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Original Article

Changes in interferon-γ and CD20$^+$ cells in acute coronary syndrome patients and its clinical significance

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Abstract: *Objective:* The changes in interferon gamma (IFN-γ) and CD20$^+$ cells in acute coronary syndrome (ACS) patients and their association with the severity of coronary artery diseases were measured. *Methods:* A total of 100 patients were divided into four groups: 30 participants in the acute myocardial infarction (AMI) group, 30 participants in the unstable angina pectoris (UAP) group, 20 participants in the stable angina pectoris (SAP) group, and 20 participants in the normal control group. From the coronary angiography (CAG) results, the Gensini scores of patients in all groups were calculated. IFN-γ and CD20$^+$ cells in all groups were evaluated using a flow cytometer. *Results:* The percentages of IFN-γ in the AMI group, UAP group, and SAP group were all significantly higher than that in the normal control group (P<0.05). After percutaneous coronary intervention (PCI), the percentages of IFN-γ in the AMI group and UAP group significantly decreased compared to those before surgery but were still higher than that in the normal control group; the differences all had statistical significance (P<0.05). The numbers of CD20$^+$ cells in the AMI group and UAP group were both higher than those in the SAP group and normal control group (P<0.05).The percentage of IFN-γ in ACS patients had a significant positive correlation with the Gensini scores (r=0.895, P<0.05), and the number of CD20$^+$ cells was positively correlated with the Gensini scores (r=0.870, P<0.05). *Conclusion:* IFN-γ and CD20$^+$ cells participated in the development of ACS, and both had a significantly positive correlation with the Gensini scores.

Keywords: CD20$^+$ cells, interferon-γ, acute coronary syndrome, Gensini score

Introduction

The pathological basis of coronary atherosclerotic heart disease (CHD) is atherosclerosis (AS) [1], and in essence, AS is a type of chronic inflammatory response [2, 3]. The inflammatory response plays important roles in the development and progression of AS [4, 5]. Inflammatory cytokines aggravate the instability of atherosclerotic plaques through induction of immune inflammatory responses to cause plaque rupture and induce the development of acute coronary syndrome (ACS). Acute coronary syndrome is a cardiovascular disease that refers to sudden reduction or interruption of blood flow in the coronary artery caused by intense coronary spasm, bleeding and thrombosis, which result from sharp changes in diseased coronary artery including atherosclerotic plaque rupture in coronary artery and surface damage or crack. Its clinical symptoms include unstable angina, acute myocardial infarction and cardiac sudden death. It accounts for about 30% in all patients with coronary heart diseases, and presents poor prognosis. In recent years, studies on its pathogenesis have revealed that inflammation plays an important role in acute coronary syndrome. Studies have shown that infiltration of local inflammatory cells and systemic inflammation is one of the leading causes of plaque rupture-caused acute coronary syndrome. Currently, the exact functions of interferon gamma (IFN-γ) and CD20$^+$ cells in ACS and their interactive mechanism are still not clear. Therefore, this study examined the percentages of IFN-γ and CD20$^+$ cells in the peripheral blood mononuclear cells (PBMCs) of all types of ACS patients to investigate the roles of IFN-γ and CD20$^+$ cells in the development of ACS.

Materials and methods

Inclusion and exclusion criteria of study subjects

A total of 100 patients hospitalized at the Department of Cardiology of the First Affiliated Hospital of School of Medicine in Shihezi University, China, between June 2014 and August 2015 were selected based on a combination of the clinical presentations and the selective coronary angiography (CAG) results. The diagnosis of CHD conformed to the diagnostic criteria of CHD by the World Health Organization (WHO) [6]: stenosis of the internal diameter of at least one coronary artery ≥50% confirmed by CAG. Based on the following diagnostic criteria, the enrolled patients were divided into 4 groups. (1) There were 20 cases in the normal control group, which had chest discomfort with unknown reasons at the same time as the patient groups. After all examinations showed no abnormal results and were confirmed by CAG, CHD were excluded. (2) There were 20 cases in the stable angina pectoris (SAP) group, which had exertional angina for 2 months without changes and positive results for the exercise test. CHD was confirmed by CAG. (3) There were 30 cases in the unstable angina pectoris (UAP) group. The diagnosis of CHD should meet the following conditions: ① Resting angina pectoris with a duration >20 min; severe new angina pectoris or worsening angina pectoris; ② When angina pectoris occurred, the electrocardiogram (EKG) showed transient ischemic changes in the ST segment; after remission, the ST segment rapidly returned to normal or was close to normal; ③ Patients had negative cardiac troponin T (cTnT) results, and patients with post-infarction angina pectoris were excluded. (4) There were 30 cases in the AMI group, which had clinical symptoms, EKG changes, and dynamic evolution. Patients with simple right ventricular infarction were excluded. Patients were diagnosed with CHD by CAG. The ages of all enrolled patients were between 30 and 70 years.

