



# Immune Checkpoint Programmed Cell Death Protein-1 (PD-1) Expression on Bone Marrow T Cell Subsets in Patients With Plasma Cell Myeloma

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**Background:** Plasma cell myeloma (PCM) is caused by immune dysregulation. We evaluated the expression of immune checkpoint programmed cell death protein-1 (PD-1) on T cell subsets in PCM patients according to disease course and cytogenetic abnormalities. This study aimed to find a target group suitable for therapeutic use of PD-1 blockade in PCM.

**Methods:** A total of 188 bone marrow (BM) samples from 166 PCM patients and 32 controls were prospectively collected between May 2016 and May 2017. PD-1 expression on BM T cell subsets was measured using flow cytometry.

**Results:** At diagnosis, the median PD-1 expression on CD4<sup>+</sup> T cells was 24.6%, which did not significantly differ from that in controls. After stem cell transplantation, PD-1 expression on CD4<sup>+</sup> T cells was higher than that at diagnosis ( $P < 0.001$ ), regardless of residual disease. PD-1 expression on CD4<sup>+</sup> T cells in patients with residual disease after chemotherapy was significantly higher than that at diagnosis ( $P = 0.001$ ) and after complete remission following chemotherapy ( $P = 0.044$ ). PD-1 expression on CD8<sup>+</sup> T cells was higher in PCM patients with cytogenetic abnormalities, including monosomy 13, 1q gain, complex karyotype, and hypodiploidy.

**Conclusions:** PD-1 blockade might have therapeutic potential in refractory PCM patients after chemotherapy, especially in those with high- or intermediate-risk cytogenetic abnormalities.

**Key Words:** Plasma cell myeloma, Immune checkpoint programmed cell death protein-1 (PD-1), Flow cytometry, T cell subset

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## INTRODUCTION

Malignant cells evade host immunity and change the tumor mi-

croenvironment milieu through activation of immune checkpoints. Immune checkpoint inhibitors block the activation of the immune system in the body, usually preventing the over-acti-

tion of T cell responses. Plasma cell myeloma (PCM) is caused by a microenvironment promoting immune escape in the bone marrow (BM) and immune dysregulation through the loss of effector T cell populations. PCM is a genetically complex disease that develops via multistep progression. The cytogenetic categories of PCM are major indicators of prognosis and form the basis for disease risk stratification.

Immune checkpoint programmed cell death protein-1 (PD-1) receptors are normally expressed on T cells, B cells, monocytes, and natural killer T cells. Programmed death-ligand 1 (PD-L1)/L2 are ligands expressed by antigen presenting cells, pancreatic islet cells, endothelial cells, and epithelial cells. PD-L1/L2 have been reported to be highly expressed on tumor cells [1]. Immune checkpoint inhibitors are effective in managing solid cancers and hematologic malignancies, and are being actively developed and used for treatment. Checkpoint inhibitors are expected to be effective in PCM treatment, and several trials involving PCM patients have been conducted; however, a clear benefit has not been observed. In a phase Ib trial, the PD-1 inhibitor nivolumab did not elicit an objective response in PCM patients [2]. The results of trials examining nivolumab in combination with other agents (NCT01592370 and NCT02726581) are not yet available. Notably, the United States Food and Drug Administration halted two randomized phase-III trials (KEYNOTE-185 trial and KEYNOTE-183 trial) owing to immune-related adverse events in 2017 [3, 4]. The limited effectiveness of immune checkpoint inhibitors in PCM suggests that the immune checkpoints functions change in different disease states. This study aimed to identify patients who may benefit from treatment with immune checkpoint inhibitors. We analyzed PD-1 expression on CD4<sup>+</sup>/CD8<sup>+</sup> T cells in PCM patients according to disease course, genetic abnormalities, disease states, and involved/uninvolved free light chain ratio. To our knowledge, no study has examined PD-1 expression in PCM according to the type of treatment received and genetic variation.

