

## Original Article

# Comparison of cytokine expressions in acute myocardial infarction and stable angina stages of coronary artery disease

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**Abstract:** Objective: To investigate the differential gene expression of cytokines and compare their impacts on the immune functions among the acute myocardial infarction patients (AMI), the stable angina patients (SA) and the controls. Methods: 20 patients with AMI, 20 patients with SA and 20 healthy volunteers were recruited into the study. Whole human genome microarray analysis was used to detect the gene expression differences in interferons, interleukins, chemokines, tumor necrosis factors and associated receptors in peripheral blood mononuclear cells (PBMCs) among three groups. Results: Compared with SA patients and the controls respectively, in AMI patients, IFN $\alpha$ 2, IFN $\alpha$ R1, IFN $\alpha$ R2, IFN $\gamma$ R1, IFN $\gamma$ R2, L1 $\beta$ , IL16, IL18, Cxcl1, Cxcl2, Cxcl6, Cxcr2, Cxcr4, LIGHT, TNFR1, LT- $\beta$ R, CD137, TRAILR, and TWEAKR mRNA expressions were significantly up-regulated ( $P < 0.05$ ), while Ccl5, Ccl24, Ccl28, Ccr5, TWEAK, CD40, CD27, and BAFFR mRNA expressions were significantly down-regulated ( $P < 0.05$ ). But, there was no significant difference in cytokine expression between the SA patients and the controls. Conclusion: In AMI patients, mRNA expression levels of cytokines were imbalanced, indicating the dysfunction of the immune system. Together with no significant change of cytokines was observed between the SA and controls, showing the different cytokine related immune activity in the AMI and SA patients.

**Keywords:** Myocardial infarction, stable angina pectoris, cytokine, cytokine receptor, gene expression

## Introduction

Coronary atherosclerosis disease (CAD) is a leading cause of morbidity and mortality in our modern world [1]. Increasing evidence has demonstrated that cytokines (CK) and associated receptors, in a form of network, play important roles in the pathogenesis of CAD, which not only involve in inflammation and thrombosis process, but also activate the innate and adaptive immune system [2, 3].

CK, an inflammatory factor, is well known in the progress of CAD [4]. However, there were few studies about their effects on the immune system in the acute myocardial infarction (AMI) and the stable angina (SA) stages of CAD [5]. Therefore, in the present study, human microarray analysis was used to systematically examine the mRNA expressions of interferons (IFN), interleukins (IL), chemokines, tumor necrosis

factors (TNF) and associated receptors in peripheral blood mononuclear cells (PBMCs) isolated from the AMI, SA patients and controls. We designed this *in vitro* study to investigate the differential gene expressions of CK and analyze the impacts on the immune functions in the patients with AMI and SA.

## Materials and methods

### Patients' information

This prospective study consisted of three groups of subjects, including 20 patients with AMI, 20 with SA, and another 20 healthy volunteers. The sample sizes, the number of subjects per group, were based on an assumed within-group variance of 0.50 and the targeted nominal power of 0.95 [6]. The baseline demographic data were displayed in **Table 1**. The AMI patients were admitted less than twelve hours

**Table 1.** Baseline demographic data in different groups ( $\bar{x} \pm s$ )

	AMI (a) (N=20)	SA (b) (N=20)	Control (c) (N=20)	P (all)	P (a vs. b)
Age (years)	57.8±11.9	63.6±9.9	28.8±3.3	0.000	0.251
Sex (M/F)	18/2	18/2	17/3	0.853	1.0
BMI (kg/m <sup>2</sup> )	23.6±2.6	22.8±2.7	21.3±1.8	0.102	0.56
Smoke (NO./d)	13.6±12.2	9.8±10.3	0	0.00	0.648
Syst (mmHg)	128.6±15.3	123.0±12.1	120.8±7.2	0.115	0.501
Diast (mmHg)	67.0±8.0	73.0±8.0	71.6±3.2	0.017	0.064
LDL-C (mmol/L)	2.5±1.0	2.1±0.8	2.9±0.5	0.327	0.548
Triglycerides (mmol/L)	1.6±1.1	1.5±1.4	1.2±0.4	0.73	0.762
HDL-C (mmol/L)	0.8±0.7	0.9±0.2	1.3±0.2	0.000	0.803
FPG (mmol/L)	5.4±0.9	5.0±0.8	4.9±0.5	0.61	0.082

Footnotes: LDL-C=low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; FPG: Fasting Plasma Glucose.

from the onset of symptoms to coronary care unit between January and June in 2013. They were 18 male and 2 female with an average age of  $58 \pm 12$  (mean  $\pm$  S.D.) years. All AMI subjects were diagnosed on the basis of following criteria [7]: rise of cardiac biomarker parameters [preferably cardiac troponin (cTn)] with at least one value above the 99th percentile reference limit and with at least one of the followings: 1) symptoms of ischemia; 2) new or presumed new significant ST-segment-T wave changes or new left bundle branch block; 3) development of pathological Q waves in the electrocardiograph; 4) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality; 5) identification of an intracoronary thrombus by angiography.