Exclusion criteria (any of the following items): patients with severe heart failure, malignant tumors, acute and chronic infection, autoimmune diseases, severe liver and kidney dysfunction, allergy to contrast agents, Bayaspirin, clopidogrel, or statins, or recent surgical and trauma history were excluded.

Specimen collection

Blood samples from the patients in the AMI group and UAP group were collected twice before and after treatment. Before emergency percutaneous coronary intervention (PCI) within 24 h of hospitalization and on the day of discharge, 5 mL of morning fasting venous blood was collected from the cubital vein of the patients in the AMI group. On the next day of hospitalization before accept PCI and on the day of discharge after accept PCI, 5 mL of the morning fasting venous blood of the patients in the UAP group was collected in sterile conditions. On the next day of hospitalization, 5 mL of the venous blood of the patients in the SAP group and the normal control groups was collected in sterile conditions. Heparin sodium was used as the anti-coagulant. Blood samples were used to determine the percentages of the IFN-γ cytokines and CD20+ cells in PBMCs.

Measurement of the IFN-γ levels in peripheral blood

Heparin sodium was used for anti-coagulation of whole blood. Whole blood was then diluted with an equal volume of PBS, and PBMCs were obtained using the density gradient centrifugation method with lymphocyte separation solution. The cell concentration was adjusted to 2×10^6/mL using Roswell Park Memorial Institute medium (RPMI) and inoculated onto 24-well plates. Cells were mixed evenly with the stimulants, including 50 μg/L phorbol myristate acetate (PMA) and 1 μmol/L ionomycin, and the protein transport inhibitor 50 μg/L monensin (the above reagents were all purchased from ALEXIS) and were incubated in a 37°C and 5% CO_2 incubator for 4 h. Cells were collected and divided into the experimental tube and the isotype control tube of the same type according to the cell counts. The cells were washed with PBS twice and incubated with fixative at room temperature in the dark for 20 min. The supernatant was discarded after centrifugation, and the cells were washed with PBS twice. Permeabilizing agent (1 mL) was added into each tube to permeabilize cells to facilitate the entry of the cytokine monoclonal antibody into cells. After centrifugation, the supernatant was discarded, and intracellular cytokines were stained. To each tube, 4 μL of fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against cytoplasmic IFN-γ was added. The

Table 1. Comparison of the baseline data in all groups ($\bar{X}\pm s$)

Item	AMI group	UAP group	SAP group	Normal group	*F* or χ^2	*P*
Male (cases)	26 (86.7%)[a]	25 (83.3%)[a]	7 (35%)	14 (70.0%)	18.73	0
Age (years)	52.17±11.94	55.6±10.27	52.6±10.27	50.55±9.49	1.576	0.215
Smoking (cases)	17 (56.7%)	16 (53.3%)	9 (45.0%)	8 (40.0%)	1.667	0.644
Hypertension (cases)	18 (60.0%)	14 (46.6%)	8 (40.0%)	9 (45.0%)	2.294	0.514
Diabetes mellitus (cases)	5 (16.67%)	5 (16.67%)	2 (10.0%)	2 (10.0%)	0.886	0.829
BUN (mmol/L)	6.04±2.02	5.38±1.29	5.86±1.70	5.78±1.93	0.768	0.515
SCr (umol/L)	85.08±25.77	83.44±19.77	81.93±4.91	80.25±7.19	0.310	0.818
TG (mmol/L)	1.74±0.58	1.58±0.66	1.56±0.57	1.53±0.57	0.641	0.59
LDL-C (mmol/L)	2.59±0.73	2.48±0.96	2.53±0.72	2.51±0.73	0.108	0.955
HDL-C (mmol/L)	0.94±0.23	0.96±0.23	0.93±0.17	0.94±0.17	0.126	0.945
TC (mmol/L)	4.60±0.84[a]	4.20±0.83[a]	3.94±0.40	3.90±0.46	5.438	0.002
Gensini Score	51.63±38.57	48.55±39.38	5.13±2.36	0.00±0.00	19.81	0