## MATERIALS AND METHODS

### Sample collection and data acquisition

We prospectively obtained 192 BM samples from 166 PCM patients at Asan Medical Center, Seoul, Korea, between May 2016 and May 2017; four samples were excluded because of lack of sample volume or patient information, and 188 samples from 162 PCM patients were included. The BM samples of PCM patients included 81 samples obtained at diagnosis and 107 obtained at follow-up. The mean value of the follow-up period after

initial diagnosis was  $31.8 \pm 36.5$  months and that after stem cell transplantation (SCT) was  $23.3 \pm 30.5$  months. PCM disease stage and treatment response were determined, according to the Revised International Staging System (R-ISS) developed by the International Myeloma Working Group in 2014 [5]. We assessed patient characteristics and medical history by chart review and performed laboratory investigations, including free light chain, M-peak, FISH, and karyotyping, as routine practice for PCM work-up. Fifty-three patients with available albumin, lactate dehydrogenase, karyotype, FISH, and  $\beta$ -2-microglobulin test results at diagnosis were classified using R-ISS index criteria; three were at R-ISS stage I; 34, at stage II; and 15, at stage III. Follow-up patients were divided into two groups: those treated with chemotherapy (CTx; N=51) and those receiving autologous SCT (N=56) (Table 1). Cytogenetic abnormalities were categorized as standard-, intermediate- and high-risk using the Mayo stratification for myeloma and risk-adapted therapy classification (mSMART) [6].

Control BM samples were collected from 32 patients who had undergone a BM study owing to cytopenia or fever of unknown origin, had no hematologic malignancy of the BM, and were reported as having normo/hypo/hypercellular marrow.

This study was approved by the Institutional Review Board of Asan Medical Center (Approval Number: 20161087); the subjects' informed consent to participate in research was waived. Written informed consent was obtained from all patients for the BM biopsy procedures and genetic testing, and the study was performed in accordance with the Declaration of Helsinki.

### Flow cytometric measurement of PD-1 expression on T cell subsets

BM aspirates were collected in EDTA tubes (BD Vacutainer; Becton Dickinson [BD] Franklin Lakes, NJ, USA). We incubated 100  $\mu$ L of phosphate-buffered saline (PBS)-diluted BM aspirate (nucleated cell number 10,000–20,000/ $\mu$ L) with fluorescent dye-conjugated antibodies (4–8  $\mu$ L each of phycoerythrin [PE]-conjugated anti-PD-1 [clone EH12.1, BD Pharmingen PE Mouse Anti-Human CD279], allophycocyanin [APC]-cyanine 7 [Cy7]-conjugated anti-CD3 [clone SK7, BD Pharmingen APC-Cy7 Mouse Anti-Human CD3], APC-conjugated anti-CD4 [clone RPA-T4, BD Pharmingen APC Mouse Anti-Human CD4], fluorescein isothiocyanate [FITC]-conjugated anti-CD8 [clone HIT8a, BD Pharmingen FITC Mouse Anti-Human CD8], and peridinin chlorophyll protein complex [PerCP]-conjugated anti-CD45 [clone 2D1, BD Pharmingen PerCP Mouse anti-human CD45] antibodies purchased from BD Pharmingen [San Diego, CA, USA] for 20

**Table 1.** Patient characteristics

	Diagnosis	Follow-up (N=107)				Patient Total
		CTx-CR	CTx-RD	SCT-CR	SCT-RD	
Samples, N	81	30	21	29	27	188
Male/female	43/38	16/14	11/10	17/13	18/9	106/86
Age, yr, median (range)	64 (37–86)	62 (38–75)	58 (42–73)	58 (26–66)	61 (47–69)	61 (26–86)
Type of M protein						
IgA	16	6	3	6	8	39
IgG	39	14	6	11	13	84
IgD	3	0	4	1	2	10
IgM	0	0	0	1	0	
Light chain	20	9	7	8	4	48
Nonsecretory	3	1	1	2	0	7
Plasma cell in BM, %, mean	41.6	1.6	27.4	0.8	29.1	25.5
Treatment						
VTD		20	5			
TD		3	7			
VMP		6	6			
HD-Dexa		1	1			
TCD		0	1			
Palmidronate		0	1			
R-ISS						
I	3					
II	34					
III	15					
Cytogenetic abnormalities						
Monosomy 13	24		5		11	40
1q gain	15		5		9	29
Complex	37		8		14	59
Hypodiploidy	12		3		5	20
Hyperdiploidy	18		3		8	29
t(11;14)	11		4		2	17
del(17p)	2				2	4

