Myeloid-derived suppressor cell accumulation induces Treg expansion and modulates lung malignancy progression

YINGHUA WAN*, XIANGDONG MU*, JINGQUAN ZHAO, LI LI, WENSHUAI XU and MINGQIANG ZHANG

Department of Respiratory and Critical Care Medicine, Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing 102218, P.R. China

Received June 20, 2023; Accepted September 1, 2023

DOI: 10.3892/br.2024.1754

Abstract. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous family of myeloid cells that suppress T cell immunity in tumor-bearing hosts. The present study aimed to examine roles of T and MDSC subsets in lung malignancy. The study analyzed 102 cases with lung malignancy and 34 healthy individuals. Flow cytometry was performed for identification of T cell and MDSC subsets and their phenotypic characteristics in peripheral blood. The lung malignancy cases exhibited lower frequencies of granulocyte-like MDSCs (G-MDSCs) expressing PD-L2 and PD-L1 than healthy controls (P=0.013 and P<0.001, respectively). Additionally, there was a higher frequency of monocyte-like MDSCs (M-MDSCs) expressing PD-L1 in the peripheral blood of patients with lung malignancy than healthy controls (P<0.001). The frequencies of G-MDSCs and M-MDSCs were positively correlated with proportions of PD-1$^+$ and CTLA-4$^+$ regulatory T cells (Tregs). *In vitro* co-culture assay demonstrated M-MDSCs of lung malignancy enhanced naive T cell apoptosis and promoted Treg subset differentiation compared with M-MDSCs of healthy controls. The findings suggested accumulation of MDSC subsets in lung malignancy and MDSCs expressing PD-L2 and PD-L1 induced Treg expansion by binding to PD-1 on the surface of Tregs.

Introduction

Lung malignancy is a common malignant tumor globally and its morbidity and mortality increase yearly due to improvements in early detection (1). In 2020, 2.2 million new cases and 1.8 million deaths of lung malignancy were estimated globally (2). Lung malignancy is a complex disease that has diverse histological and molecular types, and 85% of lung malignancy cases are non-small cell lung cancer (NSCLC) and 15% are SCLC (3,4). As tumor-induced immunosuppression is often identified in patients with cancer and contributes to cancer progression, immune checkpoint immunotherapy has attracted attention (5,6).

Myeloid cells are the most abundant nucleated hematopoietic cells in the human body and are distinct cell populations with multiple functions (7). However, during pathological processes including cancer, chronic inflammation and trauma, myeloid cells are largely converted to myeloid-derived suppressor cells (MDSCs) (8). MDSCs in the mouse represent activated Gr-1(+) CD11b(+) myeloid precursor cells (9) and then further classified into two subtypes: Monocyte like-MDSCs (M-MDSCs; CD11b$^+$Ly6C high Ly6G$^-$) and granulocyte like-MDSCs (G-MDSCs; CD11b$^+$Ly6C low Ly6G$^-$). MDSCs are usually identified based on CD11b and CD33 in humans, with low levels of major histocompatibility complex class II molecule human leukocyte antigen-D-related (HLA-DR) (10). High levels of arginase 1 are often expressed by MDSCs and reactive oxygen species and nitric oxide are produced, thereby leading to T cell depletion (11,12) and T regulatory cell (Treg) expansion (13).

Tregs are a subpopulation of T cells and can suppress the immune responses of cells, including T and B and natural killer cells, monocyte and dendritic cells, to maintain immune homeostasis and tolerance (14). Tregs in human can be sorted by CD4$^+$CD25$^+$CD127 (IL-7R)$^-$/low (15). Tregs secrete suppressive cytokines such as IL-10, TGF-B, and IL-35 to suppress T cells, can inhibit induction of IL-2 mRNA in target T cells, and interact with CTLA-4 and CD80/CD86 on antigen presenting cells (APCs) to prevent their binding to CD28 present on T cells (16). Chronic infection results in large accumulation of Tregs and expression of programmed death-1 (PD-1) (17). Immunosuppressive effects of MDSCs are achieved by expressing large amounts of immunosuppressive mediators, especially PD-ligand (L)1 (18). PD-1 binds to its ligand PD-L1 to inhibit the proliferation of T cells and promote Treg differentiation and function (19). The absence of consensus regarding involvements of T cell and MDSC subsets may limit understanding of cancer autoimmunity in lung malignancy.

Correspondence to: Professor Mingqiang Zhang, Department of Respiratory and Critical Care Medicine, Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, 168 Litang Street, Beijing 102218, P.R. China
E-mail: 200510020227tom@163.com

*Contributed equally

Key words: lung cancer, myeloid-derived suppressor cell, T regulatory cell, PD-1, PD-L1, PD-L2

The present study recruited lung malignancy cases and healthy individuals and performed a direct comparative analysis of MDSC, G-MDSCs and M-MDSCs, and T cell subsets with regard to their frequency, as well as their peripheral immunosuppressive mediators in a bid to investigate tumor-specific immune responses in lung malignancy.

Materials and methods

Study population. The present study was a case-control study involving patients with lung malignancy and healthy controls. Inclusion criteria for cases were as follows: i) Lung malignancy diagnosed according to World Health Organization classification (20); ii) TNM staging established based on the 7th edition of the TNM Classification of Lung Cancer (21) and iii) an age of 18 years or above. Exclusion criteria were as follows: i) Previous history of infectious diseases, atopy, allergic rhinitis, asthma, autoimmune disease, liver and kidney dysfunction or other malignancy and ii) use of immunomodulatory drugs. Ethics Committee of Beijing Tsinghua Changgung Hospital (Beijing, China) approved the protocol (approval no. 18190-0-01). All cases and healthy controls were informed of study background and objective and signed informed consent. A total of 102 patients with lung malignancy, consisting of 71 males and 31 females, who were admitted to Tsinghua Changgung Hospital between February 2019 and January 2020 were analyzed. These patients' age ranged from 33 to 92 years. Additionally, 34 healthy individuals (20 males and 14 females) undergoing physical examination between November 2019 and January 2020, without autoimmune diseases and/or active infection and administration of immunomodulatory or corticosteroid drugs, were included as controls. The age of healthy individuals ranged from 34 to 78 years.

Peripheral blood sampling and processing. Peripheral blood samples (5 ml) were collected and placed into heparinized tubes within 24 h of admission to the Tsinghua Changgung Hospital (Beijing, China) (for cases) or physical examination (for controls). Heparinized blood was immediately iced and centrifuged at 400 x g (30 min, 4˚C) and density-gradient centrifugation was performed to separate peripheral blood mononuclear cells (PBMCs) via Ficoll as previously described (22).

Circulating T cell subpopulation detection. Flow cytometry was performed to detect phenotypes of T cells. The functional compartments of $CD4^+$ and $CD8^+$T cells were phenotypically characterized as $CD3^+CD4^+$ and $CD3^+CD4^-$, respectively. The phenotypic characteristic of Tregs were determined according to their differential expression of CD25 and CD127 ($CD4^+CD25^+$brightCD127$^{-/+}$low expression). The subpopulations expressing PD-1 and CTLA4 were determined in each of the T cell subsets as follows: $CTLA4^+CD4^-$, $PD\text{-}1^+CD8^-$, $CTLA4^+CD8^-$, $PD\text{-}1^+$Treg, $PD\text{-}1^+CD4^-$, $CTLA4^+$Treg. Phenotypical analyses of T cells were characterized by fluorescence-activated cell sorting (FACS) using a CytoFLEX (Beckman Coulter, Inc.) with anti-human-specific antibodies against surface CD markers (all eBioscience; Thermo Fisher Scientific, Inc.) as follows: CD3 (cat. no. MHCD0327; APC-Alexa Fluor™ 750), CD4 (cat. no. 25-0049-42; PE-Cyanine), CD25 (cat. no. 12-0257-42; PE), CD127 (cat. no. 17-1278-42; APC), CTLA-4 (cat. no. 85-46-1529-42; PerCP-eFluor® 710) and PD-1 (CD279; cat. no. 61-2799-42; PE-eFluor™ 610). A total of 5 μl antibodies was added according to the manufacturer's instructions and incubated at 4˚C for 30 min. CytExpert for DxFLEX version 1.0 (Beckman Coulter, Inc.) was used for flow cytometry analysis.

Immunophenotyping of MDSC subsets by flow cytometry. MDSC subsets, M-MDSCs ($CD14^+CD15^-CD11b^+CD33^+HLA\text{-}DR^{-/low}$) and G-MDSCs ($CD15^+CD33^+CD11b^+CD14^-HLA\text{-}DR^{-/low}$) were identified by FACS using a CytoFLEX (Beckman Coulter, Inc.) with a panel of fluorescent-labeled monoclonal antibodies (all eBioscience; Thermo Fisher Scientific, Inc.) as follows: Anti-CD15 (cat. no. 11-0159-42), anti-CD33 (cat. no. 56-0338-42), anti-CD14 (cat. no. 61-0149-42), anti-CD45 (cat. no. 47-0459-42), anti-CD11b (cat. no. 46-0118-42) and anti-HLA-DR (cat. no. 25-9952-42). The frequency of cells expressing PD-2 and PD-1 was determined in MDSC subsets by flow cytometry using fluorescent-labeled monoclonal antibodies CD274-APC (cat. no. 17-5983-42, eBioscience, Thermo Fisher Scientific, Inc.) and CD273-PE (12-5888-42, eBioscience, Thermo Fisher Scientific, Inc.), allowing the identification of $PD\text{-}L1^+$G-MDSCs, $PD\text{-}L1^+$M-MDSCs, $PD\text{-}L2^+$G-MDSCs and $PD\text{-}L2^+$M-MDSCs. Samples were added to 100 μl PBS, and the cell precipitates were blown and mixed well. A total of 5 μl antibodies was added according to the manufacturer's instructions, blown and mixed well. They were incubated at 4˚C for 30min. CytExpert for DxFLEX version 1.0 (Beckman Coulter, Inc.) was used for flow cytometry analysis.

In vitro co-culture. PBMCs were isolated from leukocytes of patients with lung malignancy and healthy controls. M-MDSCs ($CD14^+CD15^-CD11b^+CD33^+HLA\text{-}DR^{-/low}$) and G-MDSCs ($CD15^+CD33^+CD11b^+CD14^-HLA\text{-}DR^{-/low}$) were independently isolated and purified (>90%) by flow cytometry as aforementioned. Naive $CD4^+$T cells were isolated from peripheral blood using a commercial kit (cat. no. 130-094-131; Miltenyi Biotec GmbH) according to the manufacturer's instructions. The purity of naive T cells ($CD45RA^+CD4^+CCR7^+$) was verified by flow cytometry as aforementioned. M-MDSCs and G-MDSCs, sourced from leukocytes of lung malignancy and healthy control were co-cultured with naive $CD4^+$T cells in 96-well plate, $2.5x10^5$ cells/well, in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) in a 5% CO_2 humid atmosphere at 37˚C. Soluble anti-CD3 (1 mg/ml; cat. no. 16-0037-81; eBioscience; Thermo Fisher Scientific, Ind.) and anti-CD28 (1 mg/ml; cat. no. 16-0289-81; eBioscience; Thermo Fisher Scientific, Inc.) in a CO_2 incubator at 37˚C for 4 h were added to achieve T cell receptor (TCR) stimulation via the TCR/CD3 complex. The cells were cultured in RPMI-1640 supplemented with 10% FBS in a 5% CO_2 humid atmosphere at 37˚C for 5 days. The proliferation and apoptosis of T cells, as well as differentiation of Tregs, were examined by using flow cytometry as aforementioned.

