Changes of peripheral blood immune cells in acute coronary syndrome

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Abstract
The objective of this study is to explore changes in main immune cells during acute coronary syndrome (ACS), including changes in subsets of monocytes, T cells, and inhibitory myeloid-derived suppressor cells (MDSCs), and to evaluate possible mechanisms. A total of 50 patients suffering from ACS were divided into two subgroups based on attacks of acute infarction, acute myocardial infarction (AMI) with infarction including ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI), and unstable angina (UA). Third, the subgroup of 19 healthy subjects was labeled the normal group (NG). CD14, CD16, and CD45 were used as markers of the subset of monocytes. CD4, CD8, and CD3 were used as markers of the subset of T cells and CD14, human leukocyte antigen—DR isotype (HLA-DR), and CD45 were used as markers of inhibitory MDSCs. Both CD11b+CD206+ and CD11b+CD68+ cells were also assayed. Our data indicated that lymphocytes/karyocytes and monocytes/karyocytes as well as those of CD3+CD4+ T cells, CD14+CD16− monocytes, CD14+CD16+ monocytes, and CD11b+CD68+ monocytes were significant in all three groups (P<0.05). The ratio of T-cell subtypes to total lymphocytes among the three subgroups can be represented as AMI>UA>NG (P<0.05). The ratios of CD14+ monocytes to total karyocytes among the three subgroups can be represented as NG>UA>AMI (P<0.05). The ratios of CD14+CD16− monocytes to total karyocytes among the three subgroups can be represented as NG>UA>AMI (P<0.05). The ratios of CD14+CD16+ monocytes to total karyocytes among the three subgroups can be represented as AMI>UA>NG (P<0.05). There were no significant differences in the proportion of MDSCs (P>0.05). Certain subsets of monocytes are closely associated with ACS, of which CD14+CD16− monocytes present a negative association, while CD14+CD16+ monocytes show a positive association. In addition, adaptive immunity is associated with unstable plaques of ACS, and CD3+CD4+ T cells may play a role in early stages of ACS.

Keywords
acute coronary syndrome, immune cells, subsets of monocytes, subsets of T cells

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Based on the latest research, atherosclerosis is believed to be a chronic disease induced by the innate immune response and adaptive immune response. It is also known that various immune cells play important roles during the development of this disease.1–3 Acute coronary syndrome (ACS), the terminal stage of coronary atherosclerotic heart disease (CAD), is considered to be a major threat to health and life.4 ACS, as a kind of acute ischemic syndrome caused by occlusion of thrombosis due to an acute coronary syndrome, is considered to be a major threat to health and life.4

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to rupture or erosion of unstable plaques, may lead
to worse clinical outcome. Coronary angiography
(CAG) has been confirmed as the golden standard
for ACS and clinically ACS can be defined when
any two items of the following three criteria are
found: (1) ischemic chest pain, (2) dynamic
changes of electrocardiography (EKG), and (3)
elevation of myocardial enzymes. Clinically,
ACS is classified into three subtypes based on
whether or not ST-segment elevation is observed:
ST-segment elevation myocardial infarction
(STEMI), non-ST-segment elevation myocardial
infarction (NSTEMI), and unstable angina (UA).
Myocardial infarction is found in both STEMI with
complete obstruction of the lumen and NSTEMI
with partial obstruction (>70%) of the lumen,
which means that positive creatine kinase MB
(CK-MB) and cardiac troponin I (cTnI) are often
found in these two subtypes. However, single myo-
cardial ischemia is found in UA with negative
CK-MB and cTnI.

It is suggested that there is an association
between heart disease and immune cells, including
T cells as well as mononuclear macrophages, and
ACS; however, few studies have focused on fur-
ther investigating this relationship and any possi-
bable mechanisms. This study aims to demonstrate
changes of immune cells in three subtypes of ACS
and explore potential mechanisms.