[a]indicates P<0.05 compared to the normal control group.

corresponding isotype control was added to the control tube. The cells were incubated at 4°C in the dark for 30 min. After being washed with PBS twice, the cells were loaded onto the machine for evaluation. After the fluorescence compensation among all channels was established, IFN-γ was detected.

Measurement of the level of CD20+ cells in peripheral blood

Heparin sodium was used for anti-coagulation of whole blood. The whole blood was then diluted with an equal volume of PBS, and PBMCs were obtained using the density gradient centrifugation method with lymphocyte separation solution. The cell concentration was adjusted to 2×10^6/mL using RPMI. Cells were collected and divided into the experiment tube and the isotype control tube based on the cell counts. According to the design, each tube 4 μl PE-labeled mouse anti-human CD20 monoclonal antibody added to it and was incubated at 4°C in the dark for 30 min. The cells were washed with PBS twice and incubated with fixative at room temperature in the dark for 20 min. After centrifugation, the supernatant was discarded, and the cells were washed with PBS twice and loaded onto the machine for evaluation. The above major reagents were all purchased from eBioscience. A FACS-Aria II type flow cytometer was used. Based on the forward-scattered light and side-scattered light, gating was established using the lymphocyte population. Cells stained with the same isotype of IgG were used in the negative control tube. After the fluorescence compensation among all channels was established, the percentages of CD20+ cells were determined.

CAG and Gensini scoring

According to the Gensini scores [7], the degree of coronary artery stenosis was scored. ① The basic score was confirmed based on the stenosis degree of the location with the most severe coronary artery stenosis: stenosis of <25% was 1 point, 25% to 50% was 2 points, 51% to 75% was 4 points, 76% to 90% was 8 points, 91% to 99% was 16 points, and 100% was 32 points. ② The scoring coefficient was confirmed based on the location of the coronary artery disease: left main disease: score ×5; anterior descending disease: proximal segment score ×2.5, middle segment score ×1.5, and distal segment score ×1; diagonal branch disease: the first diagonal branch score ×1 and the second diagonal branch score ×0.5; circumflex disease: proximal segment score ×2.5 and distal segment and posterior descending branch score ×1; right coronary artery disease: proximal, middle, and distal segments score ×1. The total score was the sum of the scores of all artery branches.

Statistical methods

Data processing and statistical analyses were performed using the SPSS 17.0 software. Measurement data were expressed as the mean ± standard deviation (SD). The comparison of mean values between two groups was

Table 2. Percentages of the IFN-γ cytokine and CD20+ cells in the PBMCs of patients in all groups ($\bar{X}$ ±s, %)

Group	AMI group	UAP group	SAP group	Normal control group
	n=30	*n*=30	*n*=20	*n*=20
IFN-γ (%)	19.92±3.35[a,b,c]	17.38±4.07[a,c]	11.35±5.27[a]	10.23±4.45
CD20+ cells (%)	8.28±2.51[a,c,e]	6.90±5.04[a,c]	3.76±1.17[d]	3.62±1.90

[a]indicates P<0.05 compared to the normal control group; [b]indicates P<0.05 compared to the UAP group; [c]indicates P<0.05 compared to the SAP group; [d]indicates P>0.05 compared to the normal control group; [e]indicates P>0.05 compared to the UAP group.