Statistical analysis. GraphPad Prism 8.0 software (GraphPad Software, Inc.; Dotmatics) was used to analyze data. Experiments were performed with three replicates in at least

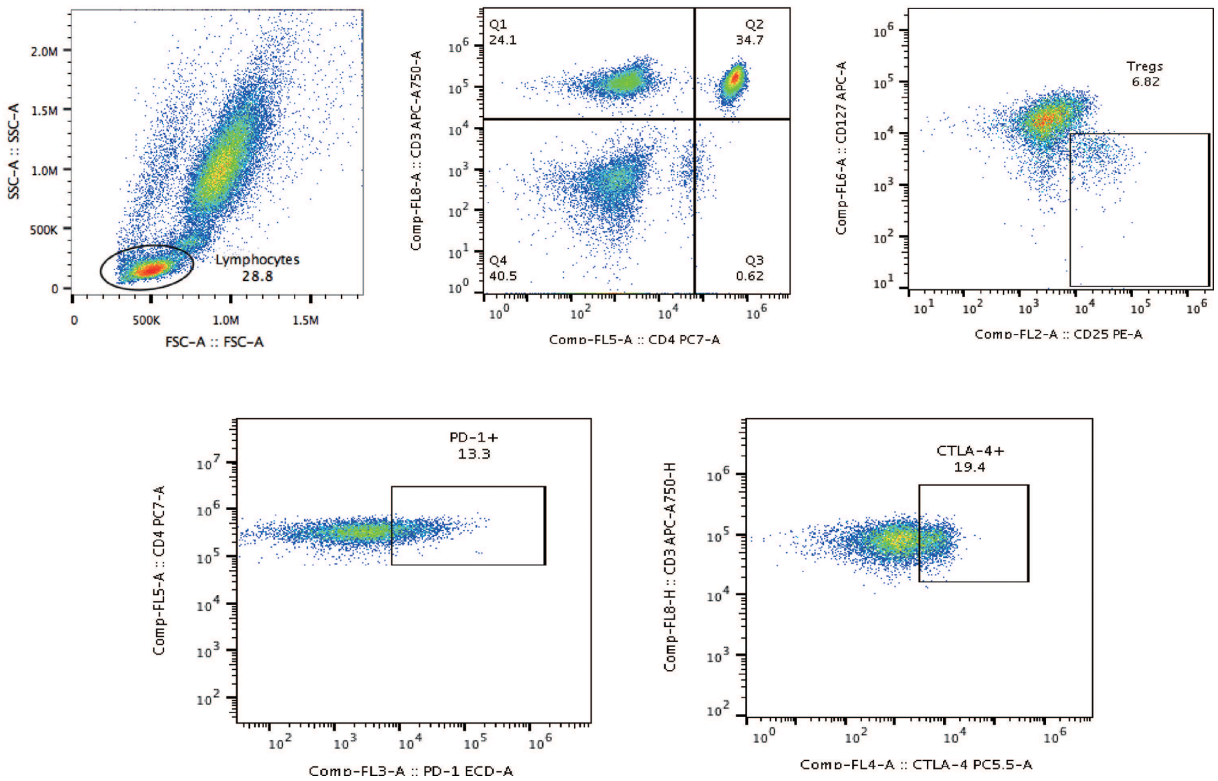

Figure 1. Flow cytometric detection to sort circulating T cell subsets. The phenotypes of T cells were determined using flow cytometry based on CD3 expression. $CD3^+CD4^+CD25^{high}CD127^{low}$ were Tregs. Tregs expressing PD-1 and CTLA4 were determined. Treg, regulatory T cell.

three independent experiments; data are presented as the mean ± standard deviation. For the data with normal distribution, one-way analysis of variance followed by Tukey's post hoc analysis or unpaired t test were performed for multiple and two group comparisons, respectively. Pearson's correlation analysis was used to evaluate the relationship of T cell and MDSC subsets. Categorical variables are expressed as proportions and analyzed using χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Demographic and clinical characteristics of the subjects. The 102 lung malignancy cases were classified as follows: Stage I (n=13), II (n=16), III (n=18) and IV (n=55); squamous cell carcinoma (n=35), adenocarcinoma (n=50), large cell carcinoma (n=7) and SCLC (n=10; data not shown). A total of 49 cases exhibited distant metastasis and 41 were non-smokers. Among these healthy individuals, there were 12 non-smokers and 22 smokers. Lung malignancy cases and healthy individuals were age-, sex- and smoking-matched.

Frequency of peripheral blood T cell subsets in lung cancer. The strategy for flow cytometric analysis to identify T cell subpopulations in the circulation is presented in Fig. 1. Flow cytometric analyses of $PD\text{-}1^+CD8^+$ T cells, Tregs, $PD\text{-}1^+$Tregs, $CTLA\text{-}4^+CD4^+$ T cells, $CTLA\text{-}4^+$Tregs, and $PD\text{-}1^+CD4^+$ and $CTLA\text{-}4^+CD8^+$ T cells are shown in Figs. 2 and 3. There were no notable differences concerning the frequencies of Tregs, $PD\text{-}1^+$Tregs and $PD\text{-}1^+CD4^+$, $CTLA\text{-}4^+CD4^+$ and $PD\text{-}1^+CD8^+$ T cells in the peripheral blood of patients with lung cancer and healthy controls. However, there were higher frequencies of peripheral $CTLA\text{-}4^+$Tregs and $CTLA\text{-}4^+CD8^+$ T cells in lung malignancy cases compared with healthy individuals ($P<0.05$; Fig. 4; Table I).

Association between clinical variables and the frequencies of $CTLA\text{-}4^+$Tregs and $CTLA\text{-}4^+CD8^+$ T cells in lung cancer. The present study investigated whether $CTLA\text{-}4^+$Tregs and $CTLA\text{-}4^+CD8^+$ T cells were associated with clinical variables of cases with lung malignancy. A lower frequency of $CTLA\text{-}4^+CD8^+$ T cells was found in cases at stage III + IV compared with those at stage I + II ($P=0.009$; Table II), indicating the proportion of $CTLA\text{-}4^+CD8^+$ T cells was associated with TNM stage. However, frequencies of $CTLA\text{-}4^+$Tregs and $CTLA\text{-}4^+CD8^+$ T cells were not significantly associated with age, sex, smoking status, histological type and distant metastasis; frequency of $CTLA\text{-}4^+$Tregs was not associated with TNM stage ($P>0.05$).

Frequencies of peripheral blood MDSC subsets in lung cancer. The strategies of flow cytometric analyses to identify G-MDSCs and M-MDSCs in the circulation are shown in Figs. 5 and 6. A greater diversity of G-MDSC and M-MDSC subpopulations was noted in lung malignancy cases compared with healthy individuals ($P<0.001$; Table III). To study interaction between MDSC subsets, PD-L1 and PD-L2 in lung malignancy, flow cytometry was used to sort G-MDSCs and M-MDSCs expressing either PD-L1 or PD-L2 in lung malignancy cases and healthy

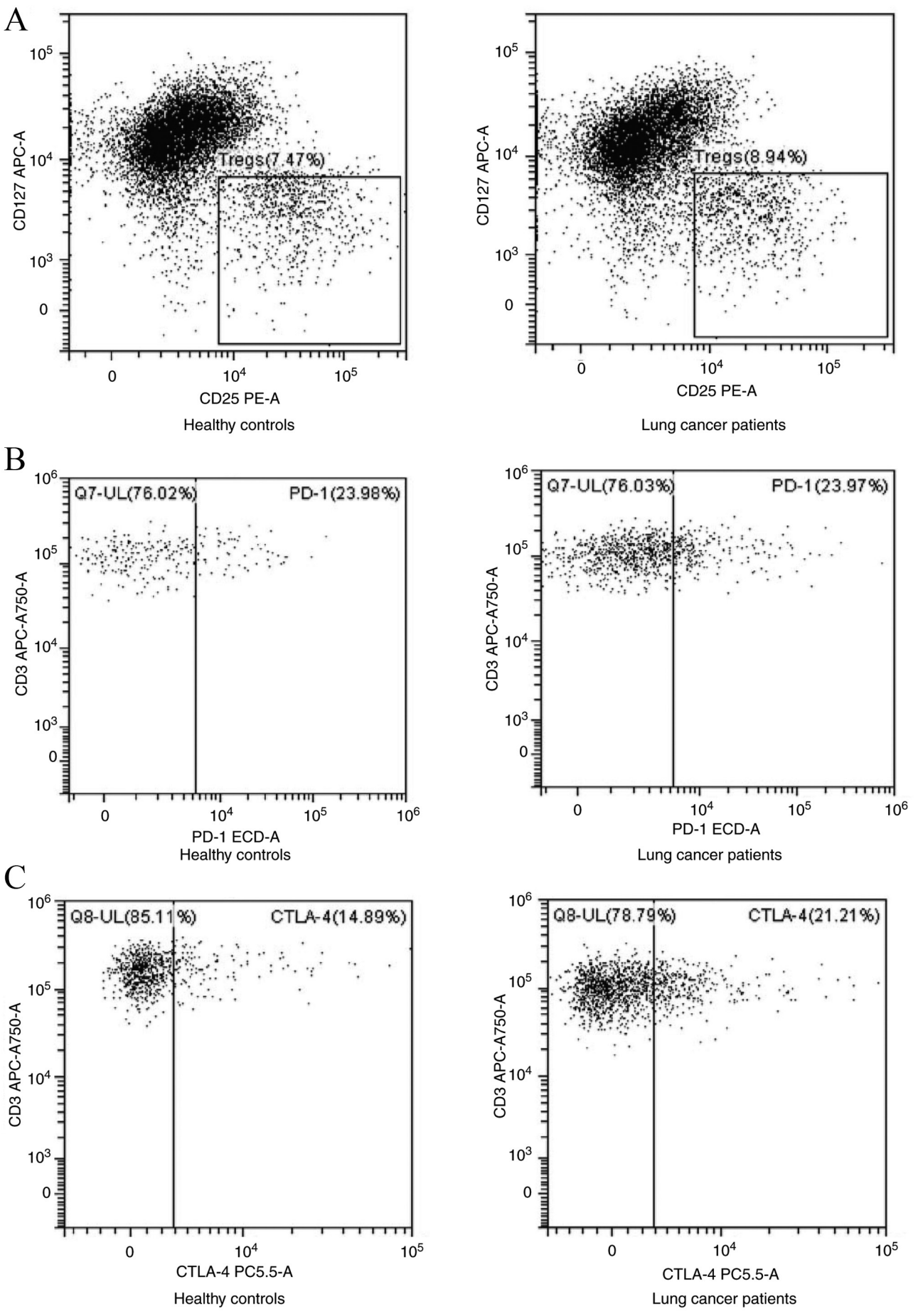

Figure 2. Flow cytometric analyses of peripheral Tregs expressing PD-1$^+$ and CTLA-4$^+$ in lung malignancy cases and healthy controls. Peripheral (A) Tregs expressing (B) PD-1$^+$ and (C) CTLA-4$^+$ in lung malignancy cases and healthy controls. Treg, regulatory T cell.

