Recruitment of myeloid-derived suppressor cells and regulatory T-cells is associated with the occurrence of acute myocardial infarction

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Abstract. The roles of myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs) in acute myocardial infarction (AMI) remain elusive. The present study aimed to analyze the proportions of the granulocytic and monocytic populations of MDSCs (G-MDSCs and M-MDSCs, respectively), and Tregs in the peripheral blood mononuclear cells (PBMCs) of patients with AMI. The present study recruited 34 patients with AMI and 37 healthy controls without clinical signs of myocardial ischemia. PBMCs were isolated from the peripheral blood samples of patients with AMI within 24 h following admission to the hospital and from those of the healthy controls during a physical examination. Two subsets of MDSCs, G-MDSCs ($CD15^+CD33^+CD11b^+CD14^-HLA\text{-}DR^{low}$) and M-MDSCs ($CD14^+CD15^-CD11b^+HLA\text{-}DR^{low}$), and Tregs ($CD3^+CD4^+CD25^{high}CD127^{low}$ T-cells) in the PBMCs derived from the patients with AMI and healthy controls were analyzed using flow cytometry. The effects of MDSCs derived from patients with AMI on naïve $CD4^+$ T-cells were examined in the co-culture system. The results revealed that the proportions of G-MDSCs and M-MDSCs were higher in the peripheral blood of patients with AMI than in that of the healthy controls. The patients with AMI had significantly higher numbers of programmed death-ligand (PD-L)1- and PD-L2-positive G-MDSCs and M-MDSCs compared with the healthy controls ($P<0.05$). The MDSCs could acquire a granulocytic phenotype following AMI, and the G-MDSCs and M-MDSCs would be more likely to express PD-L2 and PD-L1, respectively. The ratios of Tregs to $CD4^+$ T-cells and $PD\text{-}1^+$ Tregs in the peripheral blood of patients with AMI were significantly higher than those in the healthy controls ($P<0.05$). The results of flow cytometry demonstrated an increase in the numbers of inducible Tregs in the co-culture system with the G-MDSCs derived from patients with AMI compared with the G-MDSCs derived from the healthy controls ($P<0.01$). On the whole, the findings presented herein demonstrate the accumulation of MDSCs, and the upregulation of PD-L1 and PD-L2 expression on the surface of MDSCs in patients with AMI. MDSCs can induce the expansion of Tregs by binding PD-1 on the surface of Tregs, thus playing a crucial role in AMI.

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Introduction

The abrupt loss of cardiac function in patients diagnosed with heart disease is defined as cardiac arrest. The reduction of blood flow or the termination of the coronary artery of the heart, causing damage to the heart muscle, may result in myocardial infarction (MI). The high risk of the short- and long-term recurrent MI events is noteworthy, in spite of the recent development of novel therapeutic approaches for heart disease. Acute MI (AMI) involves myocardial necrosis caused by acute and persistent ischemia, and hypoxia of the coronary arteries (1). AMI is accompanied by an increased serum myocardial enzymatic activity and progressive changes in an electrocardiogram (ECG), which can be complicated by arrhythmia, shock or heart failure, and it is mainly life-threatening (2). In recent decades, the pathogenesis of atherothrombosis and coronary heart disease, including acute coronary syndrome, has been frequently studied (3). The development of AMI may be attributed to an imbalance between pro-inflammatory and anti-inflammatory responses, and

cardiac function can be promoted via the post-MI intramyocardial injection of bone marrow-derived mononuclear cells in an interleukin (IL)-10-dependent manner (4). Moreover, a notable reduction in the number of immune-suppressive regulatory T-cells (Tregs) has been noted in patients with AMI (5), and alleviating cardiac damage, and inhibiting cardiac hypertrophy and fibrosis despite sustained angiotensin II-induced hypertension in mice could result from the adoptive transfer of Tregs (6).

In healthy individuals, the rapid differentiation of immature myeloid cells (IMCs) generated in bone marrow into mature granulocytes, macrophages or dendritic cells has been reported (7). Partially, the suppression of the differentiation of IMCs into mature myeloid cells could lead to an expansion of this population under pathological conditions (e.g., cancer, infectious diseases, sepsis, etc.) (8,9). Myeloid-derived suppressor cells (MDSCs) have exhibited potent immunosuppressive functions, inducing tumor progression, angiogenesis and immune escape (10). A previous study demonstrated that the immunophenotype of MDSCs in tumor-bearing mice was protein gamma response 1 $(Gr\text{-}1)^{+}CD11b^{+}$ cells (11). The MDSCs can be classified into two subtypes: Monocyte like-MDSCs (M-MDSCs), involving $CD11b^{+}Ly6C^{high}Ly6G^{-}$ cells with a monocyte-like morphology, and granulocyte like-MDSCs (G-MDSCs), including $CD11b^{+}Ly6C^{low}Ly6G^{-}$ cells with a granulocyte-like morphology (12). However, in humans, MDSCs are mainly identified by CD11b and CD33 antibodies, with low levels of the major histocompatibility complex class II molecule human leukocyte antigen-D-related (HLA-DR). The MDSCs inhibit immune responses by expressing high levels of arginase 1 and producing nitric oxide (NO) and reactive oxygen species (13), as well as by inducing Tregs (14). MDSCs exert their suppressive effects via the expression or secretion of copious amounts of immunosuppressive mediators [e.g., programmed death-ligand 1 (PD-L1)] (14). The suppression of T-cell proliferation and the promotion of T-cell apoptosis may be attributed to MDSCs, facilitating the induction of Tregs by the release of interleukin (IL)-10 and transforming growth factor-β, which may result in reducing cell-mediated immunity (15).

The accumulation of MDSCs has been found to be associated with occurrence of cancer and chronic inflammatory and autoimmune diseases (16). Nevertheless, a few studies have concentrated on the role of MDSCs in AMI. Notably, the accumulation and activation of MDSCs can be induced by inflammation-associated factors, vascular endothelial growth factor and prostaglandin E2, and pro-inflammatory cytokines (17). The present study aimed to indicate whether the recruitment of MDSCs and Tregs is associated with the occurrence of AMI. The results may further assist cardiologists in the treatment of heart diseases, particularly AMI.

Materials and methods

Participants. A total of 34 patients who were diagnosed with AMI and admitted to the Department of Cardiology of the Beijing Tsinghua Changgung Hospital (Beijing, China) between December, 2019 and December, 2020 were enrolled in the study, including 19 males and 15 females, with an average age of 62.23±8.54 years. The diagnostic criteria of AMI were based on the Fourth Edition of the Universal Definition of MI (18). These diagnostic criteria include the rise and/or fall of cardiac troponin T with at least one value above the 99th percentile upper reference limit and satisfying at least one of the following criteria: i) The presence of symptoms of myocardial ischemia; ii) presenting new ischemic ECG changes; iii) developing pathological Q waves; iv) imaging evidence of the loss of viable myocardium or regional wall motion abnormalities; v) the identification of a coronary thrombus by coronary angiography. The diagnosis was confirmed by coronary angiography at the admission, and all patients with AMI underwent reperfusion by percutaneous coronary intervention without any delay. Additionally, 37 healthy individuals without any clinical signs of myocardial ischemia were included as the healthy controls (HCs), consisting of 21 males and 16 females, with an average age of 58.23±10.27 years. AMI patients and HCs were age- and sex-matched. The present study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Ethics Committee of the Beijing Tsinghua Changgung Hospital (Approval no. 18190-0-01). All patients and HCs were informed of the study objective, and they signed informed consent forms prior to enrollment.

Isolation of peripheral blood mononuclear cells (PBMCs). Peripheral blood samples were collected in heparin-contained tubes at 5-7 days following the onset of AMI for patients with AMI or during the physical examination for the HCs. The blood samples were immediately placed in ice and were then centrifuged at 1,200 x 6 g for 5 min at room temperature. The PBMCs were then isolated using the Ficoll-Hypaque density gradient centrifugation method (reagents used: Histopaque, 1077, Sigma-Aldrich; PBS buffer solution, SH30256.01B, HyClone; Cytiva).