Materials and methods

Subjects

A total of 50 patients with ACS admitted to the First
Affiliated Hospital of Anhui Medical University
between June and October 2016 were enrolled,
together with 19 healthy subjects. The patients were
divided into two subgroups with or without acute
myocardial infarction (AMI), AMI with infarction
including STEMI and NSTEMI, and UA with unsta-
ble angina. Third, the subgroup of remaining healthy
subjects was labeled as the normal group (NG).
ACS was diagnosed by history, CAG, EKG, echo-
cardiography, and biomarkers of myocardial injury.
All patients with ACS were older than 40 years and
and the number of heart attacks within 72 h was at least
one. All healthy subjects were also older than
40 years and without any angina history and with
normal 24h Holter recordings, echocardiography,
and cTnI. The exclusion criteria were acute and
chronic inflammatory diseases including inflamma-
tory bowel disease, rheumatoid arthritis and sys-
temic lupus erythematosus, cancer, chronic liver
injury, kidney failure, left ventricular ejection frac-
tion (LVEF)<40%, structural deformity of the
heart, and hematological disease. Any patients who
had received anticoagulation agents, antiplatelet
agglutination (including aspirin) as well as antiar-
rhythmics, immunosuppressants, antibiotics, gluco-
corticoids, or other complex Chinese traditional
medicines which could disturb the results, within
6 months before the study, were also excluded. In
addition, any patients with a history of percutaneous
coronary intervention (PCI), autoimmune diseases,
or genetic disorders and without a clear medical his-
tory were also excluded. Furthermore, patients with
UA who suffered cardiac arrest, cardiac shock,
hypotension, or pulmonary congestion were
excluded. The estimation of sample size was con-
firmed based on major effect index (total proportion
of lymphocytes) as well as comparison between the
AMI and NG groups. Intergroup expected differ-
ence of the proportion of lymphocytes was 11
and standard difference was also 11. The power of test
was 0.8 and the drop rate was 0.2. Therefore, the
minimum size for each group was 17.

This study has been approved by the Ethics
Committee of the First Affiliated Hospital of Anhui
Medical University. All patients participating in
the study have signed the informed consent.

Diagnostic criteria

Hypertension was defined as blood pressure
> 140/90mmHg or stabilization with medical
intervention. Diabetes mellitus was defined as a
level of blood glucose > 126 mg/dL or random glu-
cose > 200 mg/dL or stabilization with medical
intervention. Smoking history was defined as a
period of smoking > 6 months. STEMI was defined
as acute chest pain with a period of angina more
than 30 min and elevated ST-segment, together
with elevated biomarkers of myocardial injury and
acute obstructive lesions matching the changes of
EKG within the main coronary arteries. NSTEMI
was defined as acute chest pain, with the period of
angina lasting more than 30 min, together with
ST-segment depression. Biomarkers of myocardial
injury were elevated and acute obstructive lesions
within the main coronary arteries (>70%) were
found. UA was defined as irregular chest pain with
the period of angina lasting less than 30 min, together with ST-segment depression. Biomarkers of myocardial injury were almost normal and acute obstructive lesions within the main coronary arteries (>70%) were found.

**Material selection and sampling**

**Clinical materials**
The clinical materials of patients were history and general information including age, gender, smoking history, alcohol consumption, medical intervention, and past history (hypertension, diabetes mellitus, hyperlipidemia, coronary artery disease, surgery, family genetic disease). Ejection fraction (EF), CAG, and EKG were recorded.

**Sampling**
A 3mL sample of blood anticoagulated with ethylenediaminetetraacetic acid (EDTA) was taken from patients with ACS by EKG, history, and cTnI at the time of CAG within 6h after the onset of ACS and before invasive PCI which could disturb the results. Samples from the 19 healthy subjects were taken after normal 24h Holter recordings, echocardiography, and cTnI were confirmed. Flow cytometric assays were performed at 3h after sampling.

**Cell assays**

**Assay of monocytes**
Heparinized anticoagulated blood was placed into two tubes, 100µL per tube. Then 10µL of anti-CD206 fluorescein isothiocyanate (FITC) and 10µL of anti-CD11b PE (markers of M2 monocytes) were added to the first tube, while 10µL of anti-CD68 FITC and 10µL of anti-CD11b PE (markers of M1 monocytes) were added to the second tube. A control tube was set up and treated with the same 10µL of antibodies. All tubes were mixed with a whirlpool mixer and then placed away from light at room temperature for 15min. To each tube was added 900µL hemolytic agent, and then it was shaken evenly and placed into a water bath at 37°C for 10min before analysis by fluorescence-activated cell sorting (FACS). The number of cells in each sample was more than 10,000 and the assay data were stored in Listmode.

