

Original Article

Differential expression of T cell-related genes in AMI and SA stages of coronary artery disease

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Abstract: Objective: To identify differentially expressed T cells-related genes in peripheral blood mononuclear cells and compare their differences in T cell activation and subset functions in different stages of coronary atherosclerosis disease (CAD). Methods: 20 patients with acute myocardial infarction patients (AMI), 20 patients with stable angina pectoris (SA) and 20 healthy volunteers were recruited into the study. Whole human genome microarray analysis was used to detect the expression of T cell related genes among three groups. Results: mRNA expression of 68 genes involved in T cell was detected. 1) Antigen recognition: in the AMI patients 12 genes were down-regulated and 9 were significantly down-regulated among all 13 genes, compared with those of the SA and the control group, respectively. 2) Co-stimulators and regulators of T cell activation: among 16 genes in the AMI patients, 15 genes were lower and 8 were significantly lower than the other two groups. 3) T cell subsets, CTL: all 11 genes in the AMI patients were down-regulated, particularly GZMM and CASP8 were significantly down-regulated compared with the SA patients and controls. Th1/Th2: in the AMI patients, gene expressions including IL1 and IL18 were significantly higher, whereas GATA3 mRNA was significantly lower than those in other two groups. Th17/Treg: in the AMI group, RORC and CCR6 mRNAs were significantly down-regulated compared with the control group, while CD25 and CD127 expressions were significantly lower than SA group. There was no difference in T cell related genes between the SA patients and the controls. Conclusions: In the AMI patients, the mRNA expression of T cell antigen recognition, activation and subset functions was imbalanced or decreased, indicating the dysfunction of cellular immunity in patients with AMI. Then improving T cell mediated cellular immunity may be considered as a potential target for medical interventions in the patients with AMI.

Keywords: Myocardial infarction, stable angina pectoris, T cell, gene expression, cellular immunity

Introduction

Coronary atherosclerosis disease (CAD) is a leading cause of mortality in many countries, and increasing evidence has demonstrated that atherosclerosis is a chronic inflammatory disease, in which innate and adaptive immunity operate together in the progression of lesion [1]. T cells are a key component of the adaptive immune system and plenty of data from human beings and mice suggest an essential role for T-cell-mediated immunity in atherosclerosis [2-4]. T cells can be divided into helper T cells (T helper cells, Th), cytotoxic T cells (cytotoxic T cell, CTL) and regulatory T cells (regulatory T cell, Treg). Various subsets of T cells can be found in the plaque rupture of coronary vessels [5-7], and a number of animals models show different subsets of T cells can drive or dampen inflammatory processes [8-10] and some re-

sults were controversial. For example, CTL and the balance of Th17/Treg subsets [7, 11-14]. So quite a few issues on various T cell subset functions in human are still need to be uncovered especially in different stages of CAD, among which the acute myocardial infarction (AMI) and stable angina pectoris (SA) are most common cases [15, 16].

In this present study, we examined the expression of T cell related genes involved in T cell antigen recognition, activation and subset functions using human cDNA microarray analysis to identify gene expression differences in T cells in PBMCs isolated from AMI, SA patients and controls. We designed this *in vitro* study to investigate the differential gene expression of T cell activation and subsets functions, and then analyze the differences on cellular immunity in patients with AMI and SA.

T cell-related genes in AMI and SA

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

	AMI (a) (N=20)	SA (b) (N=20)	Con (c) (N=20)	P (all)	P (a v 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 ²)	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

Materials and methods

Patient information

This prospective study included three groups of patients with a total of 60 subjects, 20 patients with AMI, 20 with SA, and 20 healthy volunteers. The baseline demographic data are displayed in **Table 1**. The AMI patients were admitted <12 hours from the onset of symptoms to our coronary care unit between January and June 2013, included 18 males and 2 females, with an average age of 58±12 years. All AMI subjects were diagnosed on the basis of following criteria: [17] detection of a rise of cardiac biomarker values [preferably cardiac troponin (cTn)] with at least one value above the 99th percentile upper reference limit (URL) and with at least one of the following: 1) symptoms of ischemia. 2) New or presumed new significant ST-segment-T wave (ST-T) changes or new left bundle branch block (LBBB). 3) Development of pathological Q waves in the ECG. 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 males, 2 females, mean age 64±10 years) with exclusively effort-related angina were studied, with a positive exercise stress test and at least one coronary stenosis detected at angiography (>70% reduction of lumen diameter). There were no significant differences between AMI and SA patients in term of age, gender, smoking status, BMI, systolic blood pressure, diastolic blood pressure, LDL-C, triglycerides, HDL-C and fasting plasma glucose(FPG) (**Table 1**).