Phenotypic analysis. Two subsets of MDSCs, including G-MDSCs ($CD15^{+}CD33^{+}CD11b^{+}CD14^{-}HLA\text{-}DR^{low}$) and M-MDSCs ($CD14^{+}CD15^{-}CD11b^{+}HLA\text{-}DR^{low}$), and Tregs ($CD3^{+}CD4^{+}CD25^{high}CD127^{low}$ T-cells) were characterized by fluorescence-activated cell sorting (FACS) using a CytoFLEX flow cytometer (Beckman Coulter, Inc.) with a panel of anti-human-specific antibodies labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP)-cy5.5, allophycocyanin (APC) and PE-Cy7. Human antibodies specific for surface markers of T-cells included anti-CD3 (cat. no. MHCD0327; eBioscience), anti-CD4 (cat. no. 25-0049-42; eBioscience), anti-CD25 (cat. no. 12-0257-42; eBioscience), anti-CD127 (cat. no. 17-1278-42; eBioscience), anti-CTLA-4 anti-programmed cell death 1 (PD-1; cat. no. 85-46-1529-42; Invitrogen; Thermo Fisher Scientific, Inc.), and surface makers of MDSCs included anti-CD15 (cat. no. 11-0159-42; eBioscienc), anti-CD33 (cat. no. 56-0338-42; eBioscience), anti-CD14 (cat. no. 61-0149-42; eBioscience), anti-CD45 (cat. no. 47-0459-42; eBioscience), anti-CD11b (cat. no. 46-0118-42; eBioscience), anti-HLA-DR (cat. no. 25-9952-42; eBioscience), anti-PD-L1 (cat. no. 85-12-5888-42; eBioscience) and anti-PD-L2 (cat. no. 25-9952-42; eBioscience). The dilutions used for the antibodies (1:20) and the incubation conditions (at 4˚C for 30 min) were as per the manufacturer's instructions.

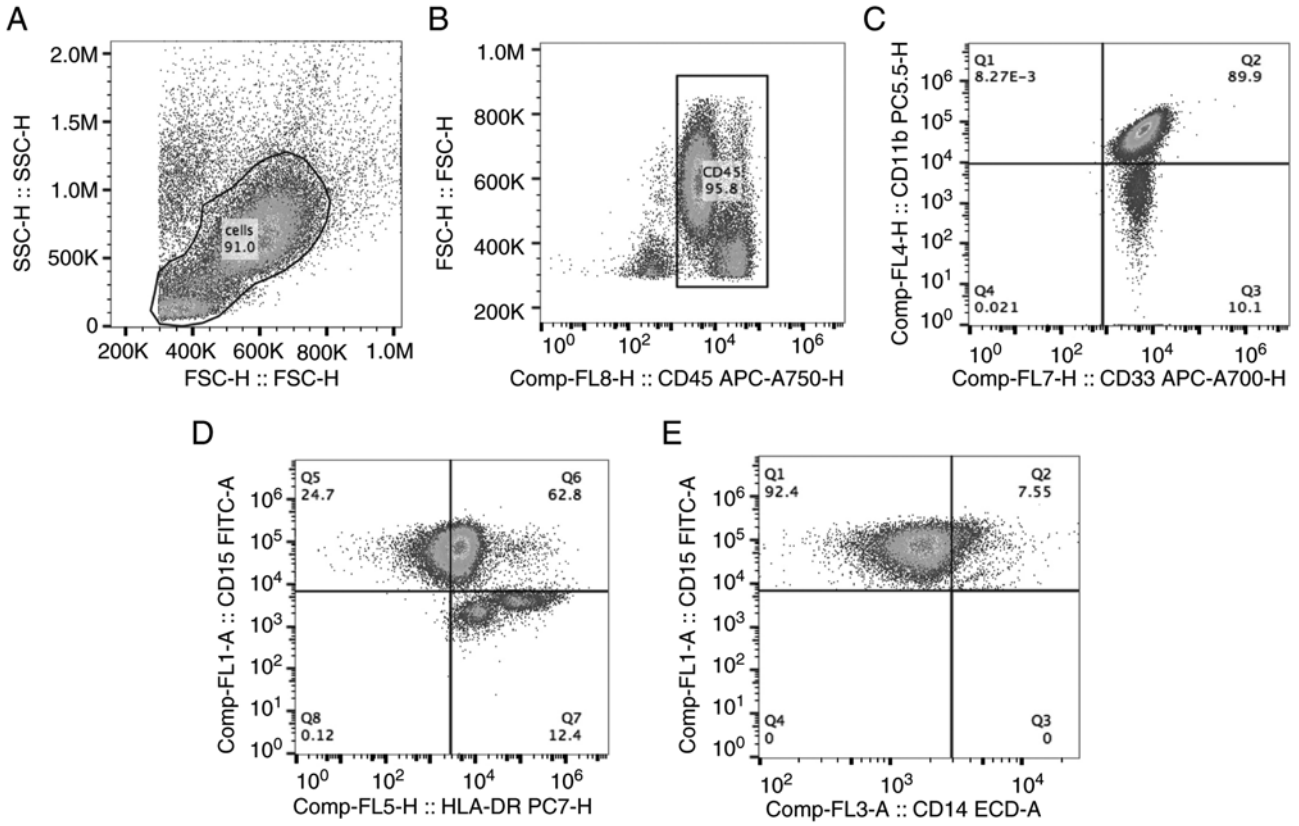

Figure 1. The gating strategy for G-MDSCs in the peripheral blood by flow cytometry. (A) Leukocytes in peripheral blood mononuclear cells were gated based on FSC-H and side scatter height. (B) FSC-H and CD45 were used to gate $CD45^+$ cells. (C) $CD11b^+CD33^+$ cells were gated. (D) $CD15^+HLA\text{-}DR^{low}$ cells were gated. (E) $CD15^+CD14^-$ cells were analyzed and sorted as G-MDSCs. G-MDSCs, granulocyte like myeloid-derived suppressor cells; FSC-H, forward scatter height; HLA-DR, human leukocyte antigen-D-related.

MDSC-inducible Tregs. The PBMCs derived from patients with AMI and HCs were classified as phenotypic types of G-MDSCs ($CD15^+CD33^+CD11b^+CD14^-HLA\text{-}DR^{-/low}$) and M-MDSCs ($CD14^+CD15^-CD11b^+CD33^+HLA\text{-}DR^{-/low}$) using flow cytometry. Well-characterized G-MDSCs and M-MDSCs were defined as to be purified >90%. The naive $CD4^+$ T-cells derived from the HCs were characterized as $CD45RA^+CD4^+$ using a commercial kit (Miltenyi Biotec GmbH) by DxFLEX flow cytometry (Beckman Coulter).

Co-culture system of MDSCs and naive $CD4^+$ T-cells. The naive $CD4^+$ T-cells were co-cultured with M-MDSCs and G-MDSCs, respectively, and they were isolated from patients with AMI and HCs, with soluble anti-CD3 (1 μg/ml; cat. no. 16-0037-81; eBioscience) and anti-CD28 antibodies (1 μg/ml; cat. no. 16-0289-81; eBioscience) (incubation conditions: at 37˚C in a CO_2 incubator for 4 h) that were added to achieve T-cell receptor (TCR) stimulation via the TCR/CD3 complex. The co-culture system used a was Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum under the humid condition plus 5% CO_2 at 37˚C for 5 days. The Tregs ($CD4^+FoxP3^+$) were characterized using DxFLEX flow cytometry (Beckman Coulter).

Statistical analysis. GraphPad Prism 8.0 software (GraphPad Software, Inc.) was utilized to perform statistical analysis. Continuous variables are expressed as the mean ± standard deviation and compared using a t-test or one-way analysis of variance followed by Tukey's post hoc test where appropriate. $P<0.05$ was considered statistically significant.

Results

Recruitment of G-MDSCs in the peripheral blood following AMI. The gating strategy for G-MDSCs is illustrated in Fig. 1. Firstly, leukocytes in PBMCs were gated based on forward scatter height (FSC-H) and side scatter height (SSC-H) (Fig. 1A). FSC-H and CD45 were used to gate $CD45^+$ cells (Fig. 1B), in which $CD11b^+CD33^+$ cells (Q2; Fig. 1C) and $CD15^+HLA\text{-}DR^{low}$ cells were gated (Q5; Fig. 1D). Finally, $CD15^+CD14^-$ cells were analyzed and sorted as G-MDSCs (Q1 in Fig. 1E). The percentages of G-MDSCs in patients with AMI and the HCs were compared using flow cytometry (Fig. 2). It was found that the mean fluorescence intensity (MFI) of the G-MDSCs was higher in the peripheral blood of patients with AMI than in the HCs ($P<0.05$, Table I). MDSCs exert their suppressive effects via the expression or secretion of copious amounts of immunosuppressive mediators, including PD-L1. PD-L1 and PD-L2 both are ligands of PD-1 and inhibit T-cell activation (19,20). To explore the interaction among MDSCs, PD-L1 and PD-L2 in AMI, flow cytometry of G-MDSCs was performed using anti-PD-L1 and anti-PD-L2 antibodies in the peripheral blood of patients with AMI and the HCs. Flow cytometry of the G-MDSCs using anti-PD-L1 and anti-PD-L2 revealed that the patients with AMI had higher proportions of $PD\text{-}L1^+$ G-MDSCs and $PD\text{-}L2^+$ G-MDSCs than the HCs

Table I. The mean fluorescence intensity of G-MDSCs and M-MDSCs in the peripheral blood of AMI patients and healthy controls.