Abbreviations: CTx, chemotherapy; CR, complete remission; RD, residual disease; SCT, stem cell transplantation; VTD, vincristine/thalidomide/dexamethasone; TD, thalidomide/dexamethasone; VMP, vincristine/melphalan/prednisolone; HD-Dexa, high-dose dexamethasone; TCD, thalidomide/cyclophosphamide/dexamethasone; R-ISS, revised international staging system; CTx-CR, with complete remission after chemotherapy; CTx-RD, with residual disease after chemotherapy; SCT-CR, with complete remission after stem cell transplantation; SCT-RD, with residual disease after stem cell transplantation; BM, bone marrow; t, translocation; del, deletion.

min in the dark, at room temperature (20–22°C). After washing with PBS, the samples were lysed with ammonium chloride-based lysing solution for 10 minutes and washed and suspended in PBS. A minimum of 100,000 stained nucleated cells per tube were acquired using a flow cytometer (FACSCanto II; BD Biosciences, Sunnyvale, CA, USA) and analyzed using the BD FACS-Diva Software (BD Biosciences). PD-1 expression on CD4<sup>+</sup> and

CD8<sup>+</sup> T cells was measured as follows: lymphocytes were gated with bright CD45 expression and low side scatter (SSC) on CD45 vs. SSC cytogram, CD3<sup>+</sup> CD8<sup>+</sup> T cells were regated on a CD3 vs. CD8 cytogram, and positive PD-1 expression was measured on a CD8 vs. PD-1 cytogram. Positive PD-1 expression on CD3<sup>+</sup> CD4<sup>+</sup> T cells was measured following the same method, using CD4 instead of CD8. The cut-off values for positivity were as-

signed according to the isotypic controls, and expression results were presented as the proportion of positive cells (%).

### Statistical analysis

Statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY, USA) and Excel 2016 (Microsoft, Redmond, WA, USA). Data were analyzed for normal distribution using the Kolmogorov-Smirnov test and presented as median with range. Statistical comparisons were performed using the Kruskal-Wallis test and Mann-Whitney test. Spearman's rank correlation coefficient was used to evaluate the association between PD-1 expression percentages and the involved/uninvolved free light chain ratio and that between PD-1 expression percentages and the follow-up period after SCT.  $P < 0.05$  was considered statistically significant.

## RESULTS

### PD-1 expression on CD4<sup>+</sup> T cells

The median expression of PD-1 on CD4<sup>+</sup> T cells at PCM diagnosis did not significantly differ from that in the controls (24.6% vs. 23.2%). PD-1 expression on CD4<sup>+</sup> T cells in patients with residual disease (RD) after CTx was significantly higher than that in patients at diagnosis ( $P < 0.001$ ), patients in complete remission (CR) after CTx ( $P = 0.025$ ), and controls ( $P = 0.001$ ).