**Assay of monocyte subtypes**
Aliquots of whole blood were placed into two tubes, 100µL per tube, and 10µL of each of the antibodies anti-CD14 FITC, anti-CD16 PE, and anti-CD45 PC5 was added to one tube. The other tube was used as the control and treated with 10µL of the same antibodies. After mixing in a whirlpool mixer, all tubes were placed away from light at room temperature for 15min. After incubation, 900µL hemolytic agent was added to each tube, shaken evenly, and then placed into a water bath at 37°C for 10min before analysis. CD14 was used to label monocytes, and the labeled monocytes were further divided into CD14+ CD16− subtype and CD14+ CD16+ subtype based on the expression level of CD16. The number of cells sampled was more than 10,000 and the assay data were stored in Listmode.

**Assay of myeloid-derived suppressor cells**
Whole blood was placed into two tubes, 100µL per tube. To one tube was added 10µL of each of the CD14 FITC, human leukocyte antigen—DR isotype (HLA-DR) PE, and CD45 PC5 antibodies. The other tube was used as the control and the same 10µL of antibodies were added. All tubes were placed away from light at room temperature for 15min after mixing in a whirlpool mixer. Each tube was treated with 900µL hemolytic agent, shaken evenly, and then placed into a water bath at 37°C for 10min. CD14+ HLA-DR was used to label myeloid-derived suppressor cells (MDSCs). The number of cells in each sample was more than 10,000 and the assay data were stored in Listmode.

**Assay of subtypes of lymphocytes**
Whole blood was placed into two tubes, 100µL per tube. Antibodies against CD4 FITC, CD8 PE, and CD3 PE-Cy5 (10µL of each) were added to one tube. The other tube was used as the control and the same 10µL of antibodies were added. All tubes were placed away from light at room temperature for 15min after mixing in a whirlpool mixer. Each tube was then treated with 900µL hemolytic agent, shaken evenly, and then placed into a water bath at 37°C for 10min. CD3+ was used to label total T lymphocytes. CD3+ CD4+ T lymphocytes represent helper T cells. CD3+ CD8+ T lymphocytes represent cytotoxic lymphocytes. The number of
cells in each sample was more than 10,000 and the assay data were stored in Listmode.

**Statistical analysis**

A data bank was established with Excel 2007 and analyzed with SPSS 23.0. Mean ± standard deviation was used to describe quantitative data with normal distribution. A chi-square test was used to compare intergroup differences. If a difference was significant, the Student–Newman–Keuls (SNK) test was used for pairwise comparison. M (P25, P75) was used to describe quantitative data with skewed distribution. A non-parametric test was used to compare intergroup differences. If a difference was significant, pairwise comparison was performed. Pearson’s correlation coefficient was used to measure correlation among variables of quantitative data with normal distribution, while Spearman’s correlation coefficient was used to measure correlation among variables of quantitative data with skewed distribution. \( P < 0.05 \) was considered significant.

**Results**

**General characteristics**

There were no significant differences in gender or age among the three groups (Table 1).

**Comparative analysis of cell subtypes**

Our results indicated that the ratios of lymphocytes:karyocytes and monocytes:karyocytes as well as those of CD3\(^+\) CD4\(^+\) T cells, CD14\(^+\) CD16\(^-\) monocytes, CD14\(^+\) CD16\(^+\) monocytes, and CD11b\(^+\) CD68\(^-\) monocytes (inflammation-promoting M1 monocytes) were significant in all three groups \( (P < 0.05) \).

**Ratio of lymphocytes to total karyocytes**

The relationship of the ratio of lymphocytes to total karyocytes among the three subgroups can be represented as NG > UA > AMI, with significant differences between the AMI and UA groups as well as between the AMI and NG groups.

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**Table 1.** General information and comparison of subtype cells.