In SA group, 20 patients (18 male and 2 female, mean age of  $64 \pm 10$  years) with exclusively effort-related angina were studied. Each of them had a positive exercise stress test and at least one coronary stenosis detected at angiography (>70% reduction of lumen diameter). There was no significant difference between AMI and SA patients (**Table 1**) in terms of age, gender, smoking status, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides and fasting glucose (FBG).

The control group consisted of 20 volunteers (17 male and 3 female, mean age of  $29 \pm 3$  years) recruited during the same period with comparable male/female ratio. Family histories, physical examination, electrocardiograph, chest radiography and routine chemical analysis showed the controls had no evidence of coronary heart diseases.

The exclusive criteria for three groups were venous thrombosis, haematological disorders, acute or chronic inflammatory diseases, intake of hormones or immuno-suppressors and malignancy.

The study protocol was approved by the ethics committee of Tongji University and informed consent form was obtained.

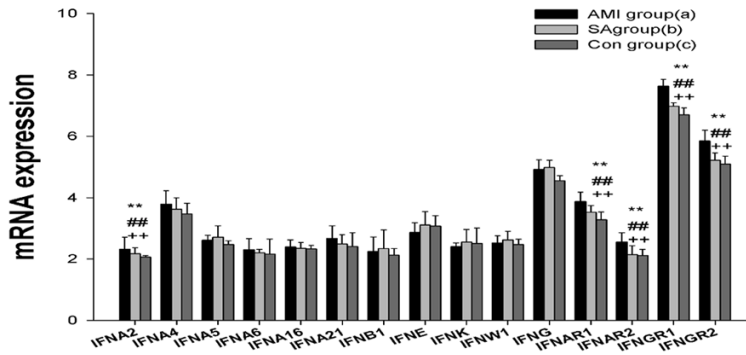
#### Gene expression chips

Agilent G4112F Whole Human Genome Oligo Microarrays were purchased from Agilent (Santa Clara, CA, USA). A microarray was composed of more than 41,000 genes or transcripts including targeted 19,596 Entrez gene RNAs. Sequence information used in the microarrays is derived from the latest databases of RefSeq, Goldenpath, Ensembl and Unigene. The functions of more than 70% of the genes in the microarray were already known. All patients were subjected to the chip analysis.

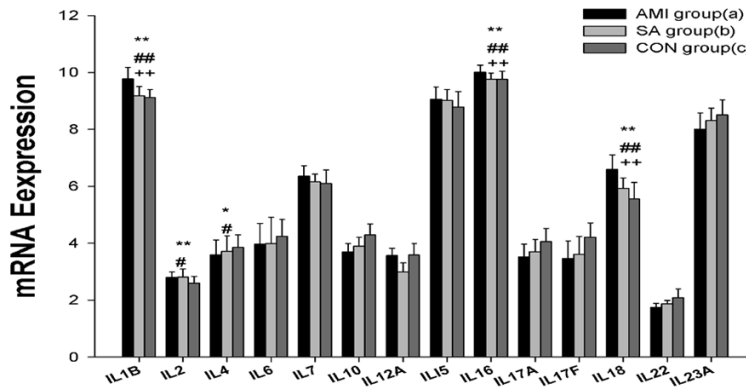
#### Total RNA isolation

5 ml of peripheral blood samples were drawn in PAXgene tube from patients of AMI and SA immediately after admission. Leucocytes were obtained through density gradient centrifugation with Ficoll solution and the remaining red blood cells were destroyed by erythrocyte lysis buffer (Qiagen, Hilden, Germany). Total RNA was extracted and purified using PAXgene™ Blood RNA kit (Cat#762174, QIAGEN, GmBH, Germany), following the manufacturer's instructions. RIN number was further checked to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa

## Cytokine expressions in AMI and SA



**Figure 1.** IFN mRNA expression levels. Comparison within three groups: \*P<0.05; \*\*P<0.01; AMI vs. Control: #P<0.05; ##P<0.01; AMI vs. SA: +P<0.05, ++P<0.01; SA vs. Control: &P<0.05, &&P<0.01.