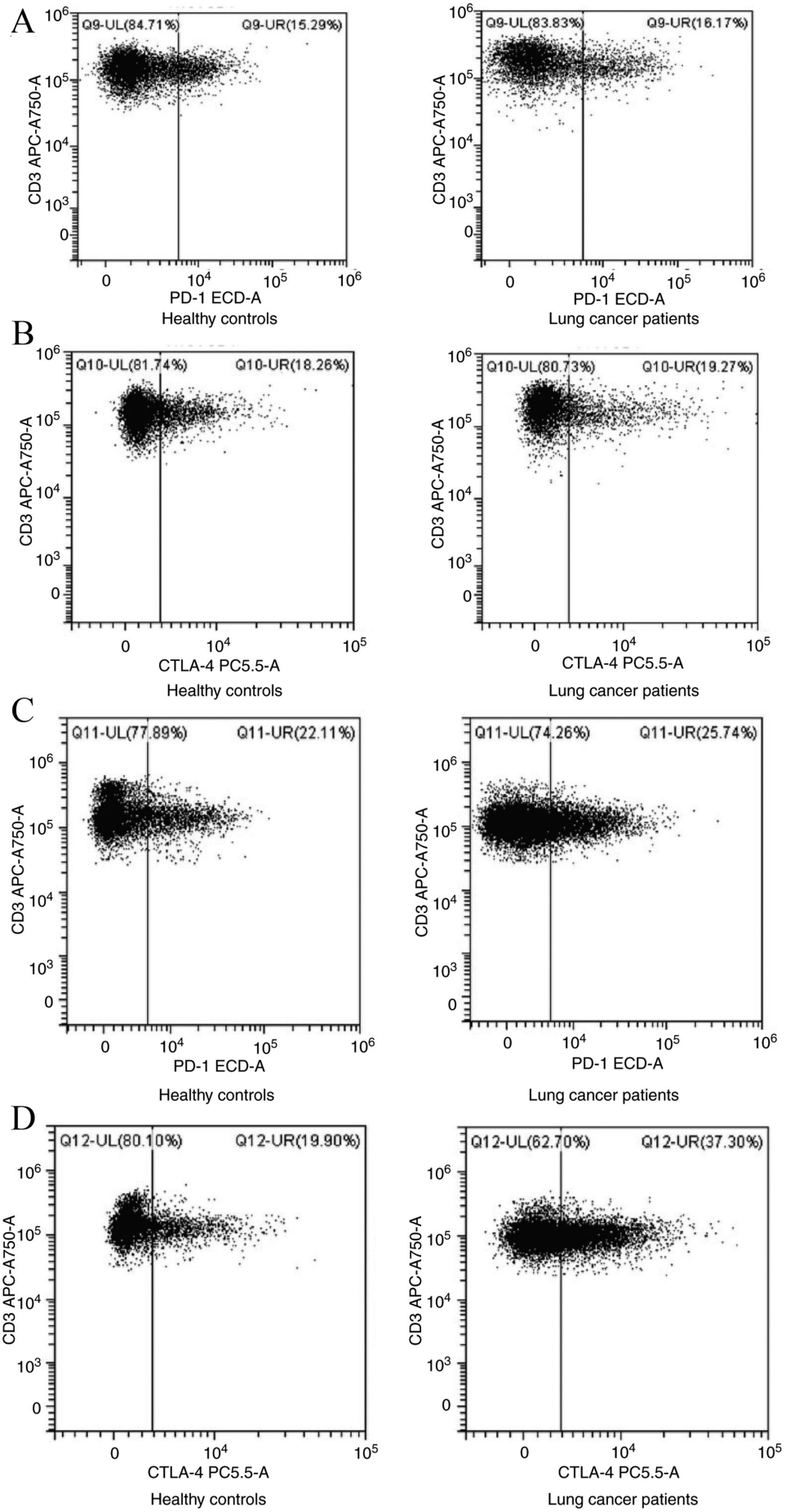

Figure 3. Flow cytometric analyses of peripheral PD-1$^+$CD4$^+$, CTLA-4$^+$CD4$^+$, PD-1$^+$CD8$^+$ and CTLA-4$^+$CD8$^+$ T cells. Peripheral (A) PD-1$^+$CD4$^+$, (B) CTLA-4$^+$CD4$^+$, (C) PD-1$^+$CD8$^+$ and (D) CTLA-4$^+$CD8$^+$ T cells between lung cancer cases and healthy controls.

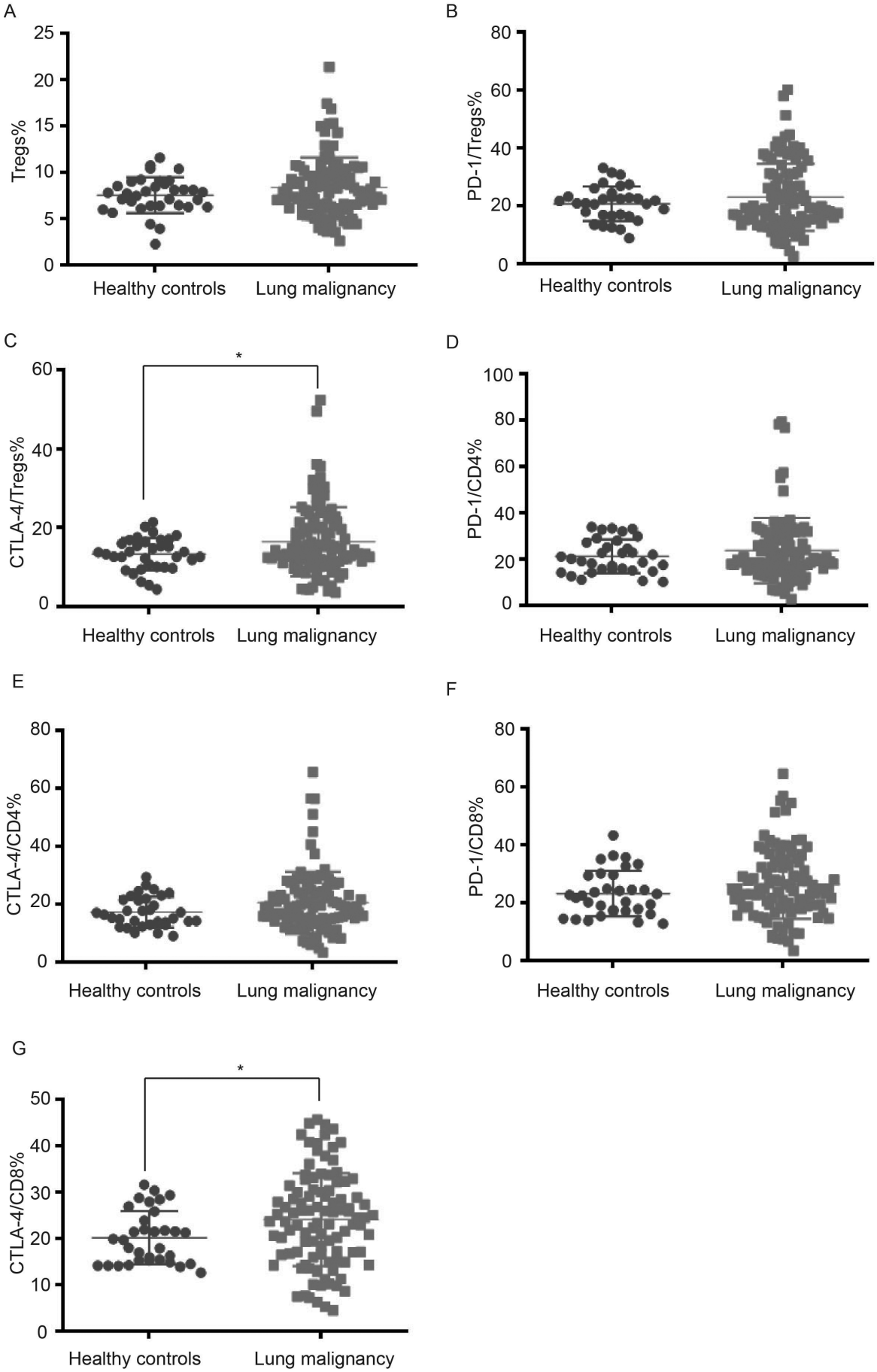

Figure 4. Flow cytometric detection of peripheral Tregs expressing PD-1$^+$ and CTLA-4$^+$ and PD-1$^+$CD4$^+$, CTLA-4$^+$CD4$^+$, PD-1$^+$CD8$^+$ and CTLA-4$^+$CD8$^+$ T cells. Frequencies of peripheral (A) Tregs expressing (B) PD-1$^+$ and (C) CTLA-4$^+$ and (D) PD-1$^+$CD4$^+$, (E) CTLA-4$^+$CD4$^+$, (F) PD-1$^+$CD8$^+$ and (G) CTLA-4$^+$CD8$^+$ T cells between lung malignancy cases and healthy controls. *P<0.05. Treg, regulatory T cell.

Table I. Frequency (%) of peripheral blood T cell subsets in the peripheral blood between patients with lung cancer and healthy controls.

T cell subset	Healthy controls (n=34)	Lung cancer (n=102)	P-value
Tregs	7.55±1.94	8.38±3.24	0.161
PD-1$^+$Tregs	20.38±6.09	22.97±11.62	0.216
CTLA-4$^+$Tregs	13.31±4.08	16.43±8.77	0.047[a]
PD-1$^+$CD4	21.02±7.21	23.68±14.10	0.295
CTLA-4$^+$CD4	17.25±5.16	20.47±10.67	0.093
PD-1$^+$CD8	23.12±7.71	26.27±11.84	0.149
CTLA-4$^+$CD8	19.98±5.74	24.06±10.05	0.026[a]

[a]P<0.05. Treg, regulatory T cell.