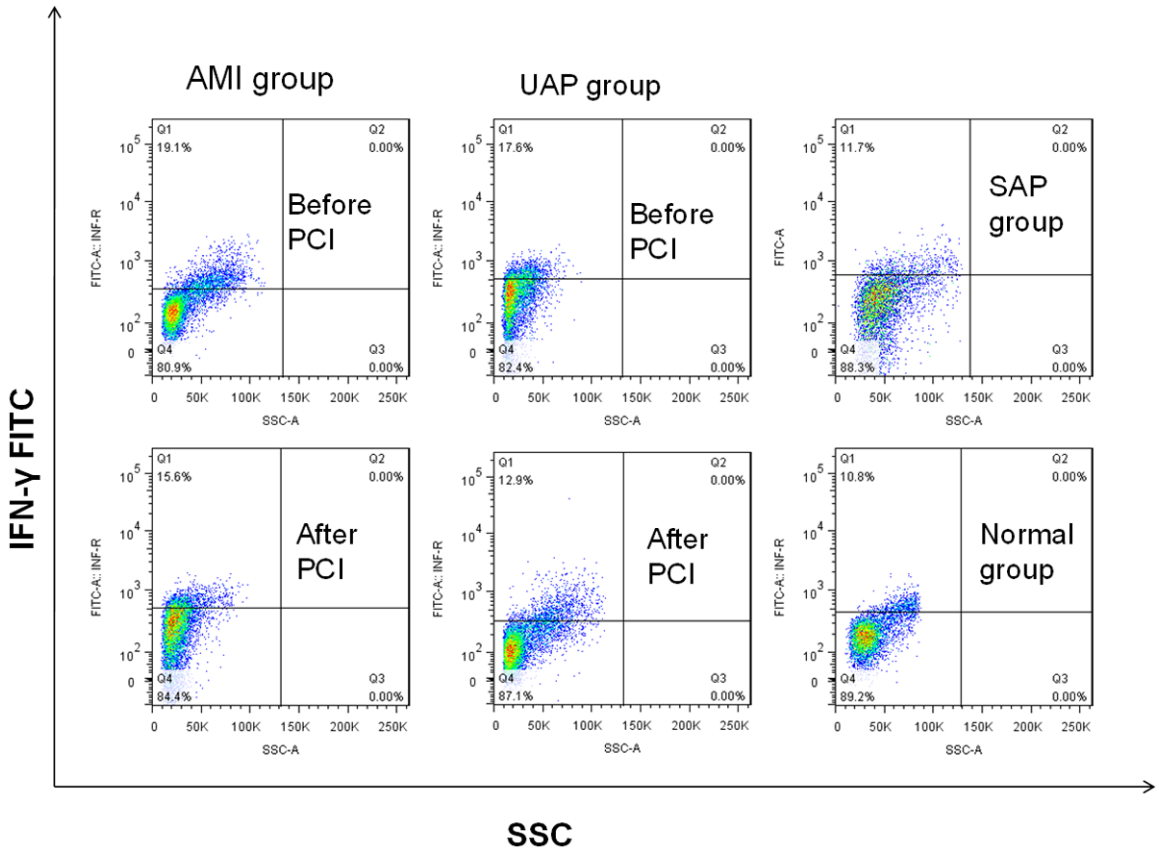

Figure 1. Measurement of the percentages of the IFN-γ cytokine in peripheral blood of all groups using flow cytometry (the Q1 quadrant shows the IFN-γ-positive percentage in the peripheral blood).

performed using the *t* test. The comparison of mean values of samples among multiple groups was performed using the analysis of variance. Further pairwise comparison was performed using the least significant difference (LSD) method. Measurement data before and after treatment were examined using the paired *t* test. The correlation between the levels of IFN-γ and CD20+ in the peripheral blood and the Gensini scores was performed using Pearson analysis. *P*<0.05 indicated a difference that had statistical significance.

