Original Paper

Expansion of Myeloid-Derived Suppressor Cells in Patients with Acute Coronary Syndrome

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Key Words

Acute coronary syndrome • Inflammation • Atherosclerosis • Myeloid-derived suppressor cells

Abstract

Aim: The aim of this study was to explore whether the circulating frequency and function of myeloid-derived suppressor cells (MDSCs) are altered in patients with acute coronary syndrome (ACS). **Methods:** The frequency of MDSCs in peripheral blood was determined by flow cytometry, and mRNA expression in purified MDSCs was analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). The suppressive function of MDSCs isolated from different groups was also determined. The plasma levels of certain cytokines were determined using Bio-Plex Pro™ Human Cytokine Assays. **Results:** The frequency of circulating CD14⁺HLA-DR^{-/low} MDSCs; arginase-1 (Arg-1) expression; and plasma levels of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-33 were markedly increased in ACS patients compared to stable angina (SA) or control patients. Furthermore, MDSCs from ACS patients were more potent suppressors of T-cell proliferation and IFN-γ production than those from the SA or control groups at ratios of 1:4 and 1:2; this effect was partially mediated by Arg-1. In addition, the frequency of MDSCs was positively correlated with plasma levels of IL-6, IL-33, and TNF-α. **Conclusions:** We observed an increased frequency and suppressive function of MDSCs in ACS patients, a result that may provide insights into the mechanisms involved in ACS.

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Introduction

Atherosclerosis is a slowly progressing multifactorial disease in which the immune response may play a pivotal role [1]. Accumulating evidence confirms the involvement of innate and acquired immunity mediators in all stages of atherosclerosis as well as during plaque destabilization, which leads to the onset of acute coronary syndrome (ACS) [1-3]. Pathologically, atherosclerosis is characterized by chronic inflammation of the arterial wall, which activates the immune response and the release of leukocytes (e.g., monocytes, neutrophils, and lymphocytes) into predisposed regions of the intima. This initial response also promotes lipid deposition, resulting in early atherosclerotic plaques and lesion formation. Immune activation is considered the most significant feature in determining atherosclerotic plaque vulnerability. Specifically, the presence of activated inflammatory cells in plaque may increase vulnerability to and induce the occurrence of acute cardiovascular events, including ACS, through the release of matrix metalloproteinase, cytokines, and other mediators of inflammation [4-9]. Although substantial advances in this field have enhanced our understanding of atherosclerosis pathogenesis, the underlying molecular mechanisms have yet to be fully elucidated.

Myeloid-derived suppressor cells (MDSCs), which were first described in cancer patients in the late 1970s [10], are a phenotypically diverse population of bone marrow-derived myeloid progenitor cells and immature myeloid cells with defects in differentiation [10-13]. Furthermore, MDSCs are the main cell type produced to negatively regulate the immune response [11] and function under a variety of pathological conditions [14-16]. The inhibitory properties of MDSCs are thought to be mediated by the depletion of L-arginine by arginase-1 (Arg-1) and inducible nitric oxidase (iNOS) [16]; cysteine sequestration [17]; reactive oxygen species (ROS) production [18]; interleukin-10 (IL-10) [19, 20]; and transforming growth factor-beta (TGF- β 1) [21] release and regulatory T-cell (Treg) induction [21]. Mouse MDSCs are positive for granulocyte (Gr1) and macrophage Mac-1 (CD11b) markers (otherwise known as Gr1⁺CD11b⁺ cells) [22, 23], but no uniform surface markers have been identified for human MDSCs. The two main subpopulations of human MDSCs are monocytic MDSCs (CD14⁺HLA-DR^{-/low}) and granulocytic MDSCs (Lin⁻CD15⁺CD11b⁺CD33⁺) [23-26]. CD14⁺HLA-DR^{-/low} cells in the peripheral blood of patients with hepatocellular carcinoma [21], melanoma [27], ovarian carcinoma [28], and inflammatory bowel disease [29] exhibit multiple characteristics of MDSCs.