Table II. Association between clinical variables of patients with lung cancer and the frequencies of CTLA-4$^+$Tregs and CTLA-4$^+$CD8$^+$ T cells in the peripheral blood.

		CTLA4/Tregs		CTLA4/CD8	
Clinical variable	n	Frequency, %	P-value	Frequency, %	P-value
Age, years			0.766		0.430
≤65	45	16.11±8.37		24.95±11.08	
>65	57	16.68±9.13		23.36±9.19	
Sex			0.354		0.508
Male	71	15.90±8.28		23.62±9.46	
Female	31	17.66±9.84		25.06±11.39	
Smoking status			0.893		0.807
Smoker	61	16.53±8.45		23.86±10.12	
Non-smoker	41	16.29±9.33		24.36±10.05	
TNM stage			0.628		0.009[a]
I + II	29	15.76±8.67		28.14±7.13	
III + IV	73	16.70±8.85		22.44±10.60	
Histological type			0.992		0.756
Squamous cell carcinoma	35	16.22±7.80		24.22±10.07	
Adenocarcinoma	50	16.53±9.80		23.60±10.49	
Large cell carcinoma	7	16.99±7.47		24.79±11.81	
Small cell lung cancer	10	17.08±7.93		27.41±7.87	
Distant metastasis			0.279		0.482
Yes	49	17.41±9.32		23.33±10.45	
No	53	15.52±8.21		24.74±9.71	

[a]P<0.05. Treg, regulatory T cell.

controls (Figs. 7 and 8). The cases with lung malignancy exhibited lower frequencies of PD-L1$^+$G-MDSCs (P=0.013) and PD-L2$^+$G-MDSCs (P<0.001) in the peripheral blood than the healthy controls. Additionally, there was a higher frequency of PD-L1$^+$M-MDSCs in peripheral blood of lung malignancy cases than healthy controls (P<0.001). However, significant differences were not found in PD-L2$^+$M-MDSCs between lung malignancy cases and healthy controls (P=0.406).

Association between clinical variables and frequency of peripheral blood MDSC subsets in lung malignancy. The present study investigated whether peripheral blood MDSC subsets were associated with clinical variables of patients with lung cancer. Patients with distant metastasis exhibited higher frequencies of G-MDSCs and M-MDSCs in peripheral blood when compared with those without distant metastasis (P<0.001; Table IV), suggesting the frequency of peripheral G-MDSCs and M-MDSCs was associated with the occurrence

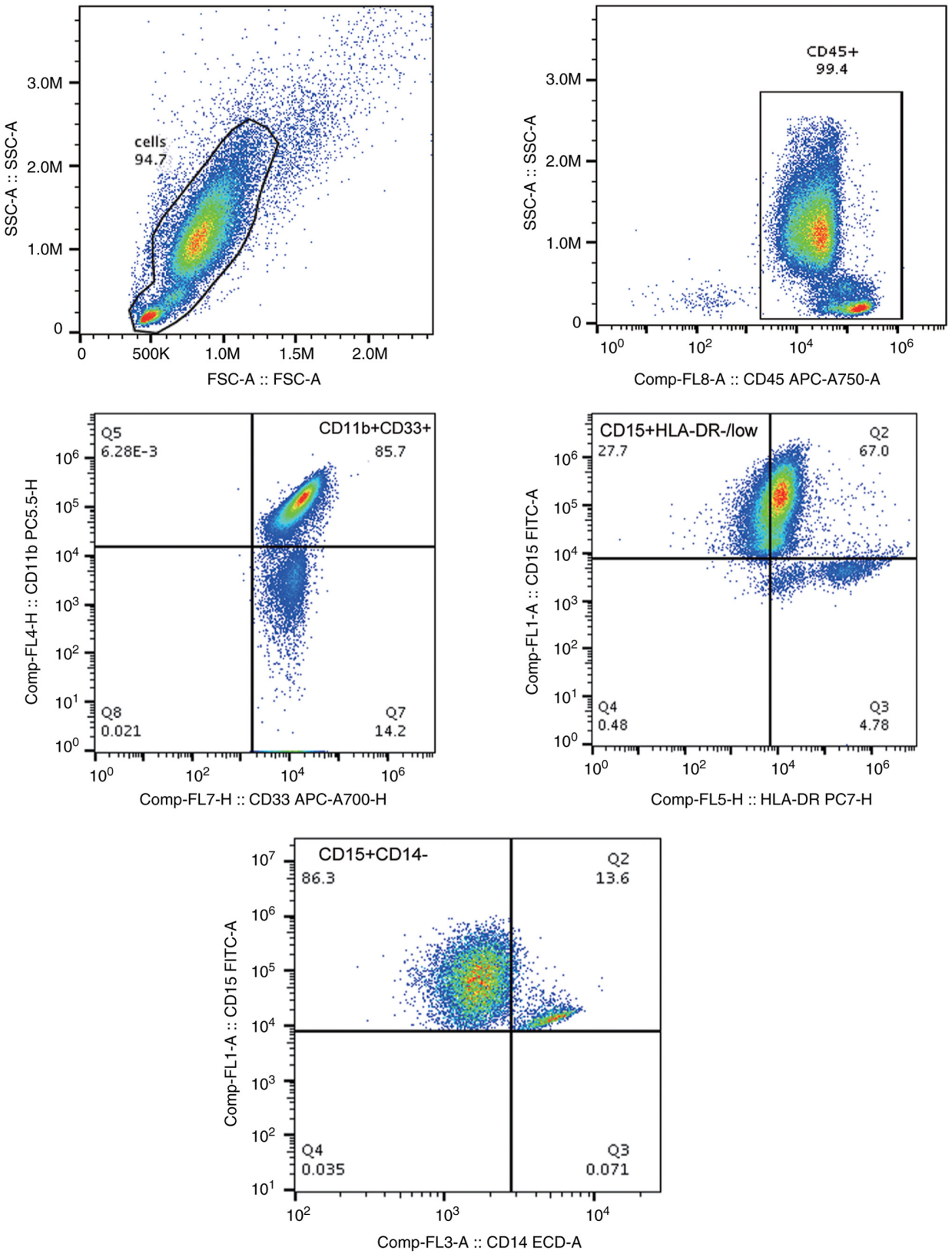

Figure 5. Flow cytometric detection to sort circulating G-MDSCs. MDSCs were determined using flow cytometry based on CD45 expression; $CD11b^{+}CD33^{+}$ cells and then CD15+HLA-DR-/low cells were gated. $CD15^{+}CD14^{-}$ cells were analyzed and sorted as G-MDSCs ($CD15^{+}CD33^{+}CD11b^{+}$CD14-HLA-DR-/low). G-MDSC, granulocyte-like myeloid-derived suppressor cell.

of distant metastasis in lung malignancy. Additionally, there was a lower frequency of peripheral $PD\text{-}L1^{+}$M-MDSCs in cases with distant metastasis when compared with those without (P=0.020; Table V), suggesting the frequency of $PD\text{-}L1^{+}$M-MDSCs in peripheral blood was associated with the occurrence of distant metastasis in lung cancer.

Table III. Frequencies (%) of peripheral blood MDSC subsets in the peripheral blood between patients with lung cancer and healthy controls.

MDSC subset	Healthy controls (n=34)	Lung cancer (n=102)	P-value
G-MDSCs	10.71±5.60	18.65±10.52	<0.001[a]
PD-L1$^+$G-MDSCs	71.18±24.30	60.49±20.30	0.013[a]
PD-L2$^+$G-MDSCs	50.84±20.08	35.03±15.77	<0.001[a]
M-MDSCs	8.91±2.96	16.68±9.63	<0.001[a]
PD-L1$^+$M-MDSCs	36.21±27.42	62.75±26.48	<0.001[a]
PD-L2$^+$M-MDSCs	48.35±32.14	53.62±31.85	0.406

[a]P<0.05. M-MDSC, monocyte-like myeloid-derived suppressor cell.

Table IV. Association between clinical variables of patients with lung cancer and the frequencies of peripheral blood MDSC subsets.

		G-MDSC		M-MDSC	
Clinical variable	n	Frequency, %	P-value	Frequency, %	P-value
Age, years			0.201		0.211
≤65	45	17.26±9.52		15.22±8.40	
>65	57	19.86±10.79		17.62±10.39	
Sex			0.879		0.878
Male	71	18.80±10.67		16.64±9.90	
Female	31	18.46±9.46		16.32±8.98	
Smoking status			0.261		0.857
Smoker	61	19.63±10.94		16.40±9.71	
Non-smoker	41	17.29±9.11		16.75±9.49	
TNM stage			0.009[a]		0.104
I + II	29	14.52±7.14		14.10±8.31	
III + IV	73	20.37±10.88		17.52±9.93	
Histological type			0.187		0.447
Squamous cell carcinoma	35	20.13±10.76		18.28±11.18	
Adenocarcinoma	50	16.91±8.57		15.27±8.43	
Large cell carcinoma	7	23.94±15.21		14.99±6.66	
Small cell lung cancer	10	16.24±8.32		18.82±12.04	
Distant metastasis			<0.001[a]		<0.001[a]
Yes	49	22.29±10.69		20.18±10.55	
No	53	15.24±8.62		13.04±7.03	

[a]P<0.05. M-, monocyte-like; G-, granulocyte-like; MDSC, myeloid-derived suppressor cell.

Correlation between frequencies of peripheral blood MDSC and T cell subsets in lung malignancy. Next, the present study investigated correlation between frequency of peripheral blood MDSC and T cell subsets and in lung cancer. First, a positive correlation was noted between frequencies of G-MDSCs and M-MDSCs (r=0.325, P=0.001; Fig. 9). In addition, G-MDSC populations shared a positive correlation with the proportion of Tregs expressing PD-1 (r=0.385, P<0.001) and CTLA-4$^+$ (r=0.337, P<0.001). Likewise, the frequency of M-MDSCs exhibited a positive correlation with the proportion of Tregs expressing PD-1 (r=0.265, P=0.01) and CTLA-4$^+$ (r=0.284, P<0.005). However, no further correlation was observed between other T cell and MDSC subsets in lung cancer.