Cell type	HC (n=37)	Patients with AMI (n=34)	P-value (HC vs. patients with AMI)
G-MDSCs (%)	16.47±13.69	33.90±18.72[a]	<0.001
PD-L1$^+$ G-MDSCs	7,485.62±1,618.75	9,007.53±2,026.47	<0.001
PD-L2$^+$ G-MDSCs	6,110.62±1,490.44	11,408.65±5,272.43[b]	<0.001
M-MDSCs (%)	8.81±2.89	18.08±7.63	<0.001
PD-L1$^+$ M-MDSCs	27,379.68±12,855.26	29,696.24±10,572.92[c]	0.45
PD-L2$^+$ M-MDSCs	10,661.59±3,801.90	13,063.97±3,726.12	0.011

[a]P<0.0001, vs. M-MDSCs in patients with AMI; [b]P<0.019, vs. PD-L1$^+$ G-MDSCs in patients with AMI; [c]P<0.0001, vs. PD-L2$^+$ M-MDSCs in patients with AMI. Statistical comparisons were performed using the ANOVA with Tukey's post hoc test. G-MDSCs, granulocyte like myeloid-derived suppressor cells; M-MDSCs, monocyte like myeloid-derived suppressor cells; AMI, acute myocardial infarction; HC, healthy controls; PD-L, programmed death-ligand.

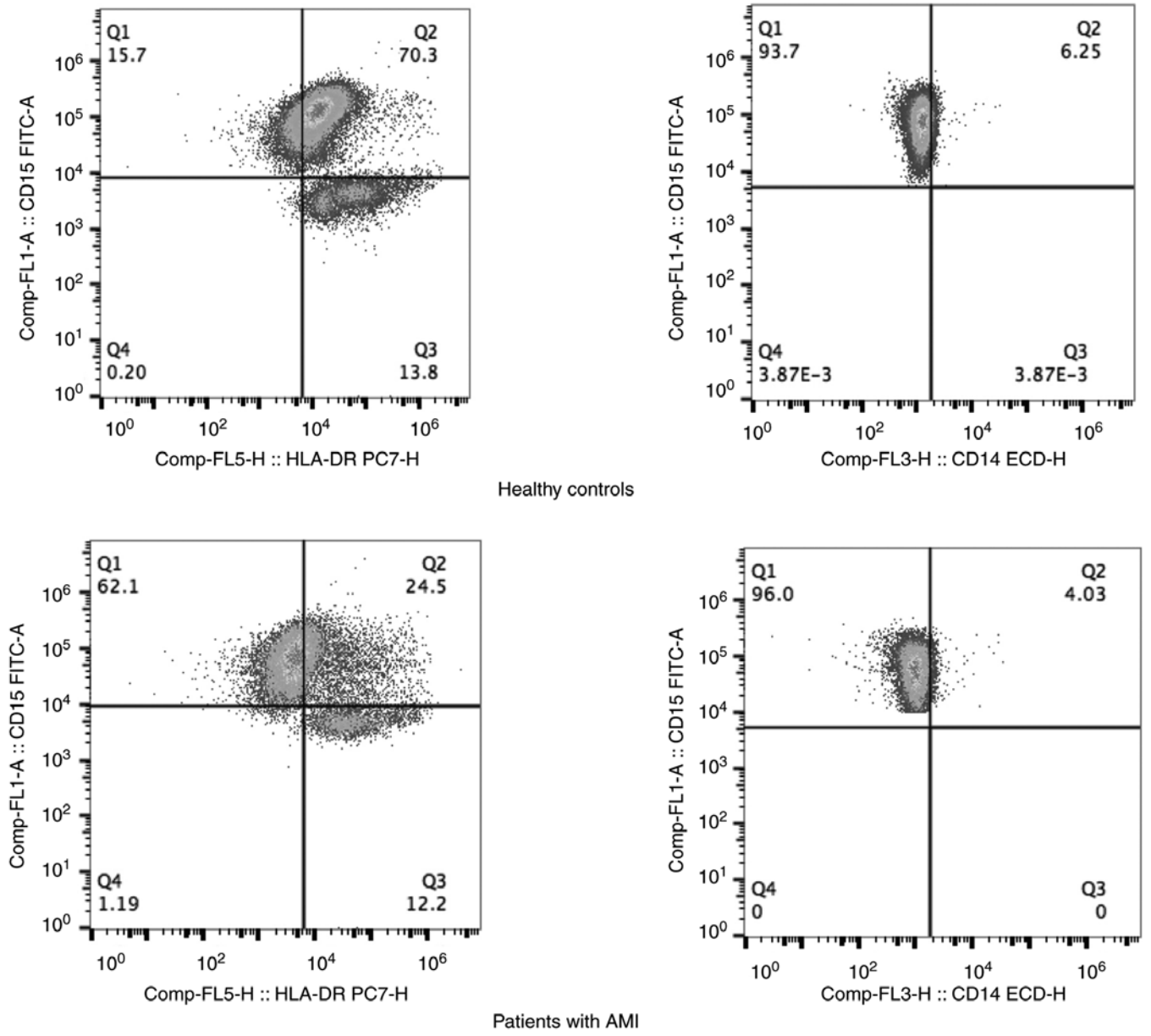

Figure 2. The proportions of granulocyte like myeloid-derived suppressor cells in the peripheral blood of patients with AMI and healthy controls were analyzed using flow cytometry. AMI, acute myocardial infarction.