Although the role of MDSCs in cancer has been intensively explored, additional work also implicates MDSCs in the pathogenesis of chronic inflammatory and autoimmune diseases, including inflammatory bowel disease [29], viral infection-related diseases [13], rheumatoid arthritis [30], and pulmonary hypertension [31]. However, little is known about the role of MDSCs in patients with ACS. In the present study, the population of MDSCs in the peripheral blood of ACS patients was characterized by flow cytometry. Furthermore, the suppressive nature of these MDSCs was also investigated in a co-culture model.

Materials and Methods

Patients

This investigation conforms to the principles of the Declaration of Helsinki. The study was approved by the ethics committee of Tongji Medical College of Huazhong University of Science and Technology, and informed consent was obtained from all subjects prior to this study. In this investigation, 169 (119 men and 50 women, 51.8 $\pm$ 0.7 years of age) patients at Union Hospital, Tongji Medical College of Huazhong University of Science and Technology, were enrolled and assigned to three groups. Patients were placed in the ACS group (n = 73, 51 men and 22 women, 53.8 $\pm$ 1.0 years of age) if they presented chest pain at rest accompanied by ischemic electrocardiographic changes, including ST-segment changes and T-wave inversions, or a significant increase in creatine kinase MB and troponin I levels. Patients were recruited to the stable angina (SA) group (n = 40, 28 men and 12 women, 51.6 $\pm$ 1.6 years of age) if they presented typical

exertional chest discomfort with down-sloping or horizontal ST-segment depression N1 mm in the exercise test. The control group (n = 56, 40 men and 16 women, 49.2 ± 1.4 years of age) was composed of patients who had normal coronary artery arteries on angiography and no history of coronary heart disease. The baseline characteristics of the enrolled participants in all groups are shown in Table 1. Eligible patients were excluded if they had been treated with anti-inflammatory drugs or if they had a medical history of collagen disease, thromboembolism, disseminated intravascular coagulation, advanced liver disease, renal failure, malignant disease, or other inflammatory diseases.

Blood Sample Preparation and Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

While in a fasting state on the morning of the admission day, blood samples were obtained via a 21-gauge needle for spotless antecubital venipuncture and collected into tubes containing 0.2 ml of sodium heparin. The samples were obtained while the subjects were in a recumbent position. The blood samples were centrifuged at $300 \times g$ for 7 min, and the plasma was then stored at -20°C . PBMCs in the blood were isolated by Ficoll-Hypaque (Sigma, USA) density gradient centrifugation and washed twice before flow cytometric analysis or magnetic cell sorting.

Flow cytometric analysis

PBMCs were harvested and stained with the following antibodies using the manufacturer's protocols: FITC-conjugated anti-human CD14 (clone: 61D3, eBioscience), PE-conjugated anti-human HLA-DR (clone: L243, eBioscience), FITC-conjugated anti-human Lin (CD3-clone: UCHT1, CD14-clone: HCD14, CD19-clone: HIB19, CD20-clone: 2H7, CD56-clone: HCD56, Biolegend), PE-conjugated anti-human CD11b (clone: M1/70, Biolegend), APC-conjugated anti-human CD33 (clone: WM53, BD Biosciences), and PE-Cy5-conjugated anti-human CD15 (clone: SSEA-1, Biolegend). The corresponding isotype antibodies were also used to enable correct compensation and to confirm antibody specificity. Then, the cells were washed with phosphate-buffered saline (PBS) and detected by flow cytometry with a FACS Calibur (BD Biosciences, USA) cell sorter. The results were analyzed using FlowJo 7.6.1 (Treestar Inc., USA). Lymphocyte and monocyte counts were determined for all patients using an automated hematology analyzer (SYSMEX, XE-5000), and the estimated absolute number of MDSCs was calculated as follows: [total lymphocyte + monocyte count (cells/ μl)] $\times$ percentage CD14 $^{+}$ $\times$ percentage MDSCs/10000 [32-35].