PD-1 expression on CD4<sup>+</sup> T cells in patients after SCT was

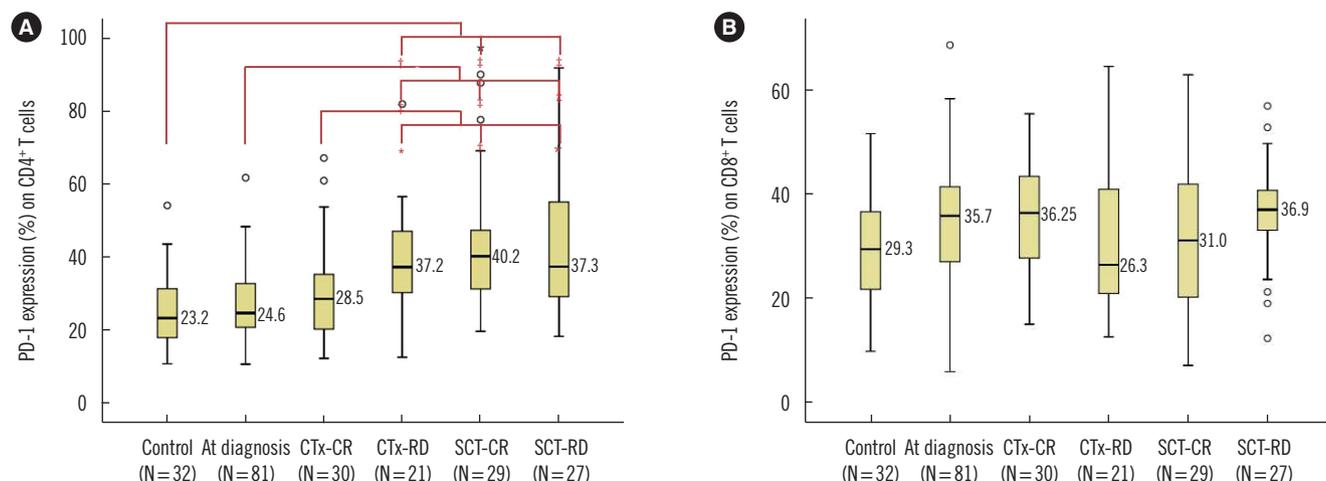
higher than that in patients at diagnosis ( $P < 0.001$ ), patients in CR after CTx (SCT-CR,  $P = 0.006$ ; SCT-RD,  $P = 0.012$ ), and controls ( $P < 0.001$ ) (Fig. 1A). Patients who had undergone SCT were divided into early (N=16) and late (N=40) phases based on a cut-off of three months after SCT; no difference in PD-1 expression was observed between the two groups (43.1% vs. 35.3%,  $P = 0.106$ ), and there was no significant correlation between the follow-up period after SCT and PD-1 expression.

No correlation was observed between PD-1 expression on CD4<sup>+</sup> T cells and the log value of involved/uninvolved free light chain ratio (Fig. 2). Moreover, there was no statistical difference in the PD-1 expression on CD4<sup>+</sup> T cells according to R-ISS stages (median values of stage II and III were 23.4% and 24.4%, respectively).

### PD-1 expression on CD8<sup>+</sup> T cells

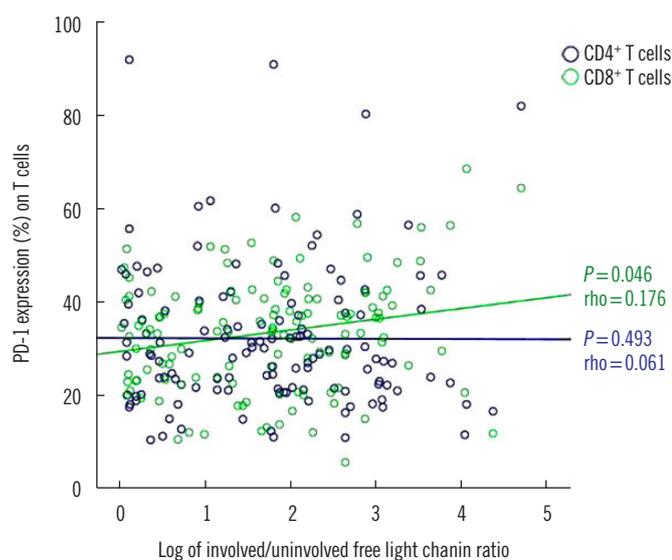
The median expression of PD-1 on CD8<sup>+</sup> T cells at PCM diagnosis did not differ from that in the controls (35.7% vs. 29.3%). No significant differences in PD-1 expression on CD8<sup>+</sup> T cells were observed between clinical states of PCM; however, the SCT-RD patients had a slightly higher median PD-1 expression than the controls (Fig. 1B). There was no difference in the PD-1 expression between the early (40.1%) and late (33.4%) phases after SCT and no correlation between the follow-up period after SCT and PD-1 expression.