The control group included 20 volunteers (17 males and 3 females, mean age 29±3 years) enrolled during the same period with comparable male/female ratio. Histories, physical examination, ECG, chest radiography and routine chemical analysis showed the controls had no evidence of coronary heart diseases.

The exclusive criteria for three groups were as follows: venous thrombosis, history of severe renal or hepatic diseases, hematological disorders, acute or chronic inflammatory diseases 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 (USA). A microarray is composed of more than 41000 genes or transcripts, including targeted 19,596 entrez gene RNAs. Sequence information used in the microarrays was derived from the latest databases of RefSeq, Goldenpath, Ensembl and Unigene. The functions of more than 70% of the genes in the microarray are already known. All patients were subjected to the chip analysis.

Total RNA isolation

5 ml of peripheral blood samples with PAXgene tube were drawn 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. It was further checked for a RIN number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). The sample was considered

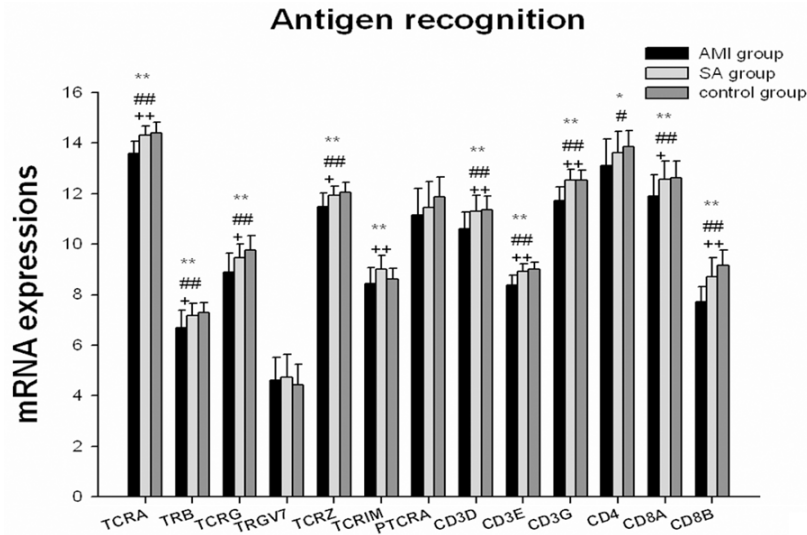


Figure 1. Expression of genes related to antigen recognition in T cell activation in PBMCs from three groups. Three groups: *P<0.05; **P<0.01. a. v c: #P<0.05; ##P<0.01. b. v c: &P<0.05;&&P<0.01. a v b: +P<0.05; ++P<0.01.

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 the 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-ΔΔCt). The melting curve and the 2-ΔΔCt-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 ± S.D. Differences between groups were examined by one-way analysis of variance (ANOVA). Pair-wise group comparisons after ANOVA were performed using Tukey's multiple comparison technique. Data were analyzed using SPSS 17.0, and p-values <0.05 were considered statistically significant.

Results

Expression of genes related to T cell receptor (TCR) antigen recognition

Expressions of 13 genes related to TCR antigen recognition in patients with AMI, SA and the control group were detected (**Figure 1**). Expressions of 12 related genes in AMI were down-regulated among three groups, and the genes encoding TCRA, TRB, TCRG, TCRZ, CD3D, CD3E, CD3G, CD8A and CD8B were significantly down-regulated (P<0.05) compared with the

qualified when 2100 RIN ≥7.0 as well as 28S/18S ≥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), following the 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 µg 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), following the manufacturer's instructions. After 17 hours of hybridization, slides were washed in staining dishes (Cat#121, Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat#5188-5327, Agilent technologies, Santa Clara, CA, US), according to 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 µm, 20 bit.