M-MDSCs in lung malignancy enhance naive T cell apoptosis and promote Treg subset differentiation. To investigate the effects of MDSC subsets derived from lung malignancy and healthy controls on naive T cell proliferation and apoptosis, as well as Treg differentiation, MDSC subsets were co-cultured with naive T cells. Results of flow cytometry demonstrated

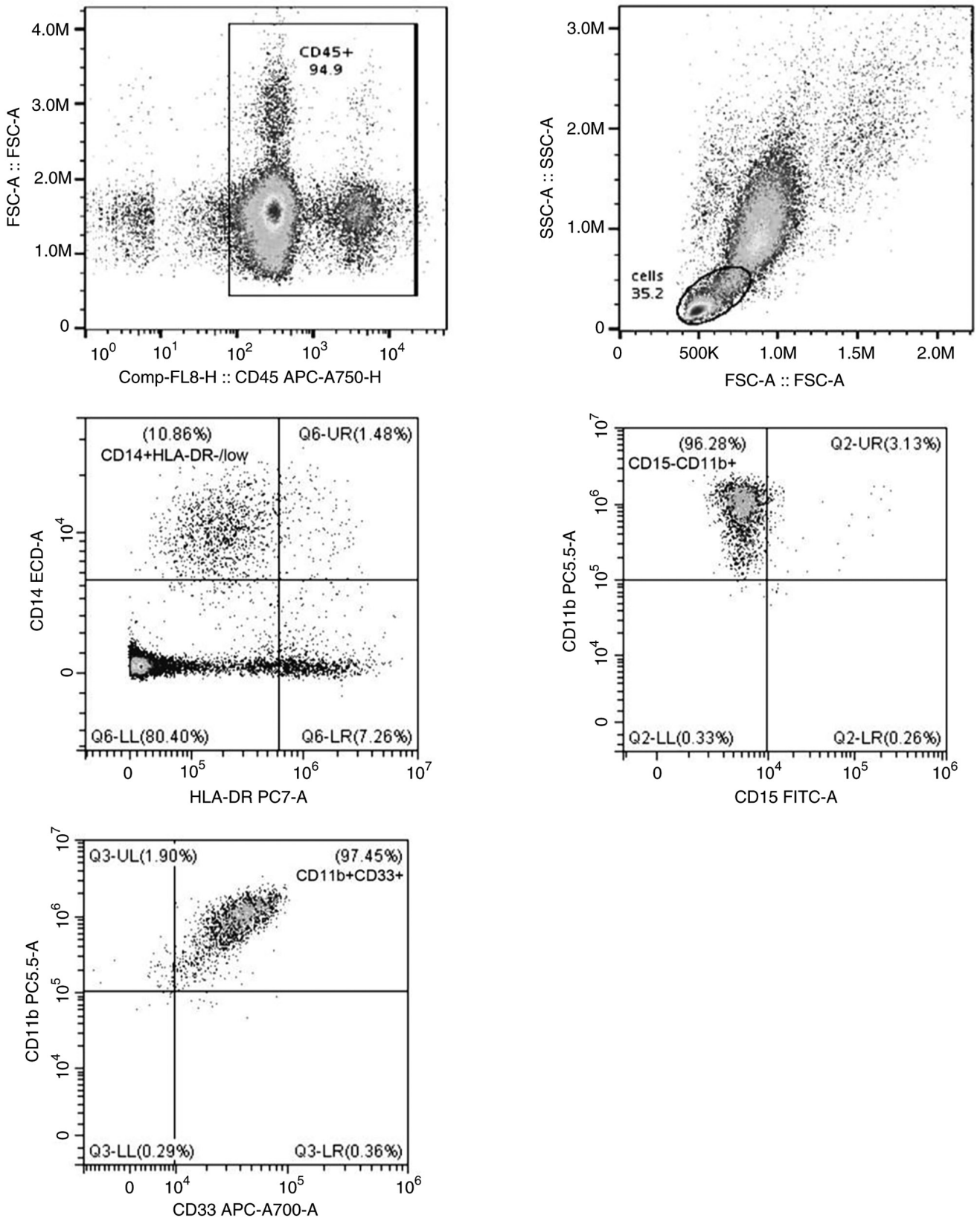

Figure 6. Flow cytometric detection to sort circulating M-MDSCs. MDSCs were determined using flow cytometry based on CD45 expression. $CD14^{+}HLA^{-}DR-/low$ cells in lymphocytes and monocytes were analyzed. M-MDSCs were sorted as $CD14^{+}CD15^{-}CD11b^{+}HLA^{-}DR-/low$. M-MDSC, monocyte-like myeloid-derived suppressor cell.

that G-MDSCs derived from lung malignancy and healthy controls exerted no significant effects on naive T-cell proliferation and apoptosis, as well as Treg differentiation (data not shown). However, compared with M-MDSCs of healthy controls, M-MDSCs from patients with lung malignancy did not notably affect naive T cell proliferation (Fig. 10A and B),

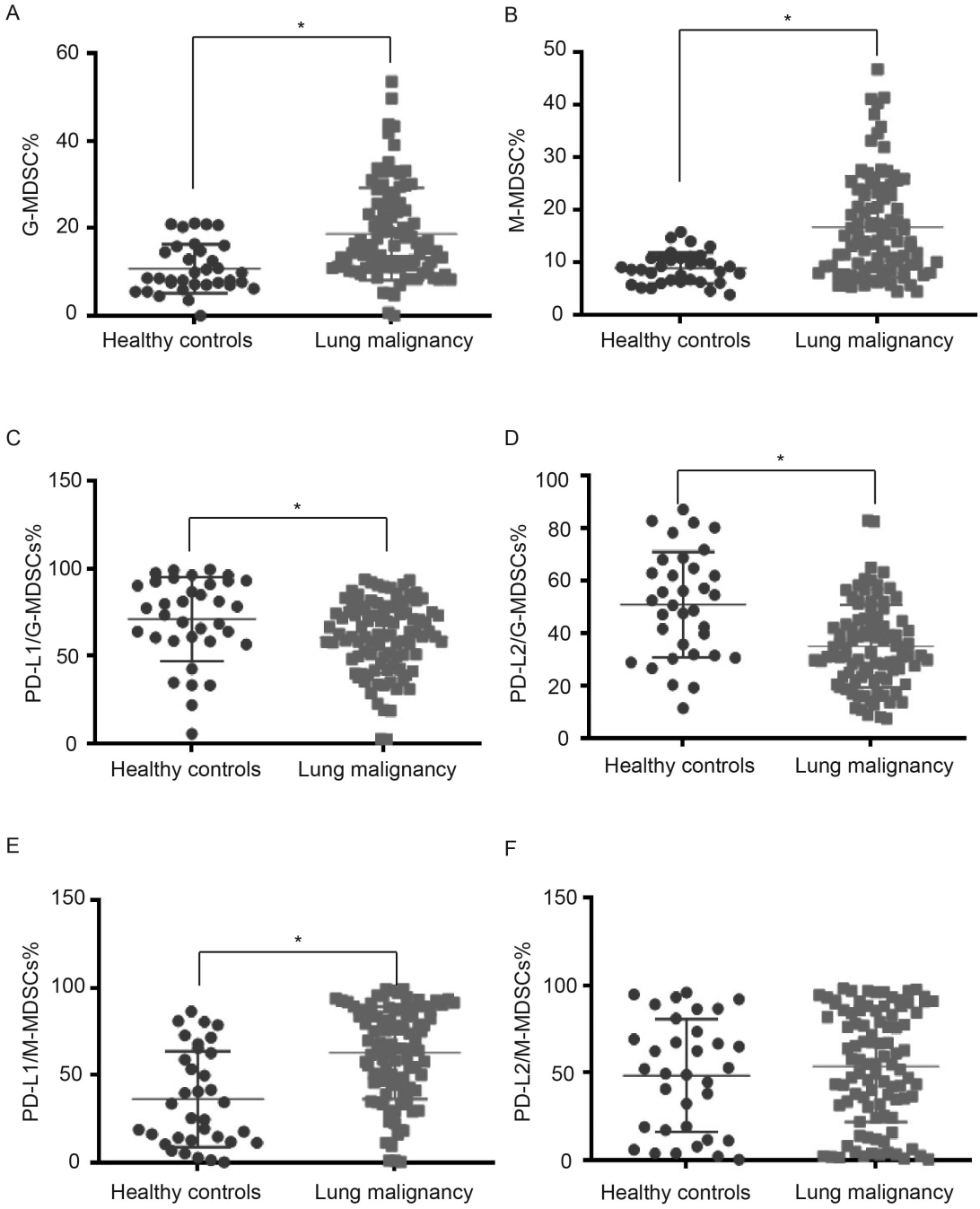

Figure 7. Flow cytometric detection of peripheral G-, M-, PD-L1$^+$G-, PD-L2$^+$G-, PD-L1$^+$M- and PD-L2$^+$M-MDSCs. Frequency of peripheral (A) G-, (B) M-, (C) PD-L1$^+$G-, (D) PD-L2$^+$G-, (E) PD-L1$^+$M- and (F) PD-L2$^+$M-MDSCs between lung malignancy cases and healthy controls. *P<0.05. MSDC, myeloid-derived suppressor cell.

but enhanced naive T cell apoptosis (Fig. 10C and D) and promoted Treg subset differentiation (Fig. 10E and F).

Discussion

The immunopathological role of MDSCs in infectious disease, inflammation and cancer by affecting T cells has been reported previously (23-25). The present study demonstrated that circulating frequencies of CTLA-4$^+$Tregs and CTLA-4$^+$CD8$^+$ T cells are increased in lung cancer, accumulation of M-MDSC and G-MDSC occurs in cases with lung malignancy and frequencies of G-MDSCs and M-MDSCs in the peripheral blood were associated with lung malignancy metastasis and positively correlated with the proportions of CTLA-4$^+$ and PD-1$^+$Tregs. These results demonstrated impaired host tumor immunosurveillance in lung cancer as MDSCs could bind to PD-1 on Treg by expressing PD-L2 and PD-L1 and induce Treg expansion.