The PD-1 expression on CD8<sup>+</sup> T cells showed weak positive



**Fig. 1.** Percentages of PD-1 expression on (A) CD4<sup>+</sup> T cells and (B) CD8<sup>+</sup> T cells in the controls and PCM patients with various disease states. In each box plot, the median value is reported beside the box. The upper end, lower end, and inner line of the boxes correspond to the 3rd quartile, 1st quartile, and median value, respectively. Error bars denote minimum and maximum values, and circles indicate outlier values. Statistically significant differences are marked with \*if  $P < 0.05$ , with †if  $P < 0.01$ , and with ‡if  $P < 0.001$ .

Abbreviations: CTx-CR, complete remission after chemotherapy; CTx-RD, residual disease after chemotherapy; PD-1, immune checkpoint programmed cell death protein-1; PCM, Plasma cell myeloma; SCT-CR, complete remission after stem cell transplantation; SCT-RD, residual disease after stem cell transplantation.



**Fig. 2.** Correlation between PD-1 expression on T cell subsets and involved/uninvolved free light chain ratio in PCM. The PD-1 expression on CD8<sup>+</sup> T cells was weakly correlated with involved/uninvolved free light chain ratio; however, the PD-1 expression on CD4<sup>+</sup> T cells was not correlated with involved/uninvolved free light chain ratio. Abbreviations: PCM, plasma cell myeloma; PD-1, immune checkpoint programmed cell death protein-1.

correlation with the log value of the involved/uninvolved free light chain ratio ( $P=0.046$ ,  $\rho=0.176$ ; Fig. 2). However, there was no significant difference in PD-1 expression on CD8<sup>+</sup> T cells according to R-ISS stages (median values of stage II and III, 36.8% and 32.5%, respectively).

#### PD-1 expression on T cell subsets according to cytogenetic abnormalities

PCM patients with monosomy 13 (37.7%), 1q gain (37.7%), complex karyotypes (37.5%), and hypodiploidy (39.6%) had higher PD-1 expression on CD8<sup>+</sup> T cells than did those with normal karyotypes (32.7%;  $P=0.020$ ,  $P=0.011$ ,  $P=0.041$ , and  $P=0.032$ , respectively). Patients with hyperdiploidy and those with t(11;14) showed no significant difference in the PD-1 expression on CD8<sup>+</sup> T cells. PD-1 expression on CD4<sup>+</sup> T cells did not differ according to cytogenetic abnormalities (Table 2). Few patients had cytogenetic indicators of high-risk myeloma, such as del(17p) (N=4), t(14;16) (N=0), and t(14;20) (N=0), and significant differences in PD-1 expression among these patients could not be evaluated.

#### Serial changes in PD-1 expression on T-cell subsets

Among the 23 patients with serial data, most showed disease status-dependent changes in PD-1 expression; the results for

**Table 2.** Differences in PD-1 expression between patients with specific cytogenetic abnormalities and those with normal karyotypes in non-CR groups (groups at time of diagnosis, after chemotherapy with RD, and after autologous SCT-RD)

Cytogenetic abnormalities	PD-1 expression	Difference in PD-1 expression
Monosomy 13 (N=40) vs. NL* (N=59)	CD4 <sup>+</sup> T cells	Not significant ( $P=0.884$ )
	CD8 <sup>+</sup> T cells	High ( $P=0.020$ )
1q gain (N=29) vs. NL (N=59)	CD4 <sup>+</sup> T cells	Not significant ( $P=0.214$ )
	CD8 <sup>+</sup> T cells	High ( $P=0.011$ )
Complex (N=59) vs. NL (N=59)	CD4 <sup>+</sup> T cells	Not significant ( $P=0.657$ )
	CD8 <sup>+</sup> T cells	High ( $P=0.041$ )
Hypodiploidy (N=20) vs. NL (N=59)	CD4 <sup>+</sup> T cells	Not significant ( $P=0.727$ )
	CD8 <sup>+</sup> T cells	High ( $P=0.032$ )
Hyperdiploidy (N=29) vs. NL (N=59)	CD4 <sup>+</sup> T cells	Not significant ( $P=0.149$ )
	CD8 <sup>+</sup> T cells	Not significant ( $P=0.162$ )
t(11;14) (N=17) vs. NL (N=59)	CD4 <sup>+</sup> T cells	Not significant ( $P=0.852$ )
	CD8 <sup>+</sup> T cells	Not significant ( $P=0.251$ )