Isolation of MDSCs and T-cells

CD14 $^{+}$ HLA-DR $^{-/\text{low}}$ MDSCs were isolated from PBMCs as previously described [27] using CD14 and HLA-DR microbeads (Milenyi Biotec, Germany). Briefly, HLA-DR $^{+}$ cells were first removed by negative selection using an LD column, followed by further separation of the CD14 $^{+}$ HLA-DR $^{-/\text{low}}$ cells using anti-CD14 microbeads. The purity of the CD14 $^{+}$ HLA-DR $^{-/\text{low}}$ cell population was $>80\%$ as assessed by FACS. These MDSCs were then used for real-time polymerase chain reaction (RT-PCR) or functional assays as described below. In addition, autologous T-cells were isolated using CD3 microbeads (Milenyi Biotec, Germany) according to the manufacturer's instructions.

Proliferation and Cytokine Secretion Assays

Quantitative functional analysis of the MDSCs was performed by co-culturing sorted CD14 $^{+}$ HLA-DR $^{-/\text{low}}$ MDSCs with autologous CD3 $^{+}$ T-cells stimulated with anti-CD3/28 antibodies (5 $\mu\text{g}/\text{ml}$) (eBioscience, USA) at different suppressor-to-responder cell ratios (0:0, 1:8, 1:4, and 1:2). The cells were grown in complete RPMI-1640 medium at 37°C and 5% CO_2 in U-bottom 96-well plates for 72 h. In some experiments, the Arg-1 inhibitor N $^{\omega}$ -hydroxy-nor-L-arginine diacetate salt (nor-NOHA, Calbiochem, 200 $\mu\text{mol}/\text{l}$), the iNOS inhibitor N $^{\text{G}}$ -methyl-L-arginine acetate salt (L-NMMA, Sigma-Aldrich, 200 $\mu\text{mol}/\text{l}$), neutralizing anti-TGF- β antibodies (R&D Biosystems, 10 $\mu\text{g}/\text{ml}$) or anti-IL-10 antibodies (R&D Biosystems, 10 $\mu\text{g}/\text{ml}$) were also added. Approximately 16 h before harvesting the cells, 1 μCi of [^3H]-thymidine (Perkin Elmer; USA) was added to each well, and the incorporation of [^3H]-thymidine [36-38] was detected using a scintillation counter. Moreover, the concentration of interferon-gamma (IFN- γ) in the culture supernatant was detected using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The minimum detectable concentration of IFN- γ (Neobioscience, China) was 8 pg/ml, and all measurements were obtained twice.

Table 1. Clinical data for the ACS, SA, and control patients enrolled in this study. The values are presented as the mean $\pm$ SEM or number (%). ACS: acute coronary syndrome; SA: stable angina; ACEI: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker

Baseline Characteristic	ACS (n = 73)	SA (n = 40)	Control (n = 56)	P-value
Sex (female/male)	22/51	12/28	16/40	0.979
Age (years)	53.8 $\pm$ 1.0	51.6 $\pm$ 1.6	49.2 $\pm$ 1.4	0.130
Risk factors, n (%)				
Hypertension	34 (47)	17 (43)	15 (27)	0.065
Diabetes mellitus	19 (26)	8 (20)	8 (14)	0.262
Hyperlipidemia	27 (37)	13 (33)	7 (13)	0.007
Current smoking	15 (21)	9 (23)	10 (18)	0.849
Obesity	20 (27)	11 (28)	10 (18)	0.393
Medications, n (%)				
Aspirin	35 (48)	18 (45)	11 (20)	0.003
ACEI/ARBs	30 (41)	20 (50)	13 (23)	0.019
Statins	32 (44)	18 (45)	6 (11)	0.000
Beta-blockers	27 (37)	17 (43)	7 (13)	0.002
Calcium blockers	16 (22)	9 (23)	9 (16)	0.651
Nitrates	14 (19)	7 (18)	1 (2)	0.009
Hypoglycemic agent	13 (18)	7 (18)	4 (7)	0.180