Tregs induce immunosuppression via contact-dependent, including the expression of PD-1, neuropilin 1, CTLA-4, lymphocyte-activation protein 3 and PD-L1, or -independent mechanisms, such as secretion of immunosuppressive molecules (26). Accumulation of immunosuppressive Tregs in lung tumor and systemic expansion enhances tumor evasion,

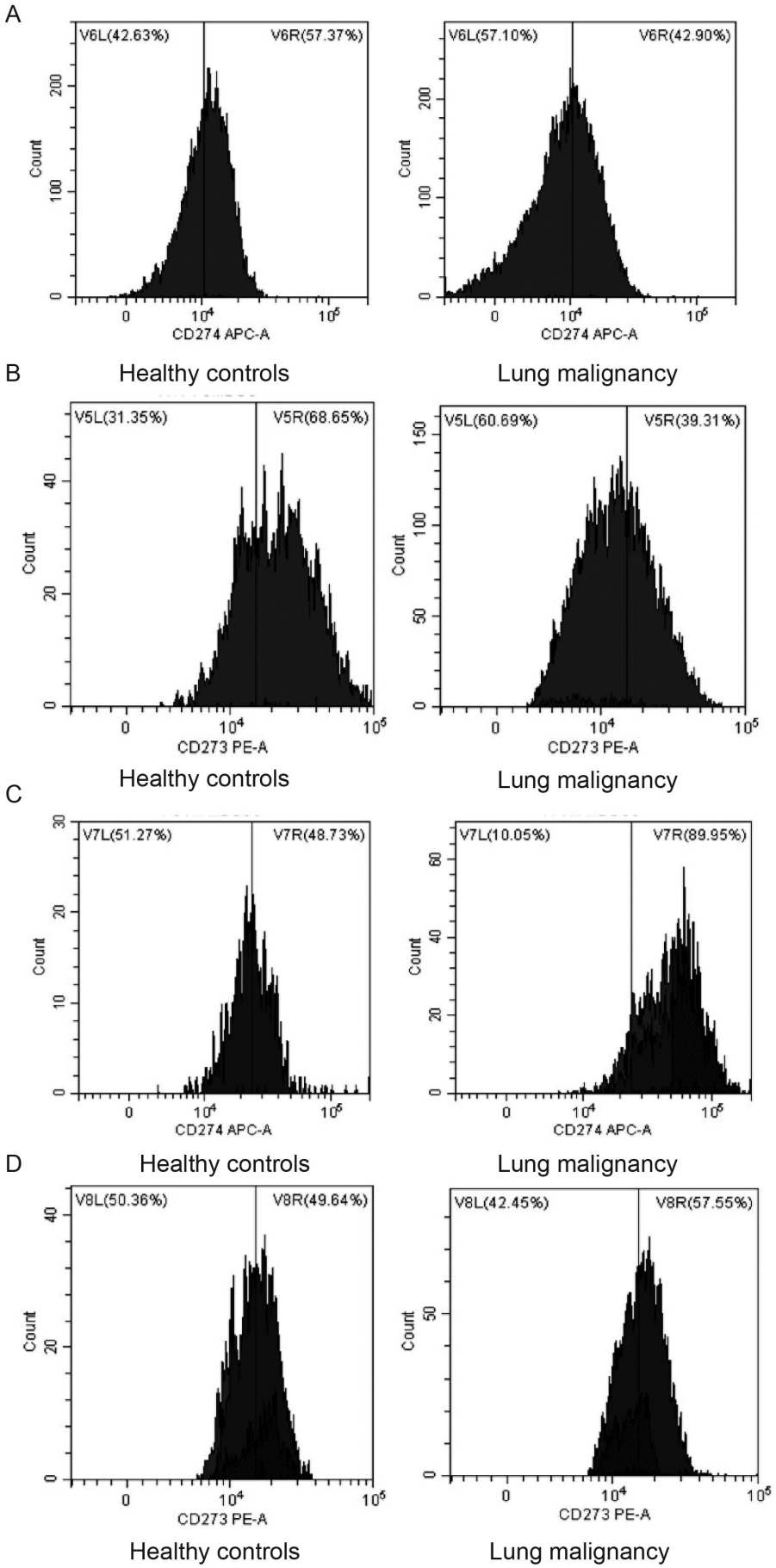

Figure 8. Flow cytometric detection of peripheral PD-L1$^+$, PD-L2$^+$G-, PD-L1$^+$M- and PD-L2$^+$M-MDSCs. Peripheral (A) PD-L1$^+$ and (B) PD-L2$^+$G- and (C) PD-L1$^+$M- and (D) PD-L2$^+$M-MDSCs between lung malignancy cases and healthy controls. G, granulocyte-like; M, monocyte-like; MSDC, myeloid-derived suppressor cell.

Table V. Association between clinical variables of patients with lung cancer and frequencies of PD-L1$^+$ and PD-L2$^+$G-MDSCs and PD-L1$^+$M-MDSCs in the peripheral blood.

		PD-L1$^+$G-MDSC		PD-L2$^+$G-MDSC		PD-L1$^+$M-MDSC	
Clinical variable	n	Frequency, %	P-value	Frequency, %	P-value	Frequency, %	P-value
Age, years			0.580		0.078		0.414
≤65	45	61.74±20.36		32.43±12.60		64.86±26.58	
>65	57	59.47±20.59		37.95±17.51		60.53±26.38	
Sex			0.300		0.960		0.240
Male	71	59.05±21.13		34.97±16.39		60.37±27.01	
Female	31	63.64±18.68		35.14±14.58		67.09±24.90	
Smoking status			0.827		0.228		0.951
Smoker	61	60.85±20.55		36.55±17.23		62.34±26.73	
Non-smoker	41	59.94±20.45		32.70±13.11		62.67±26.30	
TNM stage			0.403		0.238		0.084
I + II	29	63.16±19.27		32.12±16.23		69.57±24.79	
III + IV	73	59.39±20.90		36.22±15.54		59.55±26.70	
Histological type			0.472		0.139		0.237
Squamous cell carcinoma	35	57.94±20.89		34.26±15.07		58.73±26.83	
Adenocarcinoma	50	60.55±20.62		32.70±14.12		61.69±25.71	
Large cell carcinoma	7	71.43±14.42		43.63±19.78		80.87±24.09	
Small cell lung cancer	10	60.67±21.54		42.41±20.37		65.26±28.29	
Distant metastasis			0.296		0.088		0.020[a]
Yes	49	58.32±22.63		37.75±16.75		56.31±29.00	
No	53	62.56±18.03		32.42±14.47		68.39±22.43	

[a]P<0.05. M-, monocyte-like; G-, granulocyte-like; MDSC, myeloid-derived suppressor cell.

thus contributing to progression of numerous types of malignancy, such as lung (27), breast (28), ovary (29) and prostate cancer (30). The present study showed no notable difference in populations of peripheral blood Tregs, CTLA-4$^+$CD4$^+$ T cells, PD-1$^+$Tregs and PD-1$^+$CD4$^+$ and PD-1$^+$CD8$^+$ T cells between lung cancer cases and healthy controls. Kumagai *et al* (31) showed a profound reactivation of effector PD-1$^+$CD8$^+$ T cells rather than PD-1$^+$ Treg cells by PD-1 blockade is necessary for tumor regression, which is not consistent with the present results. The aforementioned study focused on Tregs and CD8$^+$ T cells in the tumor microenvironment rather than in the circulation. Wu *et al* (32) identified a high population of Tregs expressing PD-L1 in the tumor microenvironment of lung cancer and frequency of PD-L1$^+$Tregs was higher in cancer tissues than in normal tissue and blood, suggesting levels of these cells vary between the tumor microenvironment and circulation. However, it is unknown which immunosuppressive molecule is responsible for inducing the function of circulating Tregs in lung malignancy and whether Tregs have tumor microenvironment- or circulation-dependent phenotype? The present study observed higher frequencies of peripheral CTLA-4$^+$Tregs and CTLA-4$^+$CD8$^+$ T cells in lung cancer cases compared with healthy controls, suggesting that CTLA-4 may be the immunosuppressive molecule that induces function of circulating Tregs in lung cancer. CTLA-4 is a protein receptor that maintains peripheral tolerance by suppressing T cell activation and proliferation, thus inhibiting the immune system (33). CTLA-4 in Tregs could remove CD80/CD86 from the antigen-presenting cells and function in a cell-extrinsic fashion, thus preventing further priming of other T cells (34). Studies have identified CTLA-4 molecules as a strong inductor of Treg function (35,36). Additionally, combination of anti-PD-1/PD-L1 + anti-CTLA-4 immune-checkpoint inhibitors contributes to better survival compared with single-agent immunotherapy (37). Compared with stage I + II, the present study found a lower frequency of circulating CTLA-4$^+$CD8$^+$ T cells in patients with stage III + IV lung cancer. Similarly, Erfani *et al* (38) found an increase in circulating CD8$^+$ T cells expressing SurCTLA-4 instead of CD4$^+$ T cells expressing SurCTLA-4 in NSCLC compared with health controls. Therefore, CD8$^+$ T cells and Tregs expressing CTLA-4 might be novel immunotherapeutic targets for lung cancer.

MDSCs contribute to the immune tolerance of tumor cells by attracting Tregs into tumor sites and inhibiting proliferation of T cells (39). Here, a higher diversity of peripheral blood G-MDSC and M-MDSC populations was noted in lung cancer cases compared with healthy individuals. *In vitro* co-culture assay demonstrated M-MDSCs of lung malignancy enhanced naive T cell apoptosis and promoted Treg subset differentiation compared with M-MDSCs of healthy controls. Recruitment of MDSCs is regulated by a series of cytokines, such as IL-1β, vascular endothelial