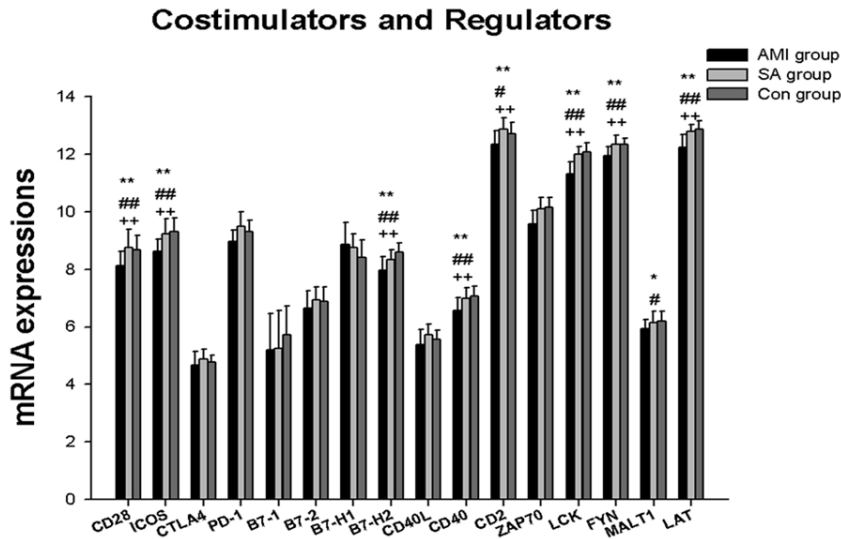


Figure 2. Expression of genes related to T cell receptors and regulators in T cell activation in PBMCs from three groups: *P<0.05; **P<0.01. a v c: #P<0.05; ##P<0.01. b v c: &P<0.05; &&P<0.01. a v b: +P<0.05; ++P<0.01.

expressions of GZMM and CASP8 in AMI patients were significantly down-regulated (P<0.05). Compared with controls, PRF1 mRNA in AMI group was significantly down-regulated (P<0.05), and GZMK expression in AMI group was significantly lower than in SA group (P<0.01). Between SA and control group, there was no statistical difference in CTL related mRNA expression.

Expression of genes related to Th1/Th2

SA patients and controls respectively. Comparing with controls, CD4 mRNA in AMI was significantly down-regulated (P<0.05), and TCRIM expression in AMI group was significantly lower than that of SA group (P<0.01). The TCR related genes in SA and control group showed no significant difference.

The results showed that mRNA expressions of 15 genes related to Th1/Th2, including cytokines, transcription factors and chemokine receptors in PBMCs from AMI, SA and the control group were detected (**Figure 3B**). Among three groups, expressions of the genes encoding IL1, IL2, IL18, CCR5, IL4, GATA3 and CRTH2 were significantly different (P<0.05). Comparing with controls, gene expressions of IL1, IL2 and IL18 in AMI group were significantly up-regulated (P<0.05), while CCR5, IL-4 and GATA3 mRNA expressions were down-regulated (P<0.05). When comparing with SA group, in AMI patients, IL1 and IL18 genes were significantly higher (P<0.01), while CCR5, GATA3 and CRTH2 genes were significantly lower (P<0.01). Between SA and control group, there was no significant difference in Th1/Th2 related mRNAs.

Expression of genes related to co-stimulators and regulators of T cell activation

Expression of genes related to Th17/Tregs

Expressions of 16 genes related to co-stimulators and regulators of T cell activation in PBMCs from patients with AMI, SA and the control group were examined (**Figure 2**). In AMI patients, 15 genes were lower and 8 encoding CD28, ICOS, B7-H2, CD2, CD40, LCK, FYN and LAT were significantly lower than the other two groups (P<0.05). MALT1 expression in AMI group was significantly lower than in controls (P<0.05). Between SA and control group, there was no significant difference in T cell activation related expressions.