RT-PCR

Total RNA was extracted from fresh bead-sorted MDSCs using Trizol reagent (Takara, Japan) according to the manufacturer's instructions. cDNA was produced by reverse transcription using a Reverse Transcriptase kit (Takara, Japan). Gene expression levels were detected using SYBR Green Nucleic Acid Gel Stains (Takara, Japan) using the $-2^{\Delta\Delta CT}$ method, and samples were analyzed with an ABI Prism 7900 Sequence Detection System (Applied Biosystems, USA). The following primer sequences were used: Arg-1 (F: 5'-GGCTGGTCTGCTTGAGAAAC-3', R: 5'-ATTGCCAACTGTGGTC TCC-3'), iNOS (F: 5'-CTTTCCAAGACACACTTCACCA-3', R: 5'-TA TCTCCTTTGTTACCGCTTCC-3'), TGF- β 1 (F: 5'-TGAGGGCTTTTCG CCTTAGC-3', R: 5'-CGGTAGTGAACCGTTGATGT-3'), IL-10 (F: 5'-GGCCAGGGCACCAGTCT-3', R: 5'-TCGAAGCATGTTAGGCA GGT-3') and GAPDH (F: 5'-CCACATCGCTCAGACACCAT-3', R 5'-GGCAACAATATCCACTTTACCAGAGT-3'). Samples were amplified in duplicate for 40 cycles, and gene expression levels were normalized to the levels of the housekeeping gene GAPDH.

Multiplex Cytokine Assays

The plasma levels of IL-6, IL-10, IL-33, IL-1 β , and tumor necrosis factor (TNF)- α were measured using a unique blend of magnetic bead-based multiplex immunoassays (Bio-Plex Pro™ Human Cytokine Assays; Bio-Rad Laboratories; USA), as previously described [39, 40]. Cytokine concentrations were determined using the Bio-Plex 200 System™ (Bio-Rad Ltd.; USA) and are expressed as pg/ml. All samples were measured twice.

Statistical Analysis

The data are presented as the mean $\pm$ standard error of the mean (SEM) or as a percentage in the text and figures. One-way ANOVA tests and Pearson chi-square tests were used to detect significant differences between the three groups. Spearman's correlation analysis was used to determine any correlation between the variables. In all cases, a p-value less than 0.05 was considered statistically significant. SPSS statistics 17.0 software was used for the statistical analyses.

Results

Baseline Patient Characteristics

The clinical data of the enrolled participants in all groups are listed in Table 1. There were no significant differences with respect to age, gender, hypertension, diabetes mellitus status, smoking status, obesity, or the use of calcium blockers or anti-diabetic drugs. However, the incidence rate of hyperlipidemia and the utilization of aspirin, angiotensin-converting enzyme inhibitor (ACEI) or angiotensin receptor blockers (ARBs), statins, β -blockers and nitrates were markedly higher in patients with ACS and SA than in the control group.

Fig. 1. The frequency and counts of circulating $CD14^+HLA-DR^{-/low}$ MDSCs are significantly increased in patients with ACS. (A) $CD14^+$ monocytes were gated by flow cytometry. (B) Representative images of the frequencies of $CD14^+HLA-DR^{-/low}$ MDSCs from a single patient in each group. (C) The percentages of $CD14^+$ monocytes among PBMCs for each group. (D) The percentages of $CD14^+HLA-DR^{-/low}$ MDSCs within the $CD14^+$ monocytes for each group. (E) The estimated absolute number of $CD14^+HLA-DR^{-/low}$ cells in the patients in each group.