\*NL is normal karyotype including loss of Y chromosome; loss of Y chromosome is considered to be related to aging.

Abbreviations: PD-1, immune checkpoint programmed cell death protein-1; CR, complete remission; RD, residual disease; SCT-RD, with residual disease after stem cell transplantation; t, translocation.

eight cases seemed discordant with the expected change in PD-1 expression. Three cases were classified as RD but had decreased PD-1 expression on CD4<sup>+</sup> T cells, and five were classified as CR but had increased PD-1 expression on CD4<sup>+</sup> T cells (Table 3). In all discordant RD cases, RD was determined based on a follow-up BM examination within five months of diagnosis. As patients usually require 2–3 cycles of treatment (six weeks per cycle) until CR, they can be considered as transitioning to CR. In four of the five CR cases with increased PD-1 expression on the CD4<sup>+</sup> T cells, the M protein reappeared (immunoglobulin isotype switches in two cases) on the protein electrophoresis at the next follow-up. In one case, the reason for the discordance between obtained and expected results could not be determined owing to a lack of follow-up.

## DISCUSSION

PCM cases in Korea has rapidly increased, from 5,566 in 2014 to 8,412 in 2019 [7]. Several therapies for PCM have emerged over the past two decades, first CTx, then autologous SCT, and, more recently, the use of proteasome inhibitors and immunomodulatory drugs; however, many patients still die from disease relapse [8,9].

A higher PD-1 expression on CD4<sup>+</sup> T cells was observed to be

**Table 3.** Changes in PD-1 expression on T-cells determined by flow cytometry with serial gating in 23 patients

Case	Gender	Age	Date of BM sampling	Disease status (Period after SCT, months)	PD-1 expression on CD4 <sup>+</sup> T cells (%)	PD-1 expression on CD8 <sup>+</sup> T cells (%)
1	F	52	2016-10-12	CTx-CR	25.4	40.6
	F	52	2016-11-07	CTx-CR	26.5	13.2
2	M	63	2016-05-04	SCT-RD (108)	35	34.2
	M	64	2016-08-27	reSCT-CR (3)	46.9	13
3	F	59	2016-10-07	SCT-CR (30)	44.3	8.2
	F	59	2017-01-11	reSCT-CR (1)	97.3	62.3
4	M	61	2016-07-29	SCT-RD (20)	33.3	33.4
	M	61	2017-01-13	SCT-RD (25)	31.4	36.9
5	F	62	2016-07-04	At diagnosis	21.2	33.7
	F	62	2016-11-16	CTx-CR	18	20.9
6	F	62	2017-01-06	SCT-CR (1)	41	41.8
	M	39	2016-06-03	SCT-CR (5)	35.4	41.9
7	M	39	2016-11-18	SCT-CR (10)	20.2	12.5
	M	43	2016-05-23	SCT-CR (6)	45.2	41.9
	M	43	2016-09-13	SCT-CR (10)	47.3	33.3
8	M	62	2016-11-04	CTx-CR	24.5	46.2
	M	62	2016-12-23	SCT-CR (1)	46.9	48.6
9	M	59	2016-10-20	At diagnosis	19.2	31.5
	M	60	2017-04-24	CTx-CR	16.1	43.3
10	F	64	2016-10-07	CTx-CR	20.2	23
	F	64	2016-11-18	SCT-CR (1)	31.4	20.1
11	F	72	2016-06-09	At diagnosis	23.6	41.2
	F	72	2017-04-17	CTx-CR	26.3	45.4
12	M	54	2016-10-07	SCT-RD (51)	58.8	56.8
	M	54	2016-11-23	SCT-RD (52)	29.8	29.3
13	M	55	2017-02-15	SCT-RD (55)	37.7	33.2
	F	59	2016-07-19	At diagnosis	21	29.1
14	F	59	2016-12-02	CTx-CR	12.2	36
	F	64	2016-05-30	CTx-CR	21.3	41.7
15	F	26	2017-02-08	SCT-CR (6)	26.5	28.3
	M	60	2016-09-10	At diagnosis	21.5	28.9
16	M	60	2017-01-25	CTx-CR	14	30.5
	F	49	2016-06-03	At diagnosis	18.2	68.5
17	F	49	2016-09-24	CTx-RD	15	17.9
	F	61	2016-11-30	At diagnosis	28.6	42.3
18	F	62	2017-04-26	CTx-RD	28.5	51.4
	F	61	2016-06-13	At diagnosis	18.3	38.9
	F	61	2016-10-21	CTx-RD	12.5	26.3
19	M	61	2016-10-26	At diagnosis	36.7	54.5
	M	61	2017-03-14	CTx-CR	45.1	55.3