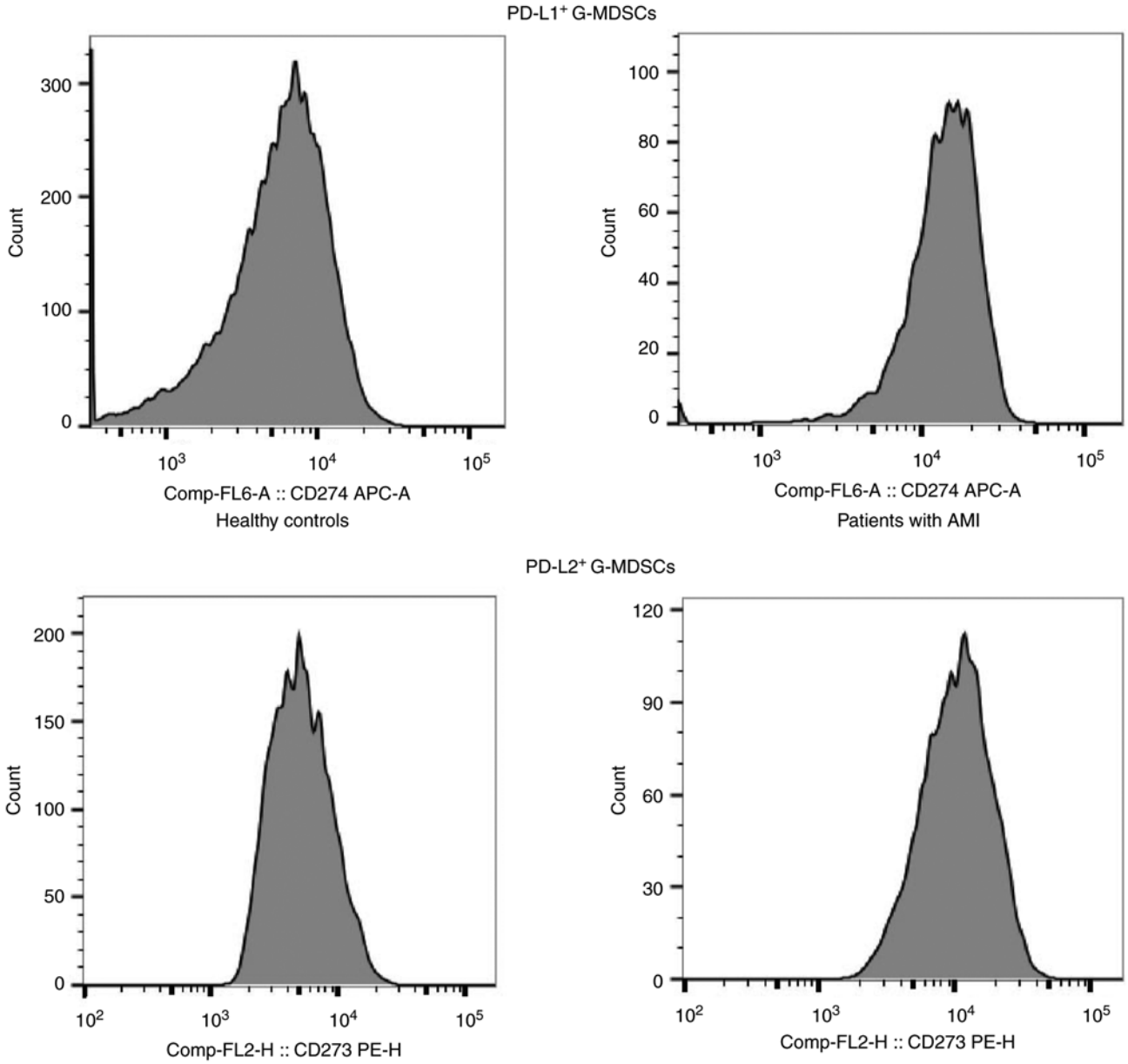

Figure 3. PD-L1+ and PD-L2+G-MDSCs in the peripheral blood of patients with acute myocardial infarction and healthy controls were analyzed using flow cytometry. PD-L, programmed death-ligand 1; G-MDSCs, granulocyte like myeloid-derived suppressor cells.

(P<0.05) (Fig. 3 and Table I). These data suggested the accumulation of G-MDSCs following AMI, as well as that these express PD-L1 and PD-L2.

Recruitment of M-MDSCs in the peripheral blood following AMI. The gating strategy for the M-MDSCs is illustrated in Fig. 4. The forward scatter area (FSC-A) and CD45 were used to gate $CD45^+$ cells (Fig. 4A). Neutrophil granulocytes were excluded, and lymphocytes and monocytes were sorted based on FSC-A and SSC-A (Fig. 4B). Finally, $CD14^+HLA\text{-}DR^{low}$ cells were analyzed in lymphocytes and monocytes (Fig. 4C). Further analysis revealed that the $CD14^+HLA\text{-}DR^{low}$ cells were $CD11b^+CD15^-$ cells, which were also known as M-MDSCs (Fig. 4D). The percentages of M-MDSCs in the patients with AMI and HCs were sorted using flow cytometry (Fig. 5). The results revealed that the patients with AMI exhibited a higher MFI of M-MDSCs in the peripheral blood than the HCs (P<0.05; Table II). Flow cytometry of the M-MDSCs using anti-PD-L1 and anti-PD-L2 antibodies indicated that the patients with AMI had higher proportions of PD-L1+ M-MDSCs and PD-L2+ M-MDSCs than the HCs (P<0.05) (Fig. 6 and Table II). These results demonstrated that the M-MDSCs could be aggregated following AMI, and that they expressed PD-L1 and PD-L2.

MDSCs acquire a granulocytic phenotype following AMI. The proportions of G-MDSCs and M-MDSCs in the peripheral blood of patients with AMI were compared, and higher proportions of G-MDSCs were found in the peripheral blood of patients with AMI than those of M-MDSCs. It was revealed that more PD-L2+ G-MDSCs were detected than PD-L1+ G-MDSCs in the peripheral blood of patients with AMI, and more PD-L1+ M-MDSCs were identified than the PD-L2+

Table II. Ratios of Tregs to $CD4^+$ T cells, PD-1^+CD4^+ T-cells, CTLA-4^+CD4^+ T-cells, PD-1^+CD8^+ T-cells, CTLA-4^+CD8^+ T cells, CTLA-4^+ Tregs and PD-1^+ Tregs in the peripheral blood of patients with AMI and healthy controls.

Cell type	Healthy controls (n=37)	AMI patients (n=34)	t	P-value
Tregs/$CD4^+$ T	6.90±2.36	8.52±1.75	3.26	0.002
PD-1^+CD4^+ T	23.62±10.49	24.69±7.08	0.50	0.619
CTLA-4^+ $CD4^+$ T	17.08±6.78	18.56±5.59	1.00	0.322
PD-1^+CD8^+ T	27.22±10.58	29.45±8.26	0.98	0.329
CTLA-4^+ $CD8^+$ T	20.76±7.42	22.05±5.83	0.81	0.421
CTLA-4^+ Tregs	13.78±5.00	15.99±5.17	1.83	0.072
PD-1^+ Tregs	23.73±7.81	28.03±6.03	2.61	0.011

Statistical comparisons were performed using the t-test. PD-1, programmed cell death 1; Tregs, regulatory T-cells; AMI, acute myocardial infarction.

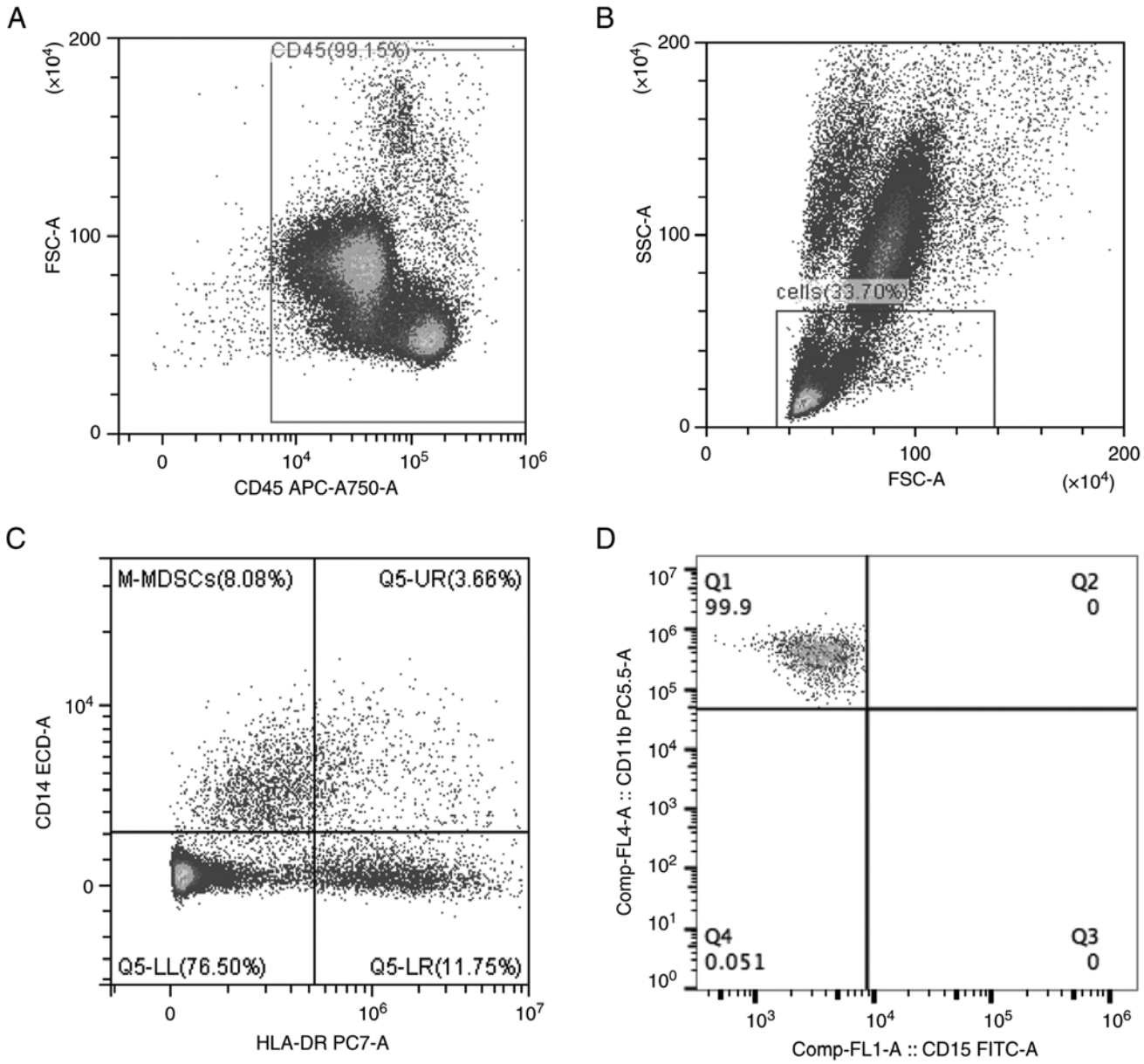

Figure 4. The gating strategy for M-MDSCs in the peripheral blood was examined using flow cytometry. (A) FSC-A and CD45 were used to gate $CD45^+$ cells. (B) Neutrophil granulocytes were excluded and lymphocytes and monocytes were analyzed based on FSC-A and side scatter area. (C) $CD14^+HLA\text{-}DR^{low}$ cells were analyzed in lymphocytes and monocytes. (D) M-MDSCs were analyzed. M-MDSCs, monocyte like myeloid-derived suppressor cells; FSC-A, forward scatter area; HLA-DR, human leukocyte antigen-D-related.