$CD14^+HLA-DR^{-/low}$ MDSC Frequency in the Peripheral Blood of ACS Patients

To determine whether MDSCs play a role in ACS pathology, we compared the MDSC frequency in the peripheral blood of patients in the ACS, SA, and control groups. The frequency of $CD14^+$ monocytes among PBMCs was higher in ACS patients ($10.39\% \pm 1.12\%$, $p = 0.007$) than in patients in the SA ($6.96\% \pm 1.24\%$) and control ($6.33\% \pm 0.53\%$) groups (Fig. 1C). Furthermore, the percentage of $CD14^+HLA-DR^{-/low}$ cells was significantly increased in $CD14^+$ monocytes of ACS patients ($23.1\% \pm 2.6\%$, $p < 0.001$) compared to those of patients in the SA ($10.1\% \pm 1.2\%$) and control ($9.4\% \pm 1.0\%$) groups (Fig. 1D). In addition, a comparative analysis using estimated absolute numbers (calculated based on the lymphocyte and monocyte counts in PBMCs and in the case of whole blood) revealed that the absolute number of $CD14^+HLA-DR^{-/low}$ cells was significantly increased in the peripheral blood of ACS patients ($50.08 \text{ cells}/\mu\text{L} \pm 9.32 \text{ cells}/\mu\text{L}$, $p < 0.001$) compared to patients in the SA ($16.92 \text{ cells}/\mu\text{L} \pm 4.78 \text{ cells}/\mu\text{L}$) and control ($10.63 \text{ cells}/\mu\text{L} \pm 1.08 \text{ cells}/\mu\text{L}$) groups (Fig. 1E). However, no difference in the percentages of $Lin^-CD15^+CD11b^+CD33^+$ cells was detected among patients in the ACS, SA and control groups (Fig. 2A and B).

The Suppressive Function of MDSCs from ACS Patients

MDSCs may suppress antigen-specific and antigen-non-specific T-cell responses. In this study, proliferation and cytokine secretion assays were conducted using a co-culture model to determine if $CD14^+HLA-DR^{-/low}$ MDSCs isolated from patients can inhibit T-cell proliferation and cytokine secretion. The regulatory action of the cells from the various groups was also compared. First, flow cytometry was performed to detect the purity of the MDSCs after cell sorting (Fig. 3A and B). As shown in Figures 4A, B and C, the $CD14^+HLA-DR^{-/low}$ cells suppressed T-cell proliferation and IFN- γ production in a dose-dependent manner. Furthermore, two of the suppressor-to-responder cell ratios (1:4 and 1:2) inhibited

Fig. 2. There were no significant differences in the frequency of circulating granulocytic Lin⁺CD15⁺CD11b⁺CD33⁺ MDSCs among the patients from the three groups. (A) Representative dot plots of Lin⁺CD15⁺CD11b⁺CD33⁺ MDSCs. (B) The percentages of Lin⁺CD15⁺CD11b⁺CD33⁺ MDSCs among PBMCs from the patients in the ACS (n=21), SA (n=13) and control (n=17) groups.

Fig. 3. Purity of the CD14⁺HLA-DR^{-/low} MDSCs isolated using magnetic selection. (A) Dot plot showing the purity of the MDSCs before isolation. (B) Dot plot showing the purity of the MDSCs after isolation.

Fig. 4. MDSCs from ACS patients exhibit increased suppressive function. CD14⁺HLA-DR^{-/low} MDSCs and CD3⁺ T-cells from ACS patients (n = 6), SA patients (n = 6) and controls (n = 6) were purified by magnetic cell sorting. (A) [³H]-Thymidine uptake was used to evaluate the effector cell proliferative indexes. (B) MDSC inhibition of the effector cells expressed as a percentage. (C) Increased suppressive function of the MDSCs from ACS patients was suggested by the IFN-γ production level. *p < 0.05 compared to the SA or control groups.

T-cell proliferation and IFN-γ production more effectively for cells from ACS patients than those from the SA and control groups (Fig. 4B and C).

Fig. 5. ARG-1 mRNA expression was increased in MDSCs purified from ACS patients. RT-PCR was used to detect the mRNA expression of Arg-1 (A), iNOS (B), TGF- β 1(C), and IL-10 (D) in purified MDSCs from patients in the ACS (n = 12), SA (n = 10), and control (n = 12) groups.