Results

Comparison of general baseline data

Comparison of the baseline data of patients in all groups showed that the age, smoking history, hypertension, diabetes mellitus, blood urea nitrogen (BUN), serum creatinine (SCr), and triglyceride (TG) did not differ significantly (*P*>0.05). The disease prevalence among males in the AMI group and UAP group were both higher than that in the control group, and the differ-

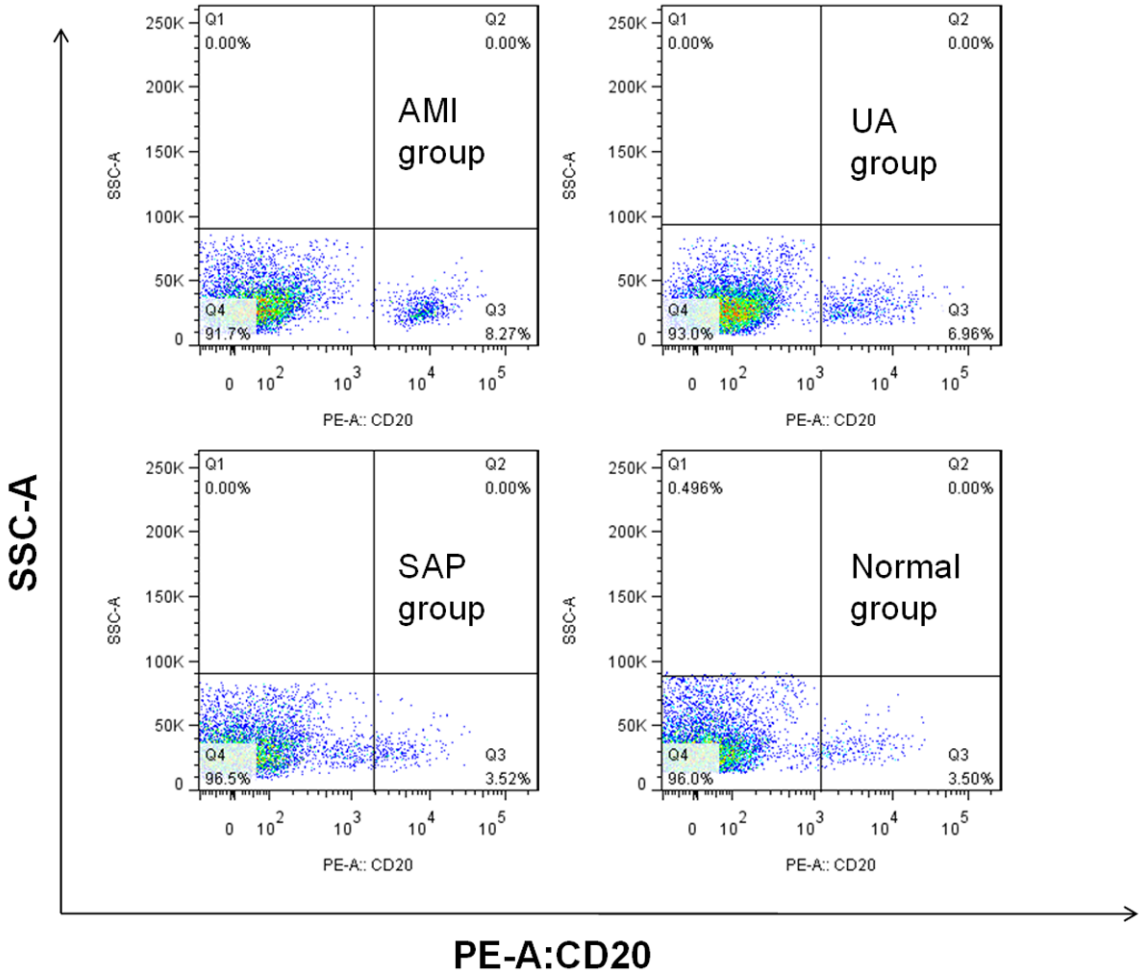

Figure 2. Measurement of the percentages of CD20+ cells in the peripheral blood of all groups using flow cytometry (the Q3 quadrant shows the percentage of CD20+-positive cells in the peripheral blood).

Table 3. Comparison of the percentages of the IFN-γ cytokine in the PBMCs of the AMI group and UAP group before and after PCI ($\bar{X}$ ±s, %)

Group	Number of cases	IFN-γ (%)	
		Before PCI	After PCI
AMI group	30	19.92±3.35[a]	15.0±3.56
UAP group	30	17.38±4.07[a]	11.69±2.51

[a]indicates P<0.05 compared to after PCI.

ences were statistically significant (P<0.05). The serum total cholesterol (TC) levels in the AMI group and UAP group were both higher than that in the control group, and the differences were statistically significant (P<0.05). These results were consistent with the pattern of the development of CHD in clinics (**Table 1**).