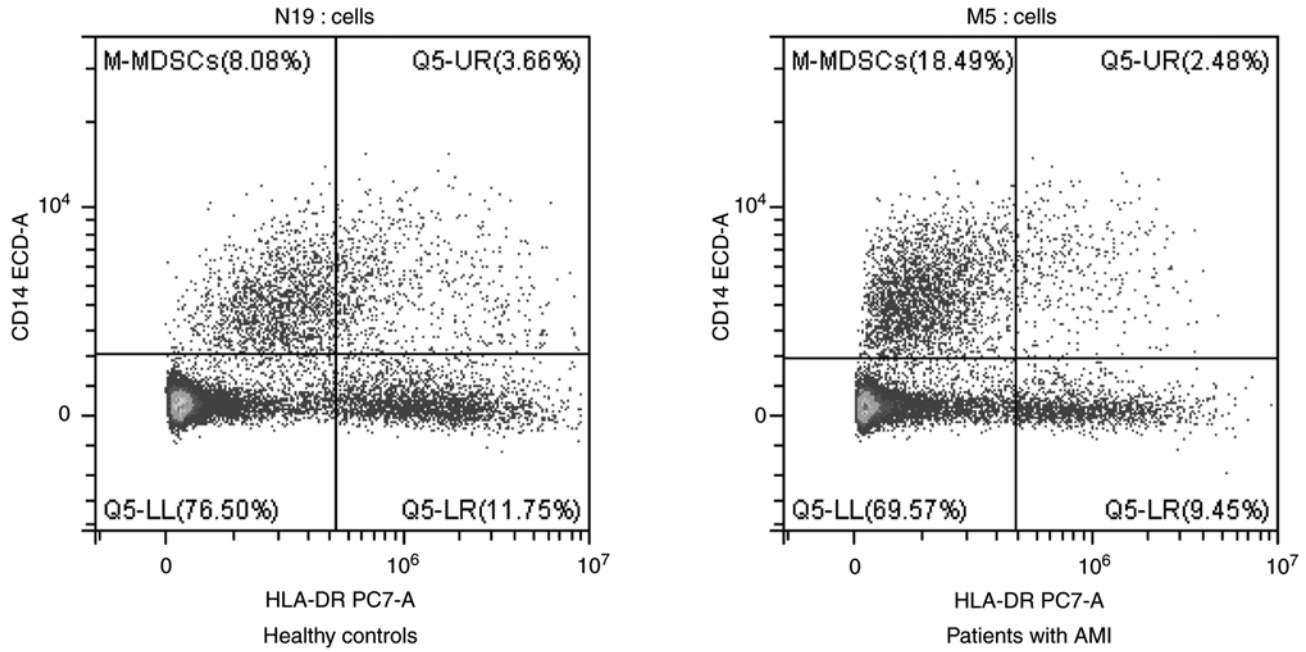

Figure 5. The proportions of monocyte like myeloid-derived suppressor cells in the peripheral blood of patients with AMI and healthy controls were analyzed using flow cytometry. AMI, acute myocardial infarction.

M-MDSCs in the peripheral blood of patients with AMI. The aforementioned findings indicated that the MDSCs acquire a granulocytic phenotype following AMI, and the G-MDSCs and M-MDSCs are more likely to express PD-L2 and PD-L1, respectively.

Treg accumulation in peripheral blood following AMI. The gating strategy for Tregs is displayed in Fig. 7. Initially, lymphocytes in PBMCs were gated based on FSC-A and SSC-A (Fig. 7A). Cells were gated using CD3 and CD4 antibodies, and $CD4^+$ and $CD8^+$ T-cells were sorted (Fig. 7B). Tregs were finally analyzed in $CD4^+$ T cells, namely $CD3^+CD4^+CD25^{high}CD127^{low}$ T-cells (Fig. 7C). The ratios of Tregs to $CD4^+$ T-cells, $PD\text{-}1^+CD4^+$ T-cells, $CTLA\text{-}4^+$ $CD4^+$ T-cells, $PD\text{-}1^+CD8^+$ T-cells, $CTLA\text{-}4^+CD8^+$ T-cells, $CTLA\text{-}4^+$ Tregs and $PD\text{-}1^+$ Tregs (Figs. 8 and 9) in the peripheral blood of patients with AMI and HCs were analyzed using flow cytometry. As presented in Table II, the ratios of Tregs to $CD4^+$ T-cells and $PD\text{-}1^+$ Tregs in the peripheral blood of patients with AMI were higher than those in the HCs ($P<0.05$). However, no significant differences were found in the numbers of $PD\text{-}1^+CD4^+$ T-cells, $CTLA\text{-}4^+CD4^+$ T-cells, $PD\text{-}1^+CD8^+$ T-cells, $CTLA\text{-}4^+$ $CD8^+$ T-cells and $CTLA\text{-}4^+$ Tregs in the peripheral blood between patients with AMI and the HCs ($P>0.05$). These results revealed the expansion of Tregs following AMI and a high PD-1 expression level on the surface of Tregs.

G-MDSCs derived from patients with AMI induce the production of Tregs. The results of flow cytometry demonstrated an increase in the numbers of inducible Tregs in the co-culture system with M-MDSCs derived from patients with AMI compared with those from the HCs (Fig. 10). However, no significant effect of the M-MDSCs from patients with AMI on the differentiation to Tregs was found.

Discussion

To date, rapid progress has been made in the understanding of MDSCs, a particular type of suppressor cells, in tumor progression and antitumor response. Previous findings have demonstrated that in addition to tumor cells, $PD\text{-}L1^+$ MDSCs may be another major source of PD-L1, inhibiting tumor-infiltrating cytotoxic T-lymphocyte activation and function in the tumor microenvironment (21). However, the role of MDSCs in AMI has not yet been fully clarified. In the present study, it was found that the proportions of G-MDSCs and M-MDSCs in patients with AMI were significantly elevated vs. those in HCs. These two subtypes of MDSCs, expressing significantly high expression levels of PD-L1 and PD-L2, could induce the expansion of Tregs by binding PD-1 on the surface of Treg, playing a pivotal role in AMI.

The aggregation and activation of MDSCs are regulated by a variety of cytokines, such as granulocyte macrophage colony stimulating factor (22), vascular endothelial growth factor, IL-1β (23), IL-6 (24), prostaglandin E2 (25) and S100A8/A9 (26). Under the action of a series of cytokines, MDSCs are recruited, migrated and expanded ~10-fold in the peripheral blood and tumor. Zhou *et al* (27) assessed the cardioprotective role of MDSCs in heart failure, and found a cardioprotective role of MDSCs in heart failure by their anti-hypertrophic effects on cardiomyocytes and anti-inflammatory effects through IL-10 and NO. The pharmacological targeting of MDSCs by rapamycin constituted a promising therapeutic strategy for heart failure (27). According to the results of the present study, the proportions of G-MDSCs and M-MDSCs were higher in the peripheral blood of patients with AMI than in the HCs. The patients with AMI had higher numbers of PD-L1- and PD-L2-positive G-MDSCs and M-MDSCs than the HCs. The findings suggested that the aggregation and