**Figure 2.** IL mRNA expression levels. Comparison within three groups: \*P<0.05; \*\*P<0.01; AMI vs. Control: #P<0.05; ##P<0.01; AMI vs. SA: +P<0.05, ++P<0.01; SA vs. Control: &P<0.05, &&P<0.01.

Clara, CA, US). The sample was considered qualified when 2100 RIN $\geq$ 7.0 as well as 28S/18S $\geq$ 0.7.

### RNA amplification and labeling

Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Cat#5190-2305, Agilent technologies, Santa Clara, CA, US) according to manufacturer's instructions. Labeled cRNA were purified by RNeasy mini kit (Cat#74106, QIAGEN, GmBH, Germany).

### Microarray hybridization

Each slide was hybridized with 1.65  $\mu$ g Cy3-labeled cRNA using Gene Expression Hybridization Kit (Cat#5188-5242, Agilent technologies, Santa Clara, CA, US) in Hybridization Oven (Cat#G2545A, Agilent technologies, Santa

Clara, CA, US) in accordance with the manufacturer's instructions. Slides were washed in staining dishes after 17 hours of hybridization (Cat#121, Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat#5188-5327, Agilent technologies, Santa Clara, CA, US) based on the manufacturer's operation manual.

### Chip scan and data acquisition

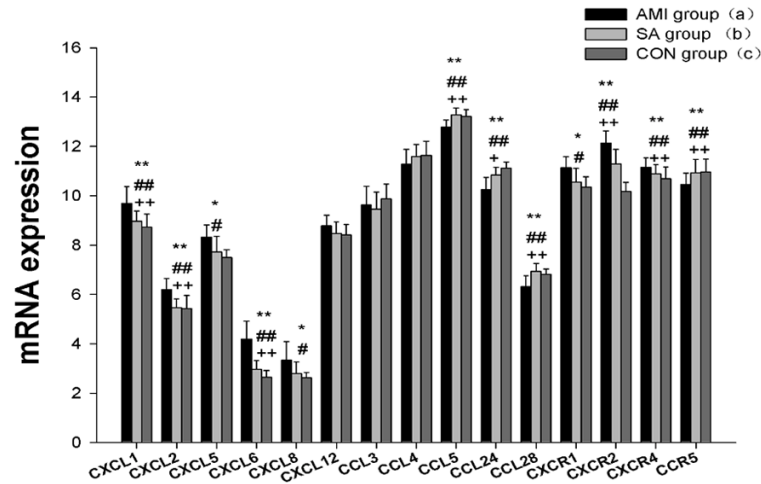
Slides were scanned by Agilent Microarray Scanner (Cat#G2565CA, Agilent technologies, Santa Clara, CA, US) with default settings. Dye channel: Green, Scan resolution=3  $\mu$ m, 20 bit. Data were extracted with Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, US).

### RT-PCR

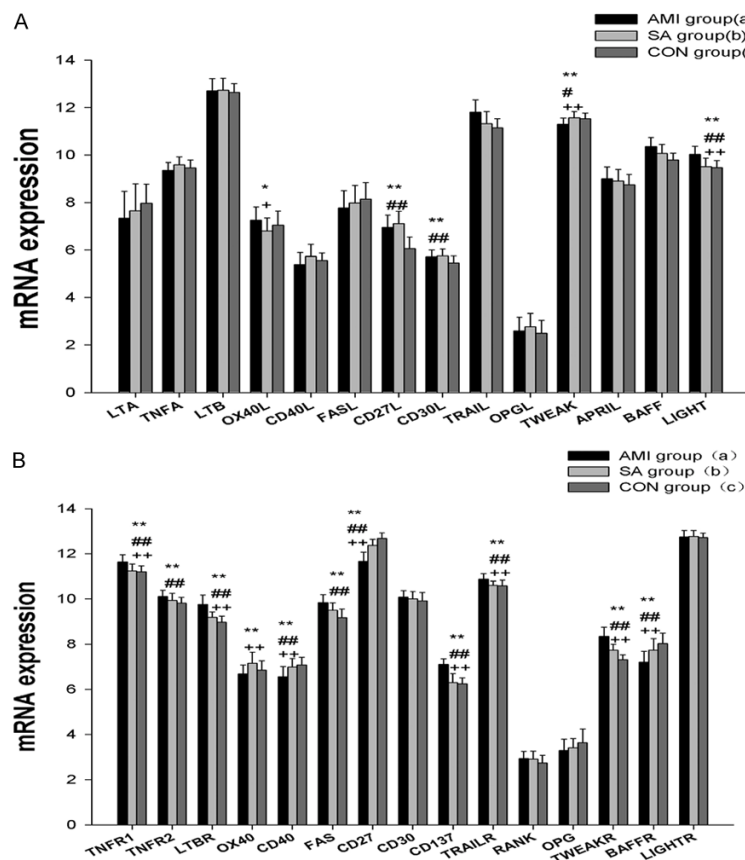
The spots in the microarray were randomly selected and their expressions were confirmed by RT-PCR. Among genes with differential expressions, 3 genes were randomly selected and these genes and related house-keeping genes (GAPDH) were subjected to RT-PCR. The relative expressions were indicated as the expression of the target genes normalized to the expression of GAPDH ( $2^{-\Delta\Delta Ct}$ ). The melting curve and the  $2^{-\Delta\Delta Ct}$ -method were used to detect the differences in the expressions among the three groups. The results from RT-PCR were consistent with the microarray analysis.

