)

12. Timperi E, Barnaba V. CD39 regulation and functions in T cells. Int J Mol Sci 2021;22:8068. (PMID: 34360833)

13. Corso A, Zappasodi P, Pascutto C, et al. Urinary proteins in multiple myeloma: correlation with clinical parameters and diagnostic implications. Ann Hematol 2003;82:487-91. (PMID: 12838370)

14. Morrison T, Booth RA, Hauff K, Berardi P, Visram A. Laboratory assessment of multiple myeloma. Adv Clin Chem 2019;89:1-58. (PMID: 30797467)

15. Woziwodzka K, Vesole DH, Malyszko J, et al. New markers of renal failure in multiple myeloma and monoclonal gammopathies. J Clin Med 2020;9:1652. (PMID: 32486490)

16. Ghali JR, Wang YM, Holdsworth SR, Kitching AR. Regulatory T cells in immune-mediated renal disease. Nephrology (Carlton) 2016;21:86-96. (PMID: 26206106)