Comparison of the percentages of the IFN-γ cytokine and CD20+ cells in the peripheral blood of the patients of all groups before and after treatment

The percentages of IFN-γ in ACS patients (including both the AMI and UAP groups) were significantly higher than those in the SAP group and normal control group. The percentages of IFN-γ in the AMI group, UAP group, SAP group, and normal control group showed a decreasing trend, and the differences were statistically significant (P<0.05). The percentages of CD20+ cells in the AMI group and UAP group were significantly higher than those in the SAP group and the normal control group (P<0.05). There was no significant difference between the AMI group and UAP group (P>0.05), and there was no significant difference between the SAP

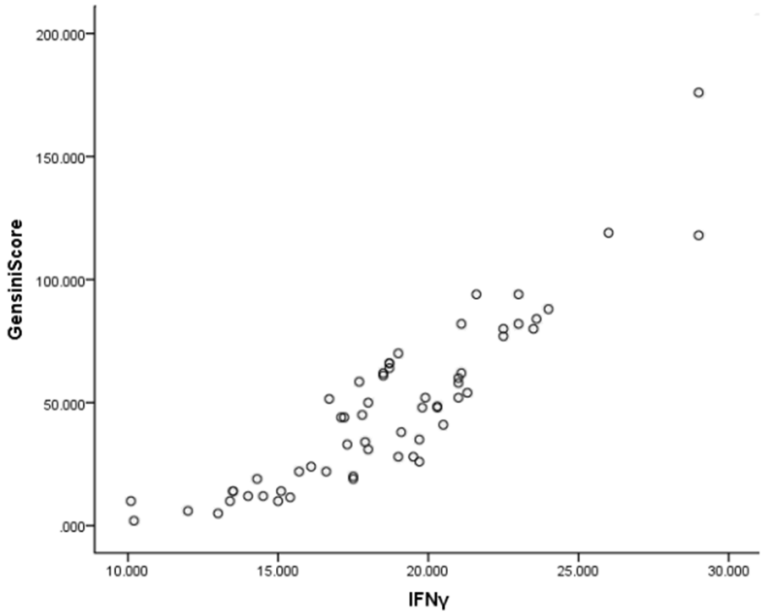

Figure 3. The correlation of interferon-γ proportion and Gensini score (r=0.895, P<0.05).

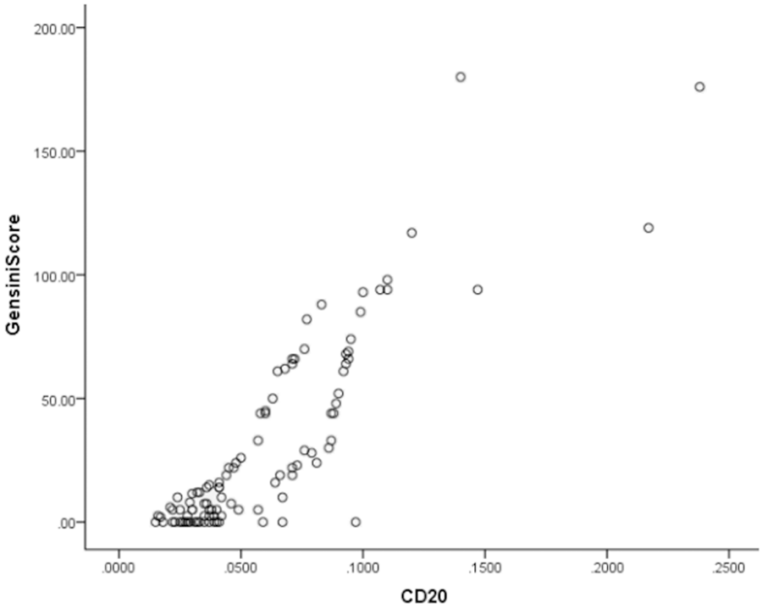

Figure 4. The correlation of CD20+ cells expression level and Gensini score (r=0.870, P<0.05).

group and the normal control group (*P*>0.05) (**Table 2** and **Figures 1** and **2**).