mRNA expressions of 13 genes related to Th17/Treg, including cytokines, transcription factors and chemokine receptors in PBMCs from AMI, SA and the control group were examined (**Figure 3C**). In PBMCs from three groups, expressions of genes including RORC, CCR6, CD25 and CD127 were significantly different (P<0.05). Gene expressions of RORC and CCR6 in AMI group were significantly down-regulated when compared with controls (P<0.05). CD25 and CD127 mRNAs in AMI were significantly lower (P<0.01) than those in SA group. Between SA and control group, there was no significant

Expression of genes related to CTL

The results showed that expressions of 11 genes related to CTL in patients with AMI, SA and the control group were detected (**Figure 3A**). In PBMCs from three groups, all the 11 genes in AMI group were down-regulated, including the genes encoding GZMK, GZMM, PRF1 and CASP8 (P<0.05). Compared with SA patients and controls respectively, mRNA ex-

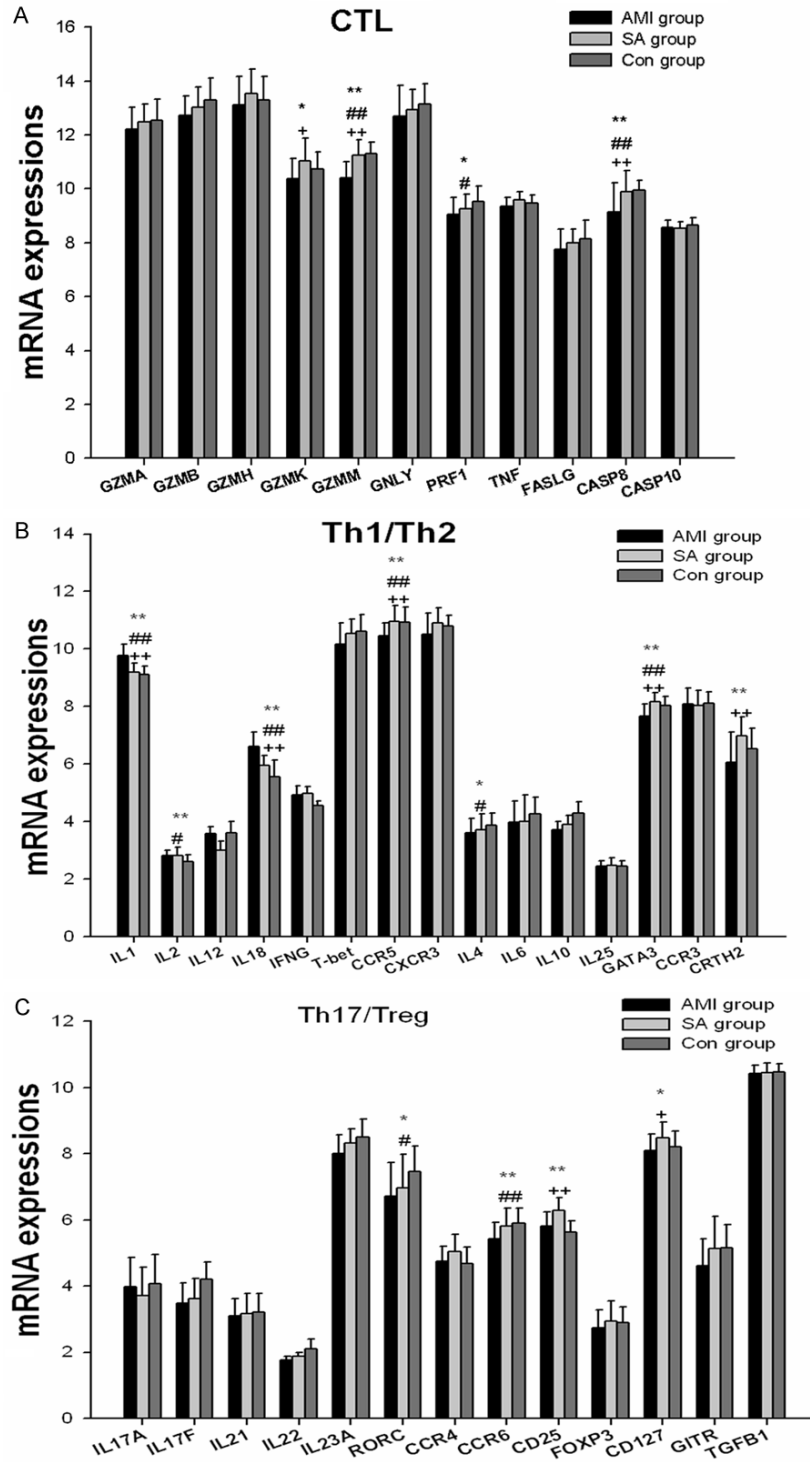


Figure 3. A. Expression of genes related to CTL in PBMCs from three groups. B. Expression of genes related to Th1 and Th2 in PBMCs from three groups. C. Expression of genes related to Th17 and Treg in PBMCs from three groups. Three groups: *P<0.05; **P<0.01. a v c: #P<0.05; ##P<0.01. b v c: &P<0.05; &&P<0.01. a v b: +P<0.05; ++P<0.01.

difference in Th17/Treg related mRNA expression.

was no significant TCR antigen recognition mRNAs difference, which revealed the different

Discussion

Expression of genes related to TCR antigen recognition

TCR is a molecule found on the surface of T lymphocytes that is responsible for recognizing antigens bounded to major histocompatibility complex (MHC) molecules. The TCR is composed of alpha (α)/beta (β) or gamma (γ)/delta (δ) heterodimers. T cells require two signals to become fully activated. The first signal, which is antigen-specific, is provided through the TCR-CD3 complex which interacts with peptide-MHC molecules on the membrane of antigen presenting cells (APC) [18, 19]. Meanwhile, this signal is enhanced by a specific co-receptor. On helper T cells, this co-receptor is CD4 who is specific for MHC class II. On cytotoxic T cells, the co-receptor is CD8 which is specific for MHC class I [20]. In our present study, gene expressions of TCRA, TRB, TCRG, TCRZ, CD3D, CD3E and CD3G were significantly lower in AMI group (Figure 1), showing the decreased ability of TCR antigen recognition. Meanwhile, the significantly down-regulated of CD4, CD8A and CD8B mRNAs suggested the T cell co-activation signal was attenuated. Taken together, the first T cell activation signal was weakened in AMI patients. Between SA and control group, there