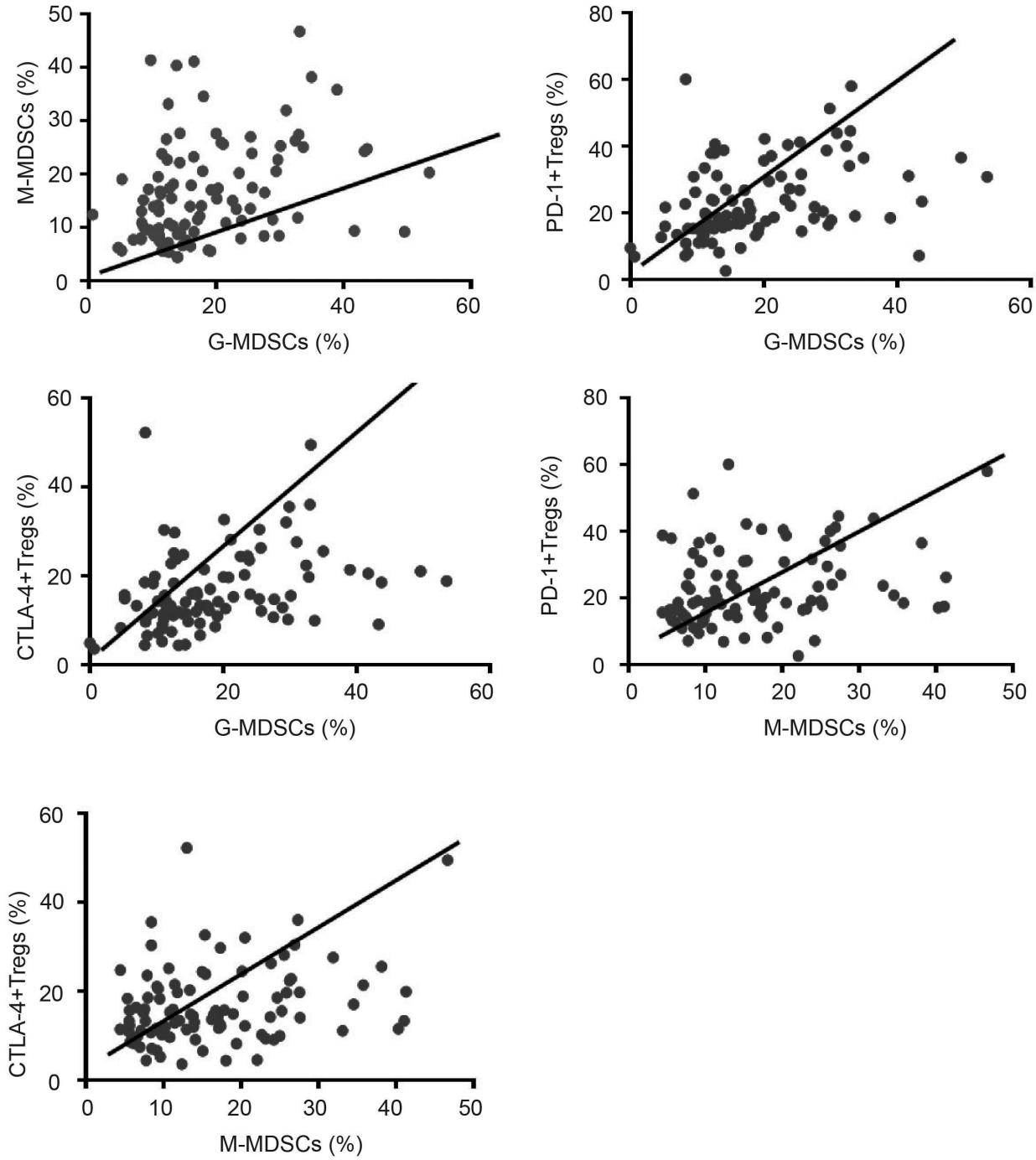

Figure 9. Pearson's correlation analyses of frequencies of peripheral blood T cell and MDSC subsets in patients with lung malignancy. Treg, regulatory T cell; G, granulocyte-like; M, monocyte-like; MSDC, myeloid-derived suppressor cell.

growth factor, prostaglandin E2, IL-6, granulocyte macrophage colony stimulating factor and S100A8/A9 (40). Most of the aforementioned cytokines activate the JAK/STAT3 signaling pathway to mediate the amplification and aggregation of MDSCs (41). In this event, MDSCs are recruited, migrate and expand ~10 times in the circulation and tumor environment. Yamauchi *et al* (42) detected a significant increase in frequency of circulating M-MDSCs in patients with NSCLC compared with healthy donors. Additionally, Li *et al* (43) demonstrated increased abundance of G-MDSCs locally within the tumor microenvironment and systemically in peripheral blood and spleen in a Live kinase B1-deficient murine model of NSCLC. Here, increased frequencies of peripheral blood G-MDSCs and M-MDSCs were observed in distant metastasis, suggesting two MDSC subsets were correlated with lung cancer metastasis. Lung-derived G-MDSCs harvested from tumor-bearing animals promote proliferation of disseminated cancer cells at metastatic sites. Tumor-infiltrated M-MDSCs induce dissemination of cancer cells by eliciting epithelial-to-mesenchymal transition/cancer stem cell phenotype (44). Hypoxia is a hallmark of the tumor microenvironment and contributes to tumor progression. Hypoxia triggers a rapid, notable selective upregulation of PD-L1 on MDSCs in tumor-bearing mice,

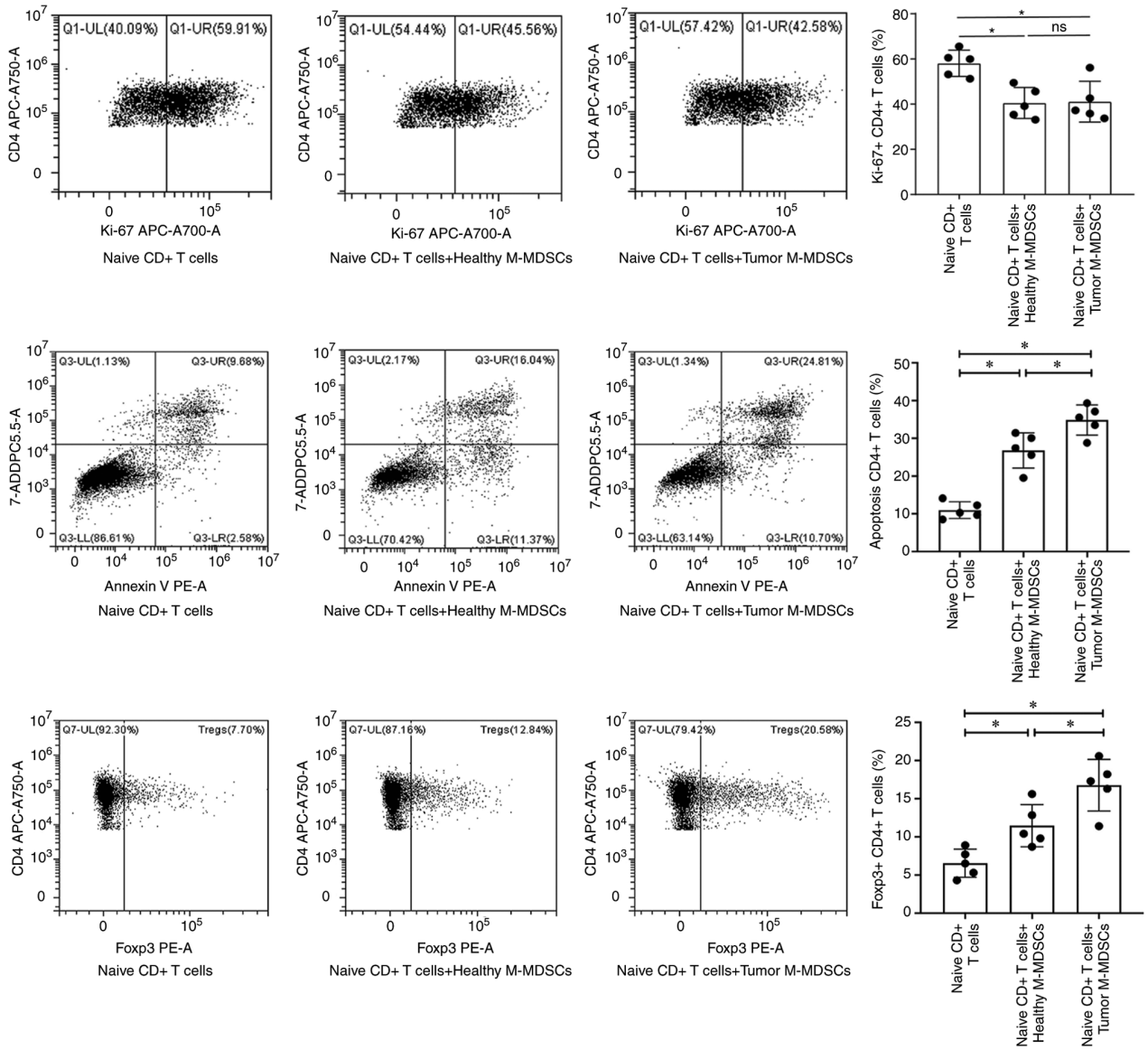

Figure 10. Flow cytometry detection of naive T cell proliferation and apoptosis, as well as Treg differentiation in *in vitro* co-culture system of MDSC subsets derived from lung malignancy and healthy controls with naive T cells. (A) Flow cytometric detection of Ki-67$^+$CD4$^+$ T cells when cocultured with M-MDSCs derived from healthy controls or lung malignancy. (B) Quantification of Ki-67$^+$CD4$^+$ T cells. (C) Flow cytometric detection of apoptotic CD4$^+$ T cells when cocultured with M-MDSCs derived from healthy controls or lung malignancy. (D) Quantification of apoptotic CD4$^+$ T cells. (E) Flow cytometric detection of Foxp3$^+$CD4$^+$ T cells when cocultured with M-MDSCs derived from healthy controls or lung malignancy. (F) Quantification of Foxp3$^+$CD4$^+$ T cells. *P<0.05. ns, not significant. Treg, regulatory T cell; MSDC, myeloid-derived suppressor cell.

inducing immunosuppressive function of MDSCs (45). MDSC-induced release of PD-L1 is also modulated by IFN-γ, which activates phosphorylated STAT1 to directly modulate IFN regulatory factor-1 (IRF1) transcription. IRF1 activation leads to PD-L1 expression in MDSCs by directly binding to an IRF-binding consensus element (46). Here, compared with controls, lung malignancy cases exhibited lower frequencies of PD-L1$^+$ and PD-L2$^+$G-MDSCs, but a high frequency of PD-L1$^+$M-MDSCs in the peripheral blood. Rather than G-MDSCs, M-MDSCs bind with PD-1 on the surface of Tregs and promote differentiation of initial CD4$^+$ T cells into Tregs; frequencies of M-MDSCs were positively correlated with proportions of CTLA-4$^+$ and PD-1$^+$Tregs. These findings support the hypothesis that notable accumulation of MDSC subsets is involved in lung cancer occurrence and progression as M-MDSCs bind to PD-1 on Tregs by expressing PD-L1 and PD-L2 on the surface and induce Treg expansion.

There were several limitations in the present study. Firstly, the mechanism behind the contribution of G-MDSCs to lung cancer should be further investigated, since a higher diversity of peripheral blood G-MDSC and M-MDSC populations was noted in cases with lung cancer compared with healthy controls. Secondly, since MDSCs are more likely to accumulate systemically in the spleen (47), more locations, such as cancerous lung tissue and spleen in lung cancer, should be used to characterize the frequencies of T cell subsets and MDSC subset. Future clinical studies and cell and animal models should be performed to demonstrate M-MDSC promotion of Tregs by PD-1/PD-L1/2 interaction.