Comparison of the percentages of the IFN-γ cytokine in the peripheral blood of the patients in the AMI group and UAP group after PCI

The percentages of IFN-γ of the patients in the AMI and UAP groups after PCI significantly decreased compared to those before PCI, and both differences were statistically significant (*P*<0.05) (**Table 3** and **Figure 1**).

Correlation analysis between the IFN-γ cytokine and CD20+ cells in peripheral blood and the Gensini scores of ACS patients

The percentages of IFN-γ in the PBMCs of ACS patients positively correlated with the Gensini scores (*r*=0.895, *P*< 0.05). The percentages of CD20+ cells in the PBMCs of ACS patients positively correlated with the Gensini scores (*r*=0.870, *P*<0.05) (**Figures 3** and **4**).

Discussion

During the pathological progression of CHD, innate and adaptive immune responses play critical roles. A large amount of immune cells, such as B lymphocytes, T lymphocytes, macrophages, and dendritic cells, are present in atherosclerotic plaque tissues [8]. These cells influence the pathological process of AS through the secretion of pro-inflammatory and anti-inflammatory cytokines and chemokines. The persistent inflammatory status aggravates the instability of plaques and eventually can result in plaque rupture to further induce ACS [9]. Therefore, studying the role of immune responses in ACS becomes more important.

In recent years, scholars showed that T lymphocytes and B lymphocytes extensively participate in the immune regulation of the cardiovascular system. During inflammation, T cells, monocytes, and NK cells enter the site of myocardial injury. The released inflammatory mediators and tissue antigens rapidly lead to systemic B lymphocyte activation. Through the surge of cytokines and the production of anti-

bodies, B lymphocytes mediate humoral immune responses [10, 11] and thus participate in the development and progression of ACS. However, the specific mechanism is still not clear. Therefore, determining how the immune pathways mediated by T cells and B cells function in ACS is very important.

CD20 is a phosphorylated protein molecule with a molecular weight of 33 to 37 kDa. CD20 is located on the surface of B lymphocytes and is the surface-specific differentiation antigen of B lymphocytes. CD20 participates in proliferation, differentiation, signal transduction, and the transmembrane transport of calcium to play important roles in the immune system [12, 13].

Tsou *et al.* [14] found that during the ischemic injury of the myocardium, mature B cells selectively produce chemokine (C-C motif) ligand 7 (CCL7) to become an important factor for the recruitment and mobilization of the monocytes in blood into injured myocardium and play important roles in the myocardial inflammatory responses in AMI. With continuing in-depth studies, Zouggari *et al.* [15] showed that after the injection of the CD20 monoclonal antibody during the acute phase of myocardial infarction (MI), mature B lymphocytes were depleted, CCL7 was reduced, the entry of monocytes into the damaged heart was prevented, and the cardiac remodeling and heart function were significantly improved. Therefore, the depletion of $CD20^+$ B lymphocytes greatly limited myocardial inflammation, reduced infarct areas, and improved myocardial function; these changes had profound influences on the ischemic injury.

Our study results showed that for the PBMCs of CHD patients, the percentages of $CD20^+$ cells in the AMI group and UAP group were higher than those in the SAP group and normal control group, and the differences were statistically significant. In addition, there was no significant difference between the AMI group and the UAP group, and there was no significant difference between the SAP group and the normal control group. These results might be associated with the immune responses in the ischemically injured myocardium of CHD patients. When atherosclerotic plaques were unstable or ruptured, many inflammatory mediators were produced that aggravated the inflammatory responses. Humoral immunity played a role, and B lymphocytes were also extensively involved; therefore, the expression of $CD20^+$ cells increased. When the atherosclerotic plaque became stable, the inflammatory response was mild; therefore, the expression of $CD20^+$ cells was lower. The results in this study were consistent with results of the study of Zouggari *et al*. [15].