### Statistical analysis

Values were expressed as mean  $\pm$  S.D. We used One-way analysis of variance (ANOVA) to examine the differences between groups. Pairwise group comparisons after ANOVA were performed using Tukey's multiple comparison technique. Data were analyzed using SPSS



**Figure 3.** Chemokines mRNA expression levels. Comparison within three groups: \* $P<0.05$ ; \*\* $P<0.01$ ; AMI vs. Control: # $P<0.05$ ; ## $P<0.01$ ; AMI vs. SA: + $P<0.05$ , ++ $P<0.01$ ; SA vs. Control: & $P<0.05$ , && $P<0.01$ .



**Figure 4.** mRNA expressions of TNF (A) and associated receptors (B). Comparison within three groups: \* $P<0.05$ ; \*\* $P<0.01$ ; AMI vs. Control: # $P<0.05$ ; ## $P<0.01$ ; AMI vs. SA: + $P<0.05$ , ++ $P<0.01$ ; SA vs. Control: & $P<0.05$ , && $P<0.01$ .

## Results

### IFN mRNA expression levels

15 gene expressions were detected in PBMCs from three groups (**Figure 1**), including type I IFN, its receptors (IFN $\alpha$ 1, IFN $\alpha$ 2), type II IFN and its receptors (IFN $\gamma$ 1, IFN $\gamma$ 2). Type I IFN consists of IFN $\alpha$ 2, IFN $\alpha$ 4, IFN $\alpha$ 5, IFN $\alpha$ 6, IFN $\alpha$ 16, IFN $\alpha$ 21, IFN $\beta$ 1, IFN $\epsilon$ , IFN $\kappa$  and IFN $\omega$ 1. PBMCs obtained from the AMI patients showed high mRNA expression levels. In the AMI group, IFN $\alpha$ 2, IFN $\alpha$ 1, IFN $\alpha$ 2, IFN $\gamma$ 1 and IFN $\gamma$ 2 mRNA expressions were significantly up-regulated ( $P<0.01$ ) than the SA and control groups. However, between the SA and control group, there was no significant difference in mRNA expression of all 15 genes.

### IL mRNA expression levels

14 gene expressions of IL in PBMCs from three groups were examined (**Figure 2**), including IL1 $\beta$ , IL2, IL4, IL6, IL7, IL10, IL12A, IL15, IL16, IL17A, IL17F, IL18, IL22 and IL23A. In the AMI group, IL1 $\beta$ , IL2, IL16 and IL18 mRNAs were significantly up-regulated, while IL4 was significantly down-regulated compared with controls ( $P<0.05$ ). Between the AMI and SA group, IL1 $\beta$ , IL16 and IL18 mRNAs in the AMI patients were significantly up-regulated ( $P<0.01$ ). However, there is no significant difference between the SA group and the controls.