Fig. 6. The inhibition of autologous T-cell proliferation and IFN- γ production by CD14⁺HLA-DR^{-low} MDSCs is partially Arg-1 dependent. The effects of variable chemical inhibitors and blocking antibodies on the CD14⁺HLA-DR^{-low} MDSC-induced suppression of autologous CD3⁺ T-cell proliferation (A) and IFN- γ production (B). nor-NOHA, N^ω-Hydroxy-nor-L-arginine diacetate salt; L-NMMA, N^G-Methyl-L-arginine acetate salt; anti-TGF- β , neutralizing anti-TGF- β antibodies; anti-IL-10, neutralizing anti-IL-10 antibodies. *p < 0.01 compared to the non-treated groups.

Arg-1 mRNA Expression is Increased in MDSCs Purified from ACS Patients

MDSCs can suppress immune responses under pathological conditions via diverse mechanisms, including increased arginase activity, nitric oxide (NO) levels, and the release of IL-10 and TGF- β 1. Here, we analyzed the mRNA expression of Arg-1, iNOS, TGF- β 1, and IL-10 in the sorted CD14⁺HLA-DR^{-low} cells by RT-PCR. Arg-1 mRNA expression was significantly increased in the sorted MDSCs from the ACS patients compared to MDSCs from the SA and control groups (p < 0.01) (Fig. 5A). No significant differences in iNOS, TGF- β 1, or IL-10 expression were observed among the three groups (p > 0.05) (Fig. 5B, C and D).

Fig. 7. Increased levels of plasma cytokines in ACS patients. The plasma concentrations of IL-1 β (A), IL-6 (B), TNF- α (C), IL-10 (D), and IL-33 (E) were determined using the Bio-Plex system.

Fig. 8. Spearman correlation analysis of the relationships between the frequency of CD14⁺HLA-DR^{-low} MDSCs and related cytokines in patients in the ACS, SA, and control groups. (A) No significant association was observed between MDSC frequency and the plasma level of IL-1 β ($r = 0.123$, $p = 0.183$). The frequency of MDSCs was positively correlated with the plasma levels of IL-6 ($r = 0.49$, $p < 0.001$) (B), TNF- α ($r = 0.281$, $p = 0.002$) (C), and IL-33 ($r = 0.33$, $p < 0.001$) (D).

MDSCs from ACS patients Suppress T-cell Responses Primarily via Arg-1

To further delineate the possible mechanisms by which CD14⁺HLA-DR^{-low} MDSCs from ACS patients inhibit T-cell function, we evaluated the suppressive function of CD14⁺HLA-

DR^{-/low} MDSCs following the addition of different chemical inhibitors or blocking antibodies. Consistent with the RT-PCR results, the suppression of T-cell proliferation and IFN- γ production by MDSCs was partially recovered after administration of the Arg-1 inhibitor nor-NOHA. No effect was observed for the iNOS inhibitor L-NMMA, neutralizing anti-TGF- β antibodies or anti-IL-10 antibodies (Fig. 6).

The Correlation of CD14⁺HLA-DR^{-/low} Cell Frequency with Inflammatory Cytokines

Recent work has demonstrated that cytokines may be key players in the systemic immune response during atherosclerosis and can enhance the accumulation, differentiation arrest, and activation of immature myeloid cells. Thus, to determine the relationship between these cytokines and CD14⁺HLA-DR^{-/low} cells, the plasma levels of several important cytokines were examined. The concentrations of IL-1 β , IL-6, TNF- α , and IL-33 were significantly higher in patients with ACS (2.64 ± 0.31 pg/ml, 20.97 ± 1.62 pg/ml, 7.82 ± 1.07 pg/ml, and 763.85 ± 66.47 pg/ml, respectively) than in patients in the SA (1.23 ± 0.12 pg/ml, 3.20 ± 0.28 pg/ml, 2.78 ± 0.23 pg/ml, and 341.11 ± 80.47 pg/ml, respectively) and control (1.15 ± 0.07 pg/ml, 3.14 ± 0.17 pg/ml, 2.83 ± 0.21 pg/ml, 252.70 ± 56.46 pg/ml, respectively) groups (all $p < 0.001$) (Fig. 7A, B, C and E). By contrast, the differences in the plasma levels of IL-10 were not statistically significant among the ACS (3.61 ± 0.36 pg/ml), SA (3.11 ± 0.34 pg/ml) and control (3.31 ± 0.28 pg/ml) patients (Fig. 7D). We next performed a correlation analysis of MDSC frequency and plasma cytokine levels in all patients. The frequency of MDSCs was significantly correlated with the plasma levels of IL-6 ($r = 0.49$, $p < 0.001$) (Fig. 8B), TNF- α ($r = 0.281$, $p = 0.002$) (Fig. 8C), and IL-33 ($r = 0.33$, $p < 0.001$) (Fig. 8D) in this cohort; no significant association was noted between MDSC frequency and IL-1 β levels ($r = 0.123$, $p = 0.183$) (Fig. 8A).