Furthermore, we also found that the percentages of $CD20^+$ cells in the peripheral blood of CHD patients positively correlated with the Gensini scores of coronary artery diseases ($P<0.05$). As the Gensini scores increased, the percentage of $CD20^+$ cells in peripheral blood increased. These results indicated that as the coronary artery disease worsened, the atherosclerotic plaque became unstable, the ischemic injury of myocardial tissues increased accordingly, and the recruitment and mobilization of monocytes by B lymphocytes was induced; therefore, the expression of $CD20^+$ cells increased. Thus, in-depth studies on the level of $CD20^+$ cells in ACS patients might provide important references for evaluating the severity of CHD in clinics.

IFN-γ is a type II interferon and is mainly produced by Th1-type $CD4^+$ lymphocytes. During the early stage of AS, IFN-γ promotes the inflammatory responses in the early stage of plaque development mainly by inhibiting smooth muscle cell growth and extracellular matrix production [16]. During the late stage of AS, IFN-γ promotes plaque rupture mainly by accelerating cell apoptosis and extracellular matrix degeneration of macrophages [17].

The results of this study showed that the percentages of IFN-γ in the PBMCs of ACS patients (including the AMI group and UAP group) before PCI treatment were significantly higher than those in the SAP group and the normal control group. The percentages of IFN-γ in the AMI group, UAP group, SAP group, and normal control group showed a sequential decreasing trend, and the differences were statistically significant ($P<0.05$). These results suggested that the secretion of the inflammatory cytokine IFN-γ increased in the body of ACS patients to cause the aggravation of vascular wall inflammation and promote increased plaque instability; these changes might be one of the major causes for the development of ACS. After PCI, the percentages of peripheral blood IFN-γ in the

ACS patients of the AMI group and UAP group significantly decreased compared to those before surgery, and the differences were statistically significant ($P<0.05$). These results indicated that immune responses in the body of ACS patients were closely associated with the instability of atherosclerotic plaques.

Liuzzo *et al*. [18] showed that the IFN-γ released by the T lymphocytes of UAP patients could activate monocytes; the latter could cause degradation of collagen and elastin to induce instability and rapture of atherosclerotic plaques. The study of Cheng *et al.* [19] also indicated that the increase in the IFN-γ cytokine increased within 24 h of disease onset in ACS patients and that the increase was the greatest for AMI, followed by UAP and SAP; which was consistent with the results of our study. In addition, animal experiments [20] showed that knockout of genes related to IFN-γ or its receptor in hypercholesterolemia mice could reduce atherosclerotic lesions, whereas the administration of recombinant IFN-γ aggravated the development of atherosclerotic plaques. In addition, we also showed that the percentages of IFN-γ in the PBMCs of ACS patients was positively correlated with the Gensini scores ($P<0.05$), indicating that when the degree of coronary artery stenosis was more severe, the percentage of IFN-γ in peripheral blood was higher. Therefore, measuring the percentage of IFN-γ in peripheral blood might provide suggestions for evaluating the disease severity of ACS and making a prognosis.

The study of Aitoufella *et al.* [21] indicated that the B lymphocyte depletion mediated by CD20 monoclonal antibodies could delay AS because B lymphocyte depletion changed the immune response, decreased Th1 cell infiltration, and limited IFN-γ secretion, thus delaying the progression of AS. These results elucidated that T lymphocytes and B lymphocytes did not affect AS through a single immune pathway; instead, these cells influenced the progression of AS through interactions among many of the cytokines that they secrete. The results of our study indicated that with the worsening of AS, the immune inflammatory responses mediated by T lymphocytes and B lymphocytes also intensified, and the levels of IFN-γ and CD20+ cells also increased accordingly. The study results were consistent with the results of Aitoufella *et al.* [21]; however, the specific mechanism still requires further studies. Therefore, in-depth studies of the interactive relationship between IFN-γ and CD20+ have importance for elucidating the immune mechanism underlying the pathogenesis of ACS.

In summary, this study examined the percentages of IFN-γ and CD20+ cells in the peripheral blood of ACS patients to deduce that IFN-γ and CD20+ cells might participate in the development and progression of ACS. Furthermore, IFN-γ and CD20+ cells promoted the instability of ACS plaques and can be used to evaluate the severity of coronary artery diseases.

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Disclosure of conflict of interest

None.

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