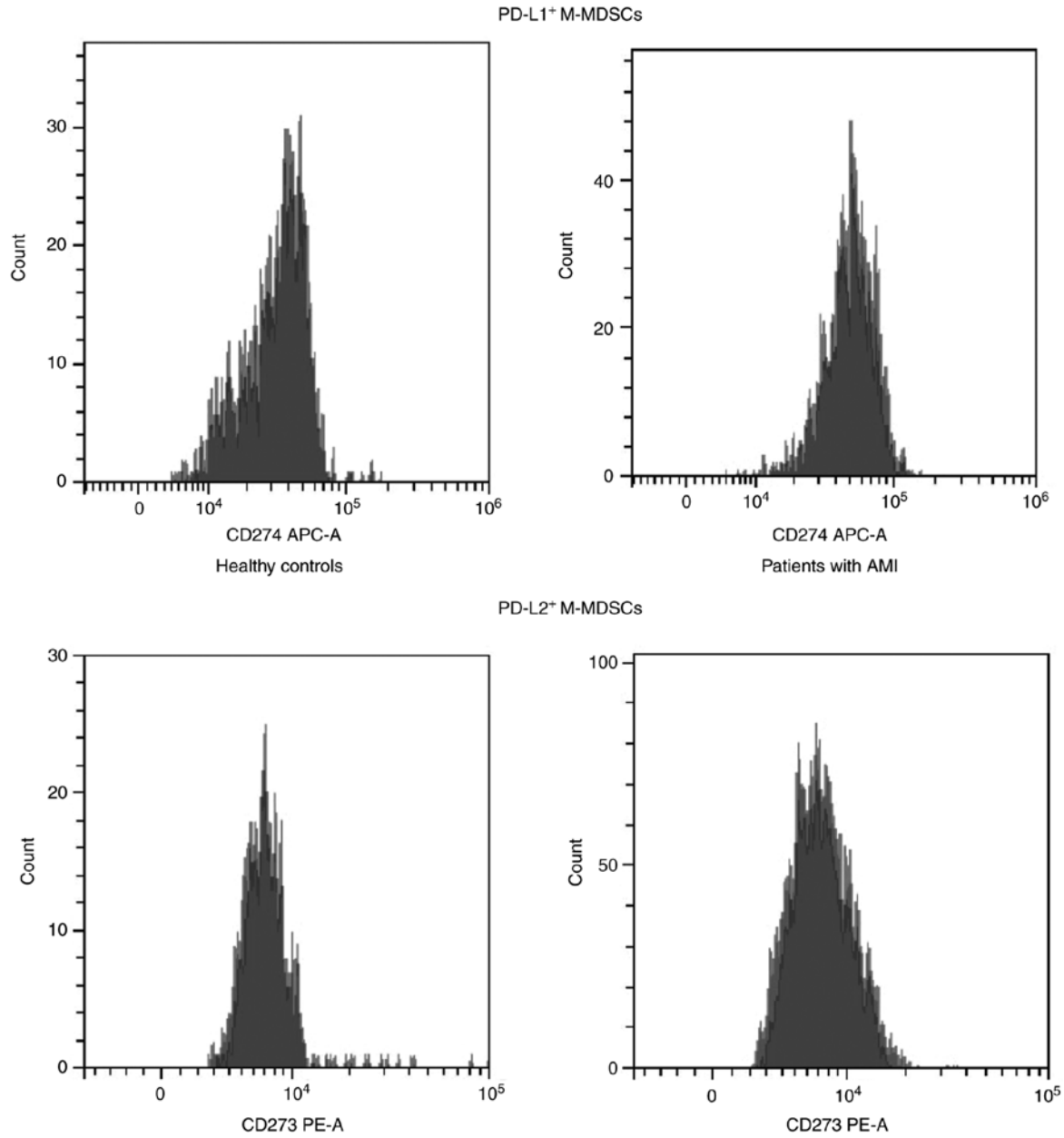

Figure 6. PD-L1$^+$ and PD-L2$^+$ M-MDSCs in the peripheral blood of patients with AMI and healthy controls were analyzed using flow cytometry. PD-L, programmed death-ligand; M-MDSCs, monocyte like myeloid-derived suppressor cells; AMI, acute myocardial infarction.

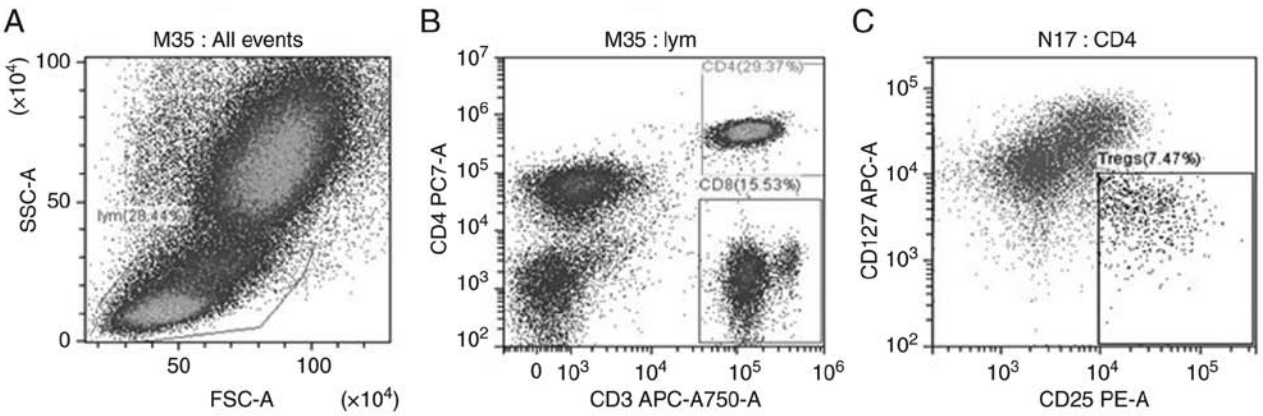

Figure 7. The gating strategy for Tregs in the peripheral blood was examined using flow cytometry. (A) Lymphocytes in peripheral blood mononuclear cells were gated based on forward scatter area and side scatter area. (B) Cells were gated using CD3 and CD4, CD4$^+$ and CD8$^+$ T-cells were analyzed. (C) $CD3^+CD4^+CD25^{high}CD127^{low}$ T-cells were Tregs.

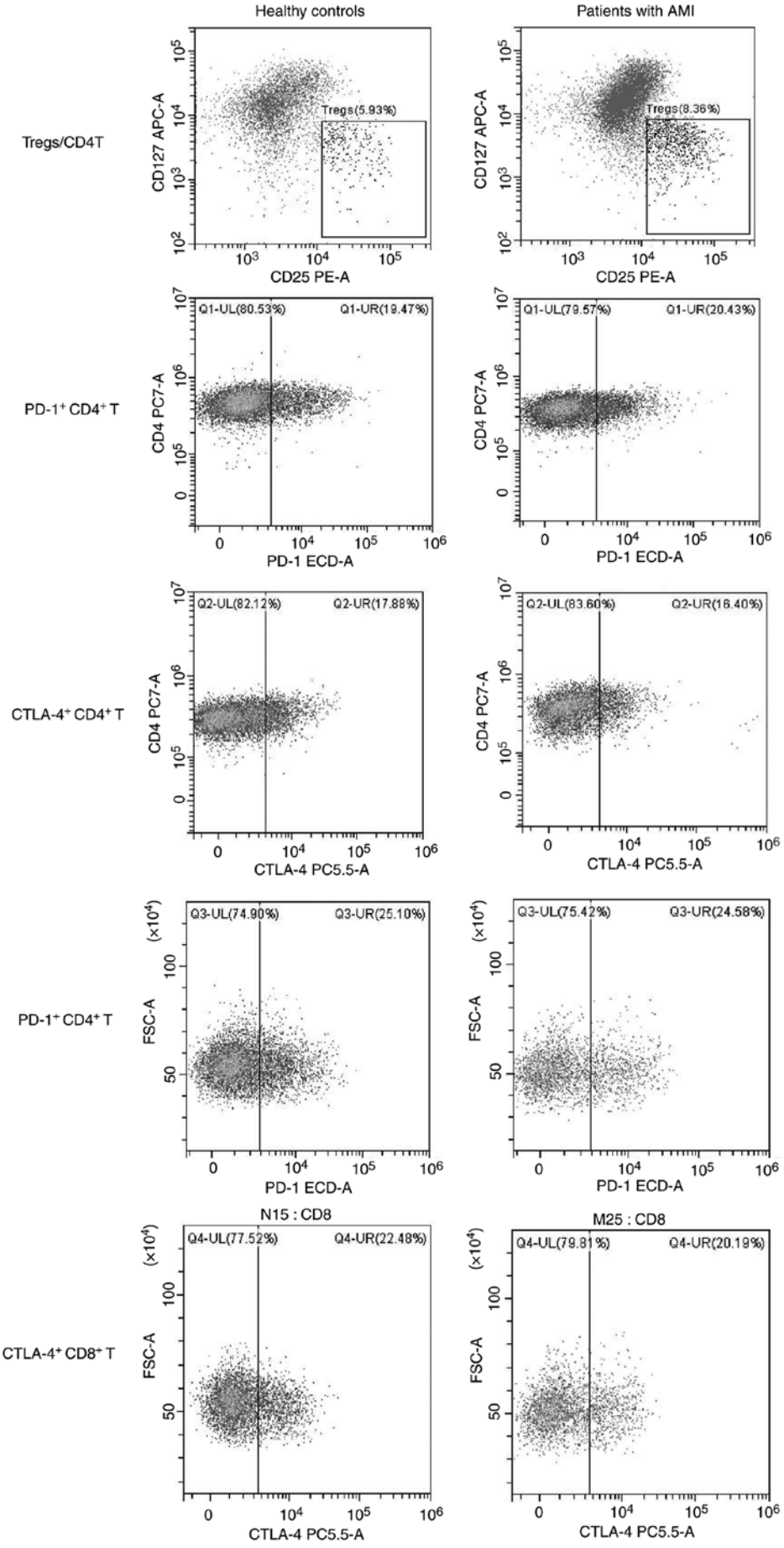

Figure 8. Tregs in $CD4^+$ T-cells, PD-1^+CD4^+ T-cells, CTLA-4^+ $CD4^+$ T-cells, PD-1^+CD8^+ T-cells and CTLA-4^+CD8^+ T-cells were analyzed using flow cytometry in the peripheral blood of patients with AMI and healthy controls. PD-1, programmed cell death 1; AMI, acute myocardial infarction.