### Chemokine mRNA expression levels

17.0. Values of  $P<0.05$  denote statistically significant changes.

The comparison of gene expression of chemokines and associated receptors in PBMCs from

three groups are shown in **Figure 3**. 15 gene expressions were detected, including CXC family, CC family, and their associated receptors. In the AMI group, Cxcl5, Cxcl8, Cxcr1 (all  $P<0.05$ ), Cxcl1, Cxcl2, Cxcl6, Cxcr2, and Cxcr4 (all  $P<0.01$ ) mRNA expressions were statistically up-regulated, while Ccl5, Ccl24, Ccl28, and Ccr5 mRNA expressions were significantly down-regulated ( $P<0.01$ ) compared with the controls. Compared with the SA group, Cxcl1, Cxcl2, Cxcl6, Cxcr2, and Cxcr4 mRNA expressions in the AMI patients were significantly up-regulated ( $P<0.01$ ), and Ccl5, Ccl24, Ccl28 and Ccr5 were significantly down-regulated ( $P<0.05$ ). Between the SA and control group, there was no statistical difference in chemokine related mRNA expressions.

## *TNF superfamily and TNF receptors superfamily mRNA expression levels*

14 mRNA expressions of TNF superfamily (**Figure 4A**) and 15 mRNA expressions of TNF receptors (**Figure 4B**) were detected in PBMC from the three groups. Compared with the control group, CD27L, CD30L, LIGHT, TNFR1, TNFR2, LT- $\beta$ R, Fas, CD137, TRAILR, and TWEAKR mRNAs in the AMI patients were significantly up-regulated ( $P<0.01$ ), while TWEAK, CD27, CD40, and BAFFR expressions were significantly down-regulated ( $P<0.05$ ). Between the AMI and the SA patients, OX40L, LIGHT, TNFR1, LT- $\beta$ R, CD137, TRAILR, and TWEAKR mRNAs in the AMI patients were significantly up-regulated ( $P<0.05$ ), while OX40, TWEAK, CD40, CD27, and BAFFR mRNAs were significantly down-regulated ( $P<0.01$ ). However, there was no significant difference between the SA and the control group in mRNA expressions of either TNF or TNF receptors.

## Discussion

IFNs are classified into two major groups: type I and type II. Type I IFNs include IFN $\alpha$ , IFN $\beta$ , IFN $\epsilon$ , IFN $\omega$  and IFN $\kappa$ . Type I IFNs use a heterodimeric receptor composed of the IFNAR1 and IFNAR2 chains, whereas type II IFNs use a receptor formed by IFNGR1 and IFNGR2 [8]. IFNs possess anti-virus and immunomodulatory effects via JAK/STAT signaling pathway after binding to corresponding receptors [9]. Researchers found that IFNs played important roles in activation, differentiation and maturation of T lymphocytes and mononuclear macrophages [10].

IFNs promoted expressions of MHC-I and MHC-II molecules on the surfaces of macrophages to kill the pathogens [11]. It was reported that both human and animal IFN receptor deficiency models were prone to various kinds of pathogens infections [12, 13]. The specific immune response to virus infections and/or autoimmune diseases is the detection of gene expressions of type I IFN in PBMC [14]. In the present study, gene expressions of IFN $\alpha$ 2 and all type I IFN and type II IFN receptors were significantly up-regulated in the AMI patients, and no patients had the histories of tumors or autoimmune diseases, which indicated the potential pathogen infections (especially virus infections) in the AMI patients, and the enhanced activation of T cells and macrophages. However, there was no significant difference between the SA patients and the controls in IFNs expressions.

The precursor T cells can be differentiated into Th1 or Th2 cells [15]. Th1 cells achieve cellular immunity mainly by secreting IL2, IL12 and IFN- $\gamma$ , while Th2 cells produce IL4, IL5, IL6 and IL10 to promote the differentiation, maturation and proliferation of B lymphocytes and the generation of antibodies. The shift in Th1/Th2 balance leads to immunity dysfunction. Our results showed the high mRNA expressions of Th1 cytokines (IFN- $\gamma$  and IL2) and low RNA expressions of Th2 cytokines (IL4 and IL10) in the AMI patients, suggesting a shift towards Th1 dominance in the AMI patients. Th17 is also a major group of Th cells and the cytokines IL17A, IL17F, IL21, and IL22 secreted by Th17 cells can promote antigen-specific immunity crucial for the defense against bacterial and fungal invasion at the skin and mucosal surfaces [16, 17]. Some researchers demonstrated that the Th1 cytokines suppress the differentiation of Th17 cells, and studies in mice showed that the differentiation of Th17 and Th1 cells was generally thought to be mutually exclusive [16]. In the present study, gene expressions of IL17A, IL17F, IL21 and IL22 were down-regulated in the AMI group, indicating the potential Th17 cells insufficiency in the AMI patients. Thus, the unbalance gene expressions of Th1, Th2 and Th17 cells suggested an immune dysfunction in the AMI patients may exist.