T cell-related genes in AMI and SA

TCR antigen recognition activity in AMI and SA patients.

Expression of genes related to co-stimulators and regulators of T cell activation

A second signal, the co-stimulatory signal for T cell activation, is antigen nonspecific and is provided by the interaction between co-stimulatory molecules expressed on the membrane of APC and T cells. The important co-stimulatory receptors expressed by T cells are the molecules of the CD28 family [21], including CD28, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), inducible co-stimulator (ICOS) and program death-1 (PD-1), which play a critical role in controlling T cell activation, proliferation and tolerance. CD28 and ICOS are both activated receptors, while CTLA4 and PD-1 are inhibitory receptors. These receptors recognize the B7 family, comprising B7-1 (CD80), B7-2 (CD86), B7-H1 (CD274) and B7-H2 (ICOSL), which are typically expressed by the APC [22]. The CD40 ligand (CD40L)/CD40 pathway is also involved in co-stimulation of T cell activation [23]. In our present study CD28, ICOS, B7-H2 and CD40 mRNAs were significantly lower in AMI patients, demonstrated the second T cell activation signal was blocked. When the TCR engages with the two activating signals, a series of regulators, including co-receptors, associated enzymes and specialized adaptor molecules are also involved in T cell activation. The binding of CD2 co-receptor with its ligand is a non-specific pathway for T cell activation [24]. Zeta-associated protein of 70 kDa (ZAP-70) is the key enzyme in early T cell enzymatic reaction signal [25]. The Src-family kinases, Lck and Fyn, are central to the initiation of TCR signaling pathways and influence T cell activation and differentiation [26, 27]. Recent findings define MALT1 (mucosa-associated-lymphoid-tissue lymphoma-translocation gene 1) as a protein with proteolytic activity that controls T cell activation by regulating key molecules in TCR induced signaling pathways [28]. The adaptor protein LAT serves as an integration node for signaling pathways that drive T cell activation [29]. In our study expressions of the genes including LCK, FYN, MALT1 and LAT were significantly lower in AMI patients than those in control group, demonstrating that T cell activation signal was inhibited in AMI patients. There was no significant gene expression difference in T cell activation between SA and controls, suggested the decreased ability

of T cells activation only occurred in the stage of AMI patients.