*(Continued to the next page)*

Table 3. Continued

Case	Gender	Age	Date of BM sampling	Disease status (Period after SCT, months)	PD-1 expression on CD4 <sup>+</sup> T cells (%)	PD-1 expression on CD8 <sup>+</sup> T cells (%)
20	F	38	2016-05-09	At diagnosis	29.9	36.9
	F	39	2017-04-17	CTx-CR	49.7	51.2
	M	54	2016-06-01	SCT-CR (9)	31.4	37.6
21	M	55	2016-08-03	SCT-CR (11)	90	62.8
	M	47	2016-05-06	SCT-RD (9)	47.1	20.7
22	M	47	2016-09-13	SCT-RD (13)	23.8	36.8
	M	47	2016-11-02	SCT-CR (14)	77.6	47.9
23	F	73	2016-11-09	CTx-RD	34.8	17.9
	F	74	2017-02-15	CTx-CR	67.1	36.5

\*Classified as RD, but PD-1 expression on CD4<sup>+</sup> T cells was decreased; <sup>†</sup>Classified as CR, but PD-1 expression on CD4<sup>+</sup> T cells was increased; <sup>‡</sup>Reappearance of M protein in the next follow-up protein electrophoresis; <sup>§</sup>Immunoglobulin isotype switch; <sup>¶</sup>Follow-up loss.

Abbreviations: M, male; F, female; CTx, chemotherapy; CR, complete remission; RD, residual disease; SCT, stem cell transplantation; reSCT, second stem cell transplantation; BM, bone marrow; PD-1, immune checkpoint programmed cell death protein-1; CTx-CR, complete remission after chemotherapy; SCT-RD, residual disease after stem cell transplantation; SCT-CR, complete remission after stem cell transplantation; CTx-RD, residual disease after chemotherap

related to CTx refractoriness. However, there was no difference in PD-1 expression on T cells between patients at diagnosis and controls, indicating that PD-1 is involved in disease exacerbation or treatment refractoriness rather than disease onset. A previous study reported that PD-1 expression on T cells was increased in minimal RD (MRD)-positive patients compared with that in newly diagnosed or MRD-negative patients [10]; this increased PD-1 expression in RD patients is similar to the results of our study. However, our study differs from the previous one in that PD-1 expression was significantly increased only on CD4<sup>+</sup> T cells. PD-1 signaling in CD4<sup>+</sup> T cells enhances the induction of the T regulatory (Treg) cell phenotype from naive T cells and participates in Treg cell function [11-13]. Treg cells accumulate in the BM microenvironment in PCM, which is increased in relapsed patients. Moreover, increased Treg cells have been associated with vulnerability to disease progression [14]. Therefore, our study shows that increased Treg cells in refractory PCM patients is due to increased PD-1 expression on CD4<sup>+</sup> T cells. The increased number and activity of Treg cells due to increased PD-1 signaling in CD4<sup>+</sup> T cells suppress tumor-specific T cell function, resulting in CTx refractoriness.