Discussion

The treatment of cardiovascular disease (CVD), a severe global epidemic associated with high prevalence, incidence, and mortality, remains a major challenge for most countries. Inflammatory processes play crucial roles in the pathogenesis of atherosclerosis plaque development, including plaque destabilization and the onset of ACS [4-7]. However, the specific pathological mechanism is unclear, and additional details are needed to understand the full effects of these processes [41].

MDSCs, which are characterized by their myeloid origin, immature state, and potent ability to suppress T-cell responses, have been studied intensively in cancer research and are increasingly recognized for their role in inflammation-mediated diseases [13, 29, 30]. Atherosclerosis involves cross-talk between the adaptive and innate immunity pathways [2, 3]. By evoking elements of the host defense system, atherosclerosis involves many of the same processes and factors that function during other chronic inflammatory diseases. MDSCs accumulate in response to chronic inflammation and have well-characterized immunomodulatory activities, leading us to hypothesize that MDSCs may be altered in patients with atherosclerosis, particularly ACS, which is closely associated with abnormal immune system activation.

In this study, we observed a significant increase in the frequency of human CD14⁺HLA-DR^{-/low} MDSCs in the peripheral blood of patients with ACS compared with those in the SA and control groups. The MDSC frequency was only slightly higher in SA patients compared with patients in the control group; however, this difference was not statistically significant. A number of studies support the view that the initiation of atherosclerosis is accompanied by inappropriate activation of the immune system in response to the accumulation and modification of lipoproteins in the arterial intima [1-3, 6]. Taken together, our observations indicate that in the context of ACS, a severe systemic inflammatory reaction, the release of important cytokines, and a modification of the local microenvironment may underlie the expansion of MDSCs. Similar to what Haile observed in IBD [29], we suspect that ACS

increases the frequency of immunosuppressive MDSCs, thereby preventing the development of a more severe and possibly fatal inflammatory response.

To determine whether ACS-derived MDSCs could suppress T-cell function, T-cell proliferation and IFN- γ production were measured in the MDSC-T-cell co-culture system. Similar to results observed for hepatitis [13], we determined that purified MDSCs suppressed T-cell proliferation and IFN- γ production in a dose-dependent manner. Furthermore, at ratios of 1:4 and 1:2, MDSCs from ACS patients inhibited T-cell proliferation and IFN- γ production more efficiently than those from the SA and control groups. These results suggest that the MDSCs of ACS patients not only occur at a drastically increased frequency but also exhibit an upregulated suppressive effect on a per cell basis compared with MDSCs from SA and control patients. The metabolism and metabolic products of L-arginine are essential for suppressive MDSCs [11, 13, 27]. Thus, to investigate the mechanism of MDSC-mediated T-cell suppression in ACS patients, we compared the mRNA expression levels of Arg-1, iNOS, TGF- β 1, and IL-10 in MDSCs isolated from patients in the three groups. We also tested the suppressive function of CD14⁺HLA-DR^{-/low} MDSCs in the presence of different chemical inhibitors or blocking antibodies. Our observations revealed that active MDSCs in ACS patients expressed higher transcript levels of Arg-1 but not iNOS, TGF- β 1, or IL-10. In addition, the Arg-1 inhibitor nor-NOHA nearly neutralized the suppressive function of MDSCs. A neutralizing effect was not observed for the iNOS inhibitor L-NMMA or neutralizing anti-TGF- β or anti-IL-10 antibodies. These results suggest that Arg-1 is one of the main suppressive mechanisms utilized during MDSC-mediated suppression of T-cell proliferation in ACS patients.