| Items                      | AMI (n = 25) | UA (n = 25) | NG (n = 19) | F/H | P   |
|----------------------------|--------------|-------------|-------------|-----|-----|
| Age                       | 58.92 ± 13.00 | 61.48 ± 11.60 | 55.11 ± 9.93 | 1.599 | 0.210 |
| Gender Male                | 19           | 17          | 10          | 2.684 | 0.261 |
| Gender Female              | 6            | 8           | 9           |     |     |
| Hypertension               | 12           | 12          | 11          | 0.539 | 0.764 |
| Diabetes                   | 0            | 5           | 2           | 5.521 | 0.063 |
| Smoking                    | 3            | 5           | 6           | 2.504 | 0.286 |
| Antihypertensive agent     | 5            | 7           | 9           | 3.929 | 0.140 |
| Antidiabetic agent         | 0            | 5           | 2           | 5.521 | 0.063 |
| Ratio of lymphocytes       | 41.48 ± 14.12 | 51.02 ± 11.25\(^a\) | 52.03 ± 12.45\(^a\) | 4.969 | 0.010 |
| Ratio of CD3\(^+\)          | 71.26 ± 13.38 | 72.63 ± 8.94 | 67.67 ± 8.76 | 1.196 | 0.309 |
| CD3\(^+\) CD4\(^+\)         | 43.19 ± 9.58 | 37.68 ± 12.41 | 35.55 ± 5.89\(^b\) | 3.581 | 0.033 |
| CD3\(^-\) CD8\(^-\)        | 24.02 ± 9.40 | 27.63 ± 11.66 | 24.97 ± 8.58 | 0.849 | 0.432 |
| Ratio of monocytes         | 1.41 ± 0.99  | 1.75 ± 0.65 | 2.19 ± 0.61\(^a\) | 5.435 | 0.007 |
| CD14\(^+\) DR\(^+\)        | 83.68 ± 25.92 | 84.11 ± 26.31 | 95.58 ± 3.12 | 1.889 | 0.159 |
| CD14\(^+\) CD16\(^-\)      | 30.99 ± 6.41 | 77.09 ± 5.93\(^a\) | 83.75 ± 3.26\(^b\) | 632.314 | <0.001 |
| CD14\(^+\) CD16\(^+\)      | 68.68 ± 6.42 | 22.71 ± 5.66\(^a\) | 15.94 ± 3.25\(^b\) | 653.468 | <0.001 |
| CD4\(^+\) CD8\(^-\)        | 0.30 (0.10, 1.15) | 0.30 (0.20, 0.40) | 0.40 (0.20, 0.50) | 0.226 | 0.893 |
| CD4\(^+\) CD8\(^-\)        | 5.80 (3.55, 8.30) | 7.60 (4.70, 13.05) | 7.00 (5.10, 14.60) | 3.447 | 0.178 |
| CD14\(^+\) DR\(^-\)        | 4.70 (1.65, 17.45) | 2.80 (2.20, 15.55) | 3.10 (2.20, 5.90) | 0.580 | 0.748 |
| CD14\(^+\)/CD8\(^+\)       | 1.73 (1.41, 2.61) | 1.36 (0.91, 2.32) | 1.52 (1.05, 1.87) | 3.793 | 0.150 |
| CD11b\(^+\) CD206\(^+\)    | 2.37 ± 1.27 | 1.92 ± 0.97 | 1.93 ± 1.20 | 1.195 | 0.309 |
| CD11b\(^+\) CD68\(^+\)     | 1.71 ± 1.04 | 1.35 ± 0.82 | 1.01 ± 0.68\(^b\) | 3.453 | 0.037 |

**AMl:** acute myocardial infarction; **UA:** unstable angina; **NG:** normal group.  
\(^a\)Significant compared with AMI \( (P < 0.05) \).  
\(^b\)Significant compared with UA \( (P < 0.05) \).
(P < 0.05); however, there was no significant difference between the UA and NG groups (P > 0.05; Figure 1 and Table 1).

**Ratio of T-cell subtypes to total lymphocytes**

The relationship of the ratio of T-cell subtypes to total lymphocytes among the three subgroups can be represented as AMI > UA > NG, with a significant difference between the AMI and NG groups (P < 0.05); however, there were no significant differences between the AMI and UA groups or the UA and NG groups (P > 0.05; Figure 2 and Table 1). There were no significant differences in the proportions of CD3+ T cells, CD3+ CD8+ T cells, CD4+/CD8+, CD4+ CD8+, and CD4− CD8− cells among the three subgroups (P > 0.05).

**Ratio of monocyte subtypes to total karyocytes**

The relationship of the ratio of CD14+ monocytes to total karyocytes among the three subgroups can be represented as NG > UA > AMI, with a significant difference between the AMI and NG groups (P < 0.05); however, there were no significant differences between the AMI and UA groups or between the UA and NG groups (P > 0.05; Figure 3 and Table 1).

The relationship of the ratio of CD14+ CD16− monocytes to total karyocytes among the three subgroups can be represented as NG > UA > AMI; there were significant differences in pairwise comparisons among the AMI, UA and NG groups (P < 0.05; Figure 4 and Table 1).

The relationship of the ratio of CD14+ CD16+ monocytes to total karyocytes among the three
subgroups can be represented as AMI > UA > NG; there were significant differences in pairwise comparisons among the AMI, UA and NG groups (P < 0.05; Figure 5 and Table 1).