Increased PD-1 expression after SCT, regardless of the SCT type, has been frequently reported [15-18]. In our study, PD-1 expression on CD4<sup>+</sup> T cells after SCT was higher than that at diagnosis, as well as that in patients with CR after CTx (CTx-CR) or controls, regardless of RD status. However, there was no significant difference between PD-1 expression on CD8<sup>+</sup> T cells before and after SCT. Simonetta, *et al.* [17] found that the PD-

1-positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells proportion increased significantly during the early phase (three months) after allogeneic SCT, followed by a progressive normalization of PD-1 expression on CD8<sup>+</sup> but not on CD4<sup>+</sup> T cells. We analyzed PD-1 expression in early and late phases after SCT based on a cut-off point of three months after SCT; however, no statistically significant difference in PD-1 expression was observed between the two phases. This could be due to the insufficient number of samples for the early phase after SCT. As the patients with follow-up after SCT included in this study were mostly in the late phase after SCT, PD-1 expression on CD8<sup>+</sup> T cells before and after SCT may appear not to differ, unlike that on CD4<sup>+</sup> T cells.

In a study by Merindol, *et al.* [18], the PD-1 expression on CD4<sup>+</sup> and CD8<sup>+</sup> T cells in patients who had undergone umbilical cord blood transplantation (UCBT) for hematologic disease treatment was higher than that in healthy controls. Although the PD-1<sup>+</sup> CD8<sup>+</sup> T cell proportion in the first three months after UCBT was high in all patients, it was significantly higher in relapsed patients than in non-relapsed patients at two and six months. Our study also showed a slight, insignificant increase in PD-1 expression on CD8<sup>+</sup> T cells in SCT-RD patients. Therefore, patient prognosis after SCT could be more related to PD-1 expression on CD8<sup>+</sup> T cells than to that on CD4<sup>+</sup> T cells.

One of the hallmarks of high-risk disease is the interaction between myeloma cells and the BM microenvironment to generate a high-risk ecosystem that facilitates both cancer cell survival and immune response failure [19]. The genetic abnormalities of malignant plasma cell clones can influence the BM micro-

environment through various mechanisms involving soluble factors, exosomes containing microRNAs, and cell-cell contact [20–23]. For example, the musculoaponeurotic fibrosarcoma (MAF) protein in t(14;16) plasma cells upregulates integrin  $\beta 7$ , thereby altering the adhesion of plasma cells to stromal cells and facilitating their growth and survival [24]. We found that PD-1 expression on CD8<sup>+</sup> T cells was higher in patients with cytogenetic abnormalities categorized as high or intermediate risk (monosomy 13, 1q gain, hypodiploidy, and complex karyotype). This indicates that high- or intermediate-risk cytogenetic abnormalities affect the T cell expression of immune checkpoints. Additionally, the genetic variation found in high-risk PCM may affect not only the plasma cells themselves but also the BM microenvironment, ultimately leading to poor prognosis or refractoriness to treatment through interactions between stromal cells and plasma cell clones.

Our study had some limitations. This was not a longitudinal study; long-term serial data are needed to determine how PD-1 expression changes according to the disease course in the same patient. Another limitation is that the control group in this study did not include healthy individuals.

In conclusion, high PD-1 expression on CD4<sup>+</sup> T cells was associated with CTx refractoriness and that on CD8<sup>+</sup> T cells was associated with high- or intermediate-risk cytogenetic abnormalities. We hypothesize that PD-1 blockade might have therapeutic potential in refractory PCM patients after CTx, especially for those with high- or intermediate-risk cytogenetic abnormalities.

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## AUTHOR CONTRIBUTIONS

Park CJ and Cho YU designed the study and Lee MY wrote the paper; Lee MY and You EK carried out data management and analysis; and Jang S, Seo EJ, Lee JH, Yoon DH, and Suh C provided study materials or patients. All authors have agreed with the final version of the paper.

## CONFLICTS OF INTEREST

None declared.

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