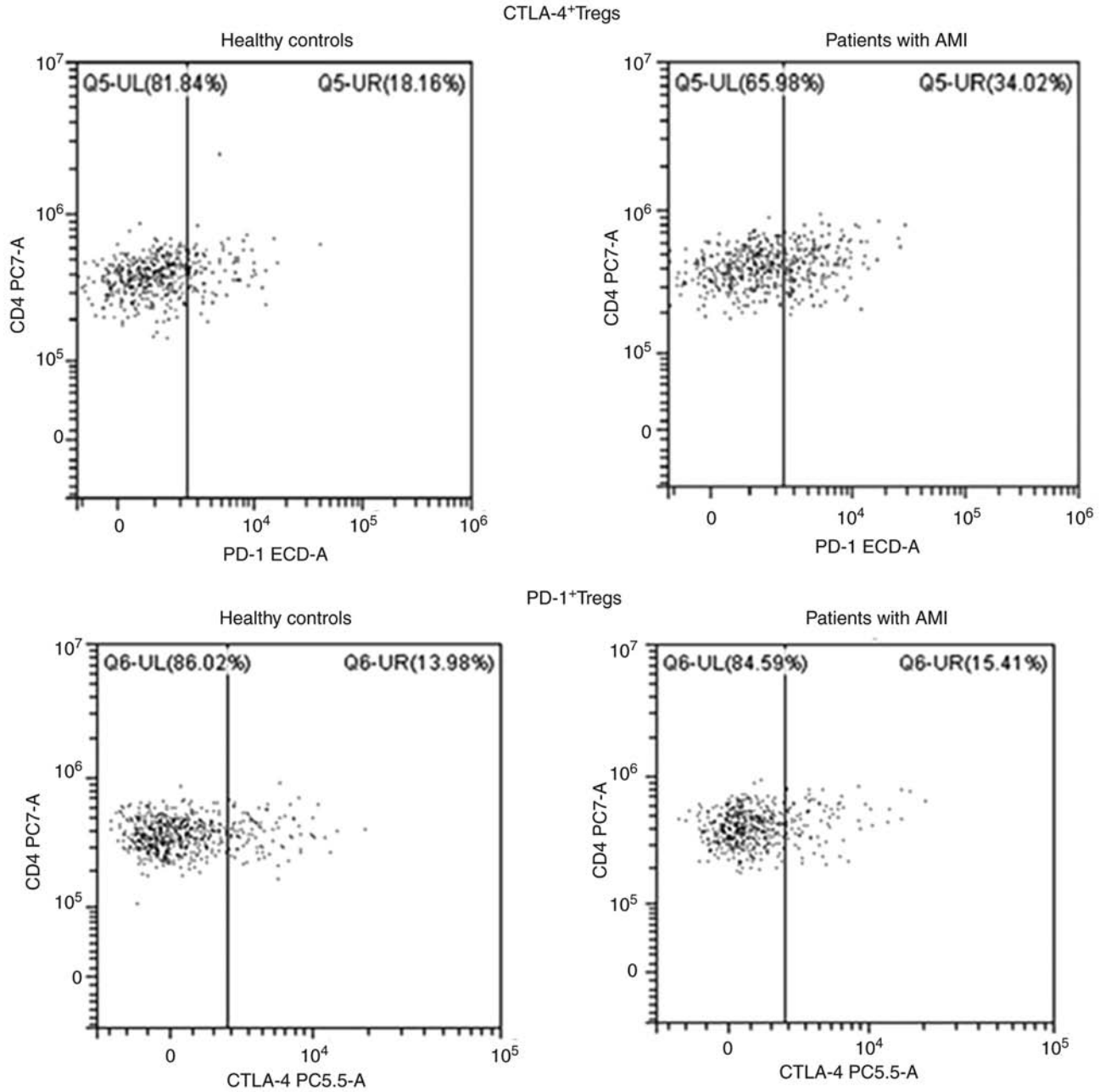

Figure 9. CTLA-4$^+$ Tregs and PD-1$^+$ Tregs were analyzed using flow cytometry in the peripheral blood of patients with AMI and healthy controls. PD-1, programmed cell death 1; AMI, acute myocardial infarction.

recruitment of MDSCs following AMI may be associated with the upregulation of PD-L1 and PD-L2 on the surface of MDSCs. In tumors, hypoxia-induced factor-1α-mediated hypoxia could upregulate the PD-L1 expression level on the surface of MDSCs, resulting in the promotion of the immunosuppressive functions of MDSCs (28). The PD-L1 expression level in MDSCs is also regulated by interferon-γ (IFN-γ). IFN-γ activates p-STAT1 to directly regulate interferon regulatory factor-1 (IRF1) transcription, and IRF1 directly binds to an IRF-binding consensus element to upregulate PD-L1 expression level in MDSCs (14). A previous study reported that the MDSC transfer facilitated immune tolerance and prevented type 1 diabetes mellitus (29). It is possible that MDSCs not only promote the resolution of inflammation, but also strengthen heart functions by unknown mechanisms in the context of AMI. Sun *et al* (30) explored the effects of G-MDSCs on the aging heart, and they found the mechanism by which G-MDSCs promote cardiac fibrosis via the secretion of S100A8/A9 and the regulation of FGF2-SOX9 signaling in fibroblasts during aging. A number of scholars have concentrated on cells of the immune system in cardiac remodeling, including main players in resolution of inflammation and repair after myocardial infarction (31). MDSCs also exhibit anti-hypertrophic and anti-inflammatory effects when co-cultured with cardiomyocytes, through the secretion of IL-10 and NO. The numbers of MDSCs are elevated in patients with AMI, and their depletion aggravates heart function (27). The fact that MDSCs from either patients with AMI or mice with AMI suppress *ex vivo* T-cell proliferation and