The neutrophil is an important component of the innate immunity. CXC chemokines and



related receptors have strong effects on chemotaxis and the activation of neutrophils to migrate to the infected or injured parts [18, 19]. In this study, CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, CXCR1, CXCR2, and CXCR4 mRNA expressions in the AMI group were significantly higher than the controls, suggesting the increased mobilization and recruitment of neutrophils in the AMI patients. However, there was no difference in CXC chemokine expressions between the SA and control group, indicating there was no chemotaxis and activation of neutrophils in the SA patients.

The CC chemokines, CCL3 (MIP-1 $\alpha$ ), CCL4 (MIP-1 $\beta$ 4) and CCL5 (RANTES), which are HIV inhibitory factors secreted by CD8<sup>+</sup>T cells, have anti-virus effects by combining with CCR5 [20, 21]. During the chronic infections, virus-specific CD8<sup>+</sup>T cells generated low level of cytokines and the cytotoxic ability of CD8<sup>+</sup>T cells was decreased in CCL5 knockout mice [22]. Recent researches showed that CCR5 promoted the NK cells proliferation and enhanced its cytotoxic ability [23], meanwhile CCR5 deficiency humans were susceptible to virus [24, 25]. As a part of the innate immune system, CCL28 takes antimicrobial activity independently [26, 27]. In the present study, CCL3, CCL4, CCL5, CCR5 and CCL28 mRNA expressions were lower in the AMI patients than the controls, which indicated the reduced anti-virus ability and increased susceptibilities to external pathogens may exist in the AMI patients. However, there was no significant difference between the SA patients and the controls in chemokine expressions.

TNF/TNFR superfamily plays a significant role in immune responses [6]. Fas, an apoptosis factor, can result in B lymphocytes direct apoptosis [28]. TNFR1 ligand pathway could induce activation-induced T cell death, and aberrant T cell activation in the stable heart transplantation patients [29]. Tsakiri found that TNFR2 signals could inhibit the activation of Treg cells in mice [30]. CD137, the co-stimulatory molecules of T lymphocytes, can inhibit the activities of both CD4<sup>+</sup>T lymphocytes and NK cells [31, 32]. In the present study, FAS, TNFR1, TNFR2 and CD137 mRNA expressions in AMI patients were significantly up-regulated, when compared with the controls, showing the decreased or suppressed activities of the immune cells and an

unbalanced immune function in the AMI patients.

Wealth of data implicated TWEAK as a disease-susceptibility gene for a humoral immunodeficiency, and a low expression of TWEAK may lead to antibody deficiency [33]. CD40/CD40L interaction was important for promoting T-cell-mediated immunity, and people with CD40 deficiency were always accompanied with both dysfunction of T cell and dendritic cells [34]. OX40 and CD27 were also co-stimulatory factors of T lymphocytes, which regulated the proliferation and survival of CD4<sup>+</sup> and CD8<sup>+</sup>T lymphocytes via binding to their ligands, so OX40 and CD27 deficiency would lead to the dysfunction of T cells immunity [35, 36]. It was reported that BAFFR deficiency could result in decrement of transitional and follicular B lymphocytes in mice [37]. In our present study, TWEAK, CD40, OX40, CD27 and BAFFR mRNA expressions in the AMI group were significantly down-regulated while compared with the SA and/or control groups, which revealed the dysfunction of immune system may exist in the AMI patients. There was no significant difference between the SA and control group in TNF/TNFR superfamily expressions.

Taken together, the present study indicated that the dysfunction of the immune system in the AMI patients may be an internal factor of AMI pathogenesis, and the pathogen infections, acting as a trigger in the process of AMI could be an external cause. The combined effect of internal and external factors may lead to the occurrence of acute myocardial infarction. However, no activation of CK network was observed in the SA patients, showing the significant differences of immune functions between the stage of AMI and SA.

### *Limitation*

First is that the statistical difference of the age in the control group. The aging may affect the immunity response, but we just consider the young people as the group with normal immunity, and also between the SA and control groups the mRNA expressions of the immunity showed no significant difference. Secondly, we proposed that impaired immunity was related with the AMI patients and may be an internal factor of AMI pathogenesis. However, proof of further in-vitro studies are required.

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## Disclosure of conflict of interest

None.

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