In conclusion, CTLA-4$^+$Tregs, M-MDSCs, CTLA-4$^+$CD8$^+$ T cells and G-MDSCs accumulated in lung cancer. MDSCs

promote Treg expansion by binding surface molecule PD-1, which is involved in lung cancer occurrence and progression. Collectively, these findings suggested a contributing role for MDSCs and Tregs in immunopathogenesis of lung cancer and these may be potential targets to design host-directed therapies for patients with lung cancer.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Sciences Foundation of China (grant no. 81900021) and Beijing Clinical Specialty (grant no. XKB2022B1002).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MQZ and XDM designed the study. YHW interpreted the data and wrote the manuscript. JQZ, LL and WSX analyzed data. LL and WSX confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of Beijing Tsinghua Changgung Hospital (approval no. 18190-0-01). All recruited patients signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Fuchs HE and Jemal A: Cancer statistics, 2021. CA Cancer J Clin 71: 7-33, 2021.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 71: 209-249, 2021.
3. Schneider F and Dacic S: Histopathologic and molecular approach to staging of multiple lung nodules. Transl Lung Cancer Res 6: 540-549, 2017.
4. Rudin CM, Brambilla E, Faivre-Finn C and Sage J: Small-cell lung cancer. Nat Rev Dis Primers 7: 3, 2021.
5. Suresh K, Naidoo J, Lin CT and Danoff S: Immune checkpoint immunotherapy for non-small cell lung cancer: Benefits and pulmonary toxicities. Chest 154: 1416-1423, 2018.
6. Law AMK, Valdes-Mora F and Gallego-Ortega D: Myeloid-derived suppressor cells as a therapeutic target for cancer. Cells 9: 561, 2020.
7. Gabrilovich DI: Myeloid-derived suppressor cells. Cancer Immunol Res 5: 3-8, 2017.
8. Hegde S, Leader AM and Merad M: MDSC: Markers, development, states, and unaddressed complexity. Immunity 54: 875-884, 2021.
9. Talmadge JE and Gabrilovich DI: History of myeloid-derived suppressor cells. Nat Rev Cancer 13: 739-752, 2013.
10. Cassetta L, Baekkevold ES, Brandau S, Bujko A, Cassatella MA, Dorhoi A, Krieg C, Lin A, Loré K, Marini O, *et al*: Deciphering myeloid-derived suppressor cells: Isolation and markers in humans, mice and non-human primates. Cancer Immunol Immunother 68: 687-697, 2019.
11. Tao J, Han D, Gao S, Zhang W, Yu H, Liu P, Fu R, Li L and Shao Z: $CD8^+$ T cells exhaustion induced by myeloid-derived suppressor cells in myelodysplastic syndromes patients might be through TIM3/Gal-9 pathway. J Cell Mol Med 24: 1046-1058, 2020.
12. Condamine T and Gabrilovich DI: Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol 32: 19-25, 2011.
13. Chang AL, Miska J, Wainwright DA, Dey M, Rivetta CV, Yu D, Kanojia D, Pituch KC, Qiao J, Pytel P, *et al*: CCL2 produced by the glioma microenvironment is essential for the recruitment of regulatory T cells and myeloid-derived suppressor cells. Cancer Res 76: 5671-5682, 2016.
14. Togashi Y, Shitara K and Nishikawa H: Regulatory T cells in cancer immunosuppression-implications for anticancer therapy. Nat Rev Clin Oncol 16: 356-371, 2019.
15. Adair PR, Kim YC, Zhang AH, Yoon J and Scott DW: Human tregs made antigen specific by gene modification: The power to treat autoimmunity and antidrug antibodies with precision. Front Immunol 8: 1117, 2017.
16. Ohue Y and Nishikawa H: Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? Cancer Sci 110: 2080-2089, 2019.
17. Park HJ, Park JS, Jeong YH, Son J, Ban YH, Lee BH, Chen L, Chang J, Chung DH, Choi I and Ha SJ: PD-1 upregulated on regulatory T cells during chronic virus infection enhances the suppression of CD8+ T cell immune response via the interaction with PD-L1 expressed on CD8+ T cells. J Immunol 194: 5801-5811, 2015.
18. Lee-Chang C, Rashidi A, Miska J, Zhang P, Pituch KC, Hou D, Xiao T, Fischietti M, Kang SJ, Appin CL, *et al*: Myeloid-derived suppressive cells promote B cell-mediated immunosuppression via transfer of PD-L1 in glioblastoma. Cancer Immunol Res 7: 1928-1943, 2019.
19. Cai J, Wang D, Zhang G and Guo X: The role Of PD-1/PD-L1 axis in Treg development and function: implications for cancer immunotherapy. Onco Targets Ther 12: 8437-8445, 2019.
20. Travis WD, Brambilla E, Nicholson AG, Yatabe Y, Austin JHM, Beasley MB, Chirieac LR, Dacic S, Duhig E, Flieder DB, *et al*: The 2015 World Health Organization classification of lung tumors: Impact of genetic, clinical and radiologic advances since the 2004 classification. J Thorac Oncol 10: 1243-1260, 2015.
21. Lababede O, Meziane M and Rice T: Seventh edition of the cancer staging manual and stage grouping of lung cancer: Quick reference chart and diagrams. Chest 139: 183-189, 2011.
22. Scrimini S, Pons J, Agustí A, Soriano JB, Cosio BG, Torrecilla JA, Núñez B, Córdova R, Iglesias A, Jahn A, *et al*: Differential effects of smoking and COPD upon circulating myeloid derived suppressor cells. Respir Med 107: 1895-1903, 2013.
23. Schrijver IT, Théroude C and Roger T: Myeloid-derived suppressor cells in sepsis. Front Immunol 10: 327, 2019.
24. Salminen A, Kauppinen A and Kaarniranta K: Myeloid-derived suppressor cells (MDSC): An important partner in cellular/tissue senescence. Biogerontology 19: 325-339, 2018.
25. Sivagnanalingam U, Beatty PL and Finn OJ: Myeloid derived suppressor cells in cancer, premalignancy and inflammation: A roadmap to cancer immunoprevention. Mol Carcinog 59: 852-861, 2020.
26. Vignali DAA, Collison LW and Workman CJ: How regulatory T cells work. Nat Rev Immunol 8: 523-532, 2008.
27. Marshall EA, Ng KW, Kung SH, Conway EM, Martinez VD, Halvorsen EC, Rowbotham DA, Vucic EA, Plumb AW, Becker-Santos DD, *et al*: Emerging roles of T helper 17 and regulatory T cells in lung cancer progression and metastasis. Mol Cancer 15: 67, 2016.
28. Plitas G, Konopacki C, Wu K, Bos PD, Morrow M, Putintseva EV, Chudakov DM and Rudensky AY: Regulatory T cells exhibit distinct features in human breast cancer. Immunity 45: 1122-1134, 2016.

29. Toker A, Nguyen LT, Stone SC, Yang SYC, Katz SR, Shaw PA, Clarke BA, Ghazarian D, Al-Habeeb A, Easson A, *et al*: Regulatory T cells in ovarian cancer are characterized by a highly activated phenotype distinct from that in melanoma. Clin Cancer Res 24: 5685-5696, 2018.
30. Erlandsson A, Carlsson J, Lundholm M, Fält A, Andersson SO, Andrén O and Davidsson S: M2 macrophages and regulatory T cells in lethal prostate cancer. Prostate 79: 363-369, 2019.
31. Kumagai S, Togashi Y, Kamada T, Sugiyama E, Nishinakamura H, Takeuchi Y, Vitaly K, Itahashi K, Maeda Y, Matsui S, *et al*: The PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. Nat Immunol 21: 1346-1358, 2020.
32. Wu SP, Liao RQ, Tu HY, Wang WJ, Dong ZY, Huang SM, Guo WB, Gou LY, Sun HW, Zhang Q, *et al*: Stromal PD-L1-positive regulatory T cells and PD-1-positive CD8-positive T cells define the response of different subsets of non-small cell lung cancer to PD-1/PD-L1 blockade immunotherapy. J Thorac Oncol 13: 521-532, 2018.
33. Gattinoni L, Ranganathan A, Surman DR, Palmer DC, Antony PA, Theoret MR, Heimann DM, Rosenberg SA and Restifo NP: CTLA-4 dysregulation of self/tumor-reactive CD8+ T-cell function is CD4+ T-cell dependent. Blood 108: 3818-3823, 2006.
34. Lang C, Wang J and Chen L: CD25-expressing Th17 cells mediate $CD8^+$ T cell suppression in CTLA-4 dependent mechanisms in pancreatic ductal adenocarcinoma. Exp Cell Res 360: 384-389, 2017.
35. Walker LSK: Treg and CTLA-4: Two intertwining pathways to immune tolerance. J Autoimmun 45: 49-57, 2013.
36. Krummey SM and Ford ML: Braking bad: Novel mechanisms of CTLA-4 inhibition of T cell responses. Am J Transplant 14: 2685-2690, 2014.
37. Chae YK, Arya A, Iams W, Cruz MR, Chandra S, Choi J and Giles F: Current landscape and future of dual anti-CTLA4 and PD-1/PD-L1 blockade immunotherapy in cancer; lessons learned from clinical trials with melanoma and non-small cell lung cancer (NSCLC). J Immunother Cancer 6: 39, 2018.
38. Erfani N, Mehrabadi SM, Ghayumi MA, Haghshenas MR, Mojtahedi Z, Ghaderi A and Amani D: Increase of regulatory T cells in metastatic stage and CTLA-4 over expression in lymphocytes of patients with non-small cell lung cancer (NSCLC). Lung Cancer 77: 306-311, 2012.
39. Kumar V, Patel S, Tcyganov E and Gabrilovich DI: The nature of myeloid-derived suppressor cells in the tumor microenvironment. Trends Immunol 37: 208-220, 2016.
40. Sinha P, Okoro C, Foell D, Freeze HH, Ostrand-Rosenberg S and Srikrishna G: Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. J Immunol 181: 4666-4675, 2008.
41. Guha P, Gardell J, Darpolor J, Cunetta M, Lima M, Miller G, Espat NJ, Junghans RP and Katz SC: STAT3 inhibition induces Bax-dependent apoptosis in liver tumor myeloid-derived suppressor cells. Oncogene 38: 533-548, 2019.
42. amauchi Y, Safi S, Blattner C, Rathinasamy A, Umansky L, Juenger S, Warth A, Eichhorn M, Muley T, Herth FJF, *et al*: Circulating and tumor myeloid-derived suppressor cells in resectable non-small cell lung cancer. Am J Respir Crit Care Med 198: 777-787, 2018.
43. Li R, Salehi-Rad R, Crosson W, Momcilovic M, Lim RJ, Ong SL, Huang ZL, Zhang T, Abascal J, Dumitras C, *et al*: Inhibition of granulocytic myeloid-derived suppressor cells overcomes resistance to immune checkpoint inhibition in LKB1-deficient non-small cell lung cancer. Cancer Res 81: 3295-3308, 2021.
44. Ouzounova M, Lee E, Piranlioglu R, El Andaloussi A, Kolhe R, Demirci MF, Marasco D, Asm I, Chadli A, Hassan KA, *et al*: Monocytic and granulocytic myeloid derived suppressor cells differentially regulate spatiotemporal tumour plasticity during metastatic cascade. Nat Commun 8: 14979, 2017.
45. Noman MZ, Desantis G, Janji B, Hasmim M, Karray S, Dessen P, Bronte V and Chouaib S: PD-L1 is a novel direct target of HIF-1α, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. J Exp Med 211: 781-790, 2014.
46. Lu C, Redd PS, Lee JR, Savage N and Liu K: The expression profiles and regulation of PD-L1 in tumor-induced myeloid-derived suppressor cells. Oncoimmunology 5: e1247135, 2016.
47. Eckert I, Ribechini E and Lutz MB: In vitro generation of murine myeloid-derived suppressor cells, analysis of markers, developmental commitment, and function. Methods Mol Biol 2236: 99-114, 2021.