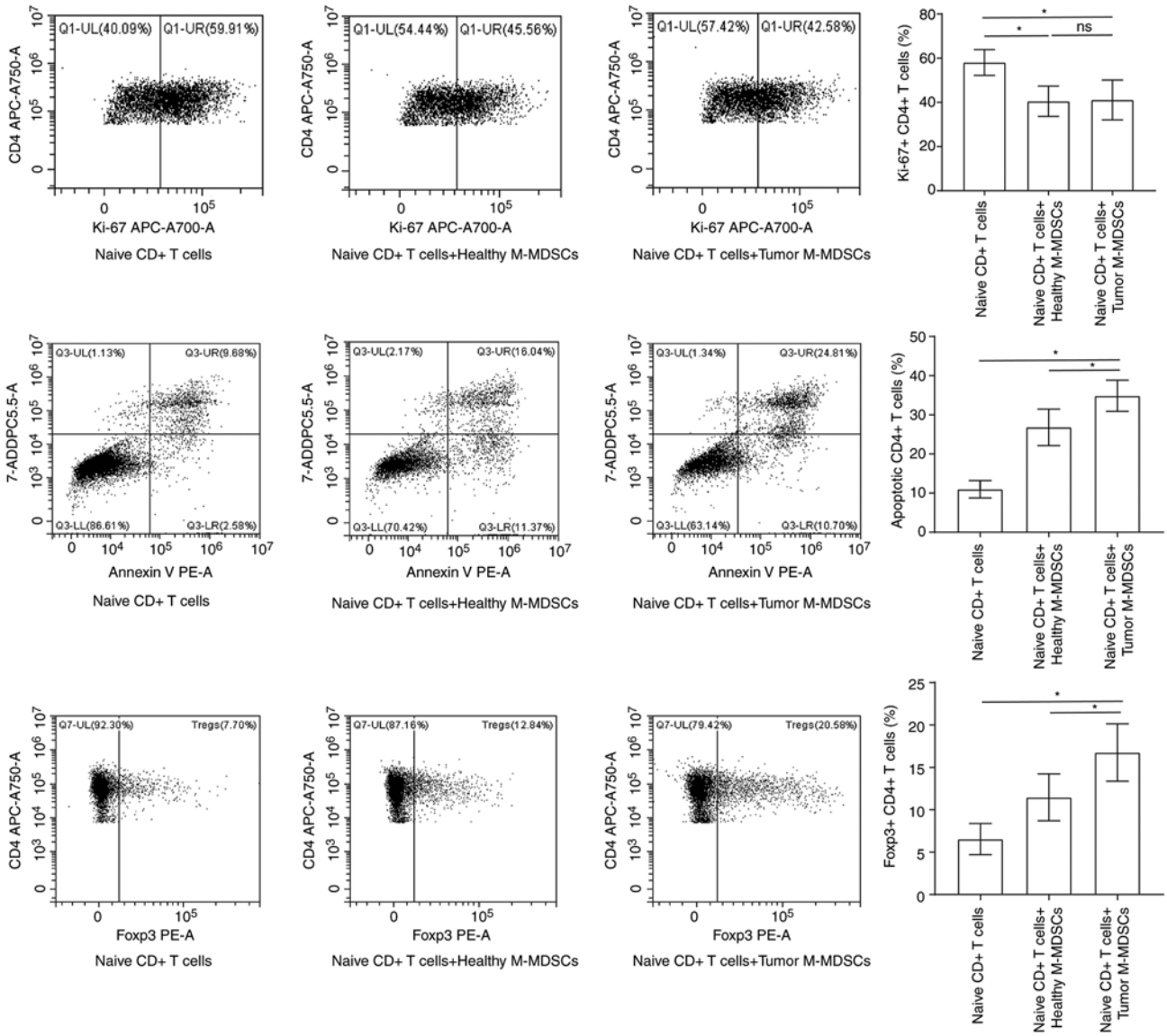

Figure 10. Treg differentiation in the *in vitro* co-culture system of M-MDSCs derived from patients with AMI with naive T-cells and the proportions of Tregs was examined using flow cytometry. *P<0.01.

IFN-γ production demonstrates their suppressive activities. Previous studies have indicated that circulating levels of inflammatory factors exhibit prognostic importance in the setting of AMI (32-34). Pro-inflammatory cytokines, such as IL-6, can recruit and activate MDSCs (35); therefore, it is unsurprising that the MDSC proportion is positively associated with levels of these pro-inflammatory cytokines in patients with AMI. More specifically, the present study indicated that MDSCs acquired a granulocytic phenotype following AMI, and G-MDSCs and M-MDSCs would be more likely to express PD-L2 and PD-L1, respectively.

Another finding of the present study was that Tregs were accumulated in the peripheral blood of patients following AMI, which was also supported by the co-culture system of MDSCs derived from patients with AMI with naive $CD4^+$ T-cells, which was consistent with previously reported findings. For instance, Xia *et al* (36) found that cardiac Tregs, which are mainly thymus-derived Tregs, were recruited from circulation that reached the peak at 7 days following AMI and lasted for 28 days. Ke *et al* (37) demonstrated that pre-treatment with rosuvastatin promoted Treg accumulation in the myocardium during myocardial ischemia-reperfusion injury, suggesting that the Treg-negative modulation of the inflammatory response could serve as therapeutic target in cardiovascular diseases. Tregs, which were traditionally considered as potent suppressors of immune response, have increasingly attracted the attention of researchers as they reside in parenchymal tissues and maintain local homeostasis. Multiple studies have demonstrated cardioprotection conferred by Tregs after MI by alleviating local inflammation, protecting cardiomyocytes against apoptosis, as well as regulating macrophage differentiation and myofibroblast activation (38-40). Hence, the clarification of the characteristic of Tregs in the context of MI is of utmost importance. Specifically, whether Tregs have local adaption with unique phenotype, how the populations accumulate after MI and what factors drive their accumulation remain to be elucidated. Park *et al* (18) demonstrated that PD-1 was upregulated on Tregs during chronic virus

infection and promoted the suppression of the $CD8^+$ T-cell immune response via the interaction with PD-L1 expressed on $CD8^+$ T-cells, indicating that PD-1 may be a mediator of the immunosuppressive function of Tregs. Turnquist *et al* (41) demonstrated that IL-33 expanded functional MDSCs, in which CD11b(+) cells exhibited intermediate levels of Gr-1 and Tregs, including $ST2L^+Foxp3^+$ cells, and they mediated the Treg-dependent promotion of cardiac allograft survival, leading to an interaction between Tregs and MDSCs. Previous studies have indicated that the accumulation of MDSCs, in addition to their ability to inhibit T-cell proliferation, can trigger the development of Tregs (42,43). In human immunodeficiency virus disease, the expansion of MDSCs has been found to promote the differentiation of Tregs and inhibit T-cell function, which is a hallmark of several chronic infectious diseases (44). A previous study indirectly indicated that Tregs contribute to rosuvastatin-induced cardioprotection against AMI (34). In the present study, the comparative analysis revealed that patients with AMI had higher numbers of PD-L1- and PD-L2-positive G-MDSCs and M-MDSCs than the HCs, and the numbers of $PD\text{-}1^+$ Tregs in the peripheral blood of patients AMI were higher than those in the HCs. The upregulation of PD-L1 and PD-L2 in MDSCs could bind with PD-1 on the surface of Tregs and then promote the differentiation of initial $CD4^+$ T-cells into Tregs following AMI. The interaction between PD-1 and PD-L1 can phosphorylate two structural motifs, an immunoreceptor tyrosine-based inhibitory motif and an immunoreceptor tyrosine-based switch motif, in the cytoplasmic region of PD-1, recruit src-homology 2 domain-containing tyrosine phosphatase-2, inhibit signaling downstream of T-cell receptor by dephosphorylation, and block T-cell activation (45,46). Similarly, Jaworska *et al* (47) found that Tregs protected the kidneys from ischemia reperfusion-induced inflammation and injury, and PD-1 on the surface of Tregs, prior to adoptive transfer, attenuated their renoprotective effects against ischemic injury. More specifically, they confirmed that the administration of PD-L1 or PD-L2 blocking antibodies markedly exacerbated the loss of renal function, renal inflammation and acute tubular necrosis during acute kidney injury, which revealed that the renoprotective role of Tregs was achieved by its immunomodulator PD-1 binding to the known ligands, PD-L1 and PD-L2 (47).

The present study has some limitations. Firstly, the sample size was not sufficient, hindering the generalization of the findings. Secondly, the absence of dynamic changes in MDSC subsets and the number of Tregs following AMI, particularly at 12, 24 and 48 h following the onset of symptoms of AMI, needs to be determined. Finally, no cellular and animal models were used to demonstrate the role of MDSCs in the promotion of the expansion of Tregs via the PD-1/PD-L1/PD-L2 interaction. Thus, further large-scale multi-center studies are required to eliminate the aboremention limitations and to verify the findings of the present study.

In conclusion, the present study demonstrated that both subtypes of MDSCs, G-MDSCs and M-MDSCs, were accumulated following AMI and expressed high levels of PD-L1 and PD-L2. MDSCs promoted the expansion of Tregs by binding PD-1 on the surface of Tregs, playing a pivotal role in AMI. Although immunosuppression caused by the involvement of MDSCs and Tregs could benefit tumor immune escape and penalize immunotherapy for diverse types of cancer, on the basis of inflammatory responses compromising microcirculation during reperfusion, it is essential to determine whether the accumulation of MDSCs and Tregs in the myocardium from circulation can confer cardioprotective effects against AMI; thus, further studies are warranted. The exploration of a potent signaling mechanism is also suggested to manipulate cellular heterogeneity in the immune response during AMI. Molecular AMI-related studies will be advantageous to obtain a time-dependent equilibrium in immune regulation through metabolic and epigenetic rearrangement in the future. Further research is also required to determine whether the accumulation of MDSCs and Tregs in the myocardium from the circulation can confer cardioprotective effects against AMI, particularly in the lymph nodes, bone marrow and tumor sites, as well as in the survival of cardiac allografts.

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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

MZ was involved in the design of the study and in the drafting of the manuscript. XS performed the experiments. JZhao was involved in data analysis. WG was involved in the study design, manuscript revision and grammatical corrections. JZhou conceived and supervised the study. MZ and JZhou confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Beijing Tsinghua Changgung Hospital (Approval no. 18190-0-01). All patients and healthy controls signed informed consent forms.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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