A substantial body of evidence suggests the involvement of the systemic immune response in the pathogenesis of atherosclerosis and a possible role for cytokines in the development, progression, and complications of this disease [42-44]. Cytokines are defined as lymphocyte-derived proteins that affect the growth or function of immune cells and that are secreted from a variety of cell sources. In this study, we measured the levels of numerous cytokines in plasma samples isolated from ACS, SA, and control patients. Our data suggest that plasma IL-1 β , IL-6, TNF- α , and IL-33 levels are all elevated in ACS patients, further validating the conclusions above. Several studies have demonstrated that certain cytokines can promote the accumulation, differentiation arrest, and activation of immature myeloid cells [18, 45, 46]. Thus, we sought to examine the possible correlations between these cytokines and MDSC frequency in all patients. A higher frequency of circulating MDSCs was positively correlated with IL-6, TNF- α , and IL-33 levels, indicating that these cytokines, which are likely responsible for plaque destabilization, may also contribute to the release of additional MDSCs from bone marrow.

The dramatic expansion of the CD14⁺HLA-DR^{-/low} MDSC population in response to ACS is very similar to that previously described in patients with other inflammatory diseases [13, 29-31]. Although the direct mechanism of MDSC generation has not been fully elucidated, several lines of evidence offer clues as to how the high frequency of circulating MDSCs might occur in the context of ACS. First, MDSCs are a heterogeneous population of cells, and the local inflammatory microenvironment greatly affects their tissue recruitment, retention, and immunomodulatory capabilities [45]. ACS occurs as a consequence of coronary plaque erosion or rupture, and severe systemic inflammation is thought to play an important role in these coronary events. Therefore, the high frequency of circulating MDSCs may occur because systemic inflammation during coronary plaque erosion or rupture stimulates the bone marrow to release more MDSCs into the peripheral blood. Second, it has been reported that MDSCs can mediate Treg induction through various mechanisms [21, 47, 48]. However, downregulation of Treg cells occurs in ACS patients [8, 9]. This relationship between Treg cells and MDSCs suggests that multiple mechanisms and molecules are involved in the expansion of MDSCs in ACS. Notably, it has been reported that IL-6 potentially activates phosphorylated signal transducer and activator of transcription 3 (pSTAT3) and augments MDSC survival, thereby promoting MDSC expansion [49]. In addition, IL-33 stimulates the activation of macrophages that produce G-CSF, boosting the number and survival of MDSCs [50]. In this study, the plasma levels of IL-6 and IL-33 were elevated in ACS patients, and the

increased levels of these cytokines were positively correlated with MDSC frequency. These findings support the hypothesis that several cytokines, such as IL-6 and IL-33, may also be involved in the process of MDSC expansion in ACS patients.

This study is subject to several limitations. First, the study population was relatively small, and additional studies with more patients are necessary for a more in-depth comparison. Second, our *in vitro* results indicated a potential role of MDSCs in ACS patients. However, further studies using animal models of atherosclerosis are required to fully investigate the *in vivo* function of MDSCs and identify the precise effects and mechanisms of MDSC expansion in plaque progression and destabilization.

In summary, this is the first study to demonstrate an expansion of CD14⁺HLA-DR^{-/low} MDSCs with immunosuppressive function in patients with ACS. The expansion of these cells into the peripheral blood may be induced by severe systemic inflammation to maintain immune homeostasis during the onset of ACS; however, additional studies are needed to elucidate the mechanistic link between MDSCs and the pathogenesis of ACS. Further elucidation of the role of MDSCs in patients with ACS will may provide additional treatment targets for the clinical management of atherosclerosis and ACS.

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Disclosure Statement

The authors have no competing interests to declare.

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