**Subtypes of monocytes**

The relationship of the ratio of CD11b+ CD68+ monocytes (inflammation-promoting M1 monocytes) to total karyocytes among the three subgroups can be represented as AMI > UA > NG; there was a significant difference between the AMI and NG groups (P < 0.05); however, there were no significant differences between the AMI and UA groups or between the UA and NG groups (P > 0.05; Figure 6 and Table 1). As shown in Table 1, the ratios of CD11b+ CD206+ monocytes (inflammation-inhibiting M2 monocytes) to total karyocytes were not significantly different among the AMI, UA, and NG subgroups (P > 0.05).

**MDSCs**

There were no significant differences in the proportion of MDSCs, identified as CD14+ HLA-DR−, among the AMI, UA, and NG subgroups (P > 0.05; Table 1).

**Discussion**

Atherosclerosis is a chronic non-degenerative aseptic inflammation of the arterial wall, in which inflammatory plaques containing a large number of immune cells play a critical role in the cascade response of plaques from vulnerability to rupture.8–10 It has been indicated that, among patients at higher risk of cardiovascular diseases, the percentage of both intermediate and non-classical subtypes is relatively higher, which means that these two subtypes are potential biomarkers of disease severity.11

In this study, the relationship of the ratios of CD14+ monocytes/total karyocytes in the three subgroups was NG > UA > AMI, among which, compared with NG, the ratio in AMI decreased significantly. It has been demonstrated that there is a dramatic association between monocytes and the development and severity of ACS during the acute stage of MI. The relationship of the ratio of CD14+ CD16− monocytes to total karyocytes among the three subgroups was NG > UA > AMI; for the ratio of CD14+ CD16+ monocytes to total karyocytes, the relationship was AMI > UA > NG. In fact, the sampling in this study was performed at the CAG point after regular diagnosis of ACS including EKG, past history, cTnI, and before PCI. The hyperacute stage of inflammation had been delayed at the sampling point, and consequently the inflammation-promoting and transient classical subtypes had decreased. Nevertheless, compared with healthy subjects, the proportion of CD14+ CD16+ monocytes among patients in both the STEMI and NSTEMI groups increased significantly and was positively associated with the severity of ACS. Meanwhile, the number of differentiated M1 macrophages derived from CD14+ CD16− and CD14+ CD16+ monocytes increased during advanced ACS; hence, the results indicated that the number of CD14+ CD16− subtype monocytes decreased and that of CD14+ CD16+ subtype monocytes increased in patients with STEMI. Recently and interestingly, it has been found that there is a conversion effect of different subtypes of monocytes.
during the inflammation stage, demonstrating a drift from the classical subtype partly to the intermediate and mostly to the non-classical subtype, based on the results of flow cytometry.\(^\text{12}\)

The relationship of the ratio of CD11b\(^+\) CD68\(^+\) monocytes (inflammation-promoting M1 monocytes) to total karyocytes among the three subgroups was AMI > UA > NG; compared with healthy subjects, the proportion of CD11b\(^+\) CD68\(^+\) monocytes to total karyocytes among patients in the AMI group increased significantly, which indicates, first, that a special functional cell without a clear mechanism between monocytes and macrophages may exist in the peripheral blood, and, second, that monocytes may preferentially differentiate into M1 macrophages during the acute inflammation stage. However, in our study, there were no significant differences in the MDSC population.

Meanwhile, the relationship of the ratio of CD3\(^+\) CD4\(^+\) T cells to total lymphocytes among the three subgroups was AMI > UA > NG, and, compared with healthy subjects, the proportion of CD3\(^+\) CD4\(^+\) T cells among patients with AMI increased significantly. There were no significant differences in the proportions of CD3\(^+\) T cells, CD3\(^+\) CD8\(^+\) T cells, CD4\(^+\)/CD8\(^+\), CD4\(^+\) CD8\(^+\), and CD4\(^-\) CD8\(^-\) cells. Based on our findings, we suggest that the adaptive immune response could be closely associated with the instability of ACS plaques.

In summary, CD14\(^+\) CD16\(^+\) monocytes are positively associated with the severity of ACS, while CD14\(^+\) CD16\(^+\) monocytes are negatively associated with the severity of ACS. M1 macrophages are positively associated with the acute stage of ACS. The adaptive immune response is associated with the instability of ACS plaques and CD3\(^+\) CD4\(^+\) T cells may play an important role in the early stage of ACS.

**Declaration of conflicting interests**

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