Expression of genes related to CTL

CTLs kill virus-infected cells and tumor cells, and play a critical role in immune protection. Specific CTL killing involves three steps including antigen recognition, activation and fatal attack [30]. CTL is activated by TCR and CD8 binding (the first signal) and co-stimulatory molecules (the second signal). The cytoplasmic granules in CTLs contain proteins perforins and granzymes. When the CTLs bind to their targets, the contents of the granules are discharged by exocytosis [31]. Another way of CTLs killing is through the FasL expressed on CTL surface and the secretion of TNF- α . When CTL recognize their targets, they bind with the Fas and TNF receptors on the surface of target cells and lead to their apoptosis through caspase signals [32]. The presence of CTL cells in atherosclerotic lesions is widely demonstrated but studies investigating their role in atherogenesis have yielded contradictory results [7, 11]. In our study, all 11 genes related to CTL killing ability in AMI patients were down-regulated, especially GZMK, GZMM, PRF1 and CASP8 were significantly down-regulated while compared with SA and/or controls. As mentioned before, the TCR antigen recognizing and two signals of T cell activation were weakened in AMI patients. However, there was no significant difference in CTL related mRNA expression between SA and control group. All the results suggested the significantly different CTLs killing ability in the stages of AMI and SA.

Expression of genes related to Th1/Th2

After stimulation by several cytokines, naive CD4+T cells differentiate into effector T cells, such as the T helper type 1 (Th1), T helper type 2 (Th2), and T helper type 17 (Th17) lineages [33]. Th1 cells achieve cellular immunity mainly by secreting IL1, IL2, IL12, IL18 and IFN- γ . T-bet is a Th1 transcription factor for regulating Th1 development [34], and CCR5 and CXCR3 are specific Th1 lymphocytes chemokine receptors [35, 36]. Th2 cells produce IL4, IL5, IL6, IL10 and IL25 to activate B lymphocytes and generate antibodies. GATA3 is the Th2 specific transcription factor, and CCR3 together with CCR4 are chemokine receptors of Th2 [37-39]. The shift in Th1/Th2 balance leads to immunity dysfunction. Our results showed in AMI patients,

the high mRNA expressions of Th1 cytokines (IL1, IL2 and IL18) and low RNA expressions of Th2 cytokines, transcription factor and chemokine receptors (IL4, GATA3 and CCR2), suggesting a shift towards Th1 dominance in AMI. And this result is consistent with the experiments of Soltesz and Zhang [40, 41].

Th17 cells mainly produce cytokines IL17A, IL17F, IL21 and IL22, and have been observed in atherosclerotic plaques both in humans and animals [42]. Th17 cells can promote antigen-specific immunity crucial for the defense against bacterial and fungal invasion at the skin and mucosal surfaces [43, 44]. RORC is the specific transcription factor of Th17 [45], and CCR4 and CCR6 are chemokine receptors of Th17 [46, 47]. It has been demonstrated that the Th1 cytokines suppress the differentiation of Th17 cells, and studies in mice showed that the differentiation of Th17 and Th1 cells is generally thought to be mutually exclusive [43]. In our present study, in AMI group gene expressions of IL17A, IL17F, IL21, IL22, IL23A, RORC, CCR4 and CCR6 were down-regulated, especially RORC and CCR6 were significantly down-regulated, while no significant difference were observed between SA and control, which indicated the potential insufficiency of Th17 activity in the stage of AMI.

Tregs that possess anti-inflammatory properties have also been reported in atherosclerotic plaques [11, 12]. Tregs are a minor subpopulation of T lymphocytes that maintain self-tolerance to autoantigens and suppress the activity of proatherogenic effector T cells taking an atheroprotective effect [48]. CD25, FOXP3 and CD127 are the specific surface markers for Tregs [49, 50]. In addition, other various surface markers such as CD3, CTLA-4 and GITR are also involved in the function of Tregs [51]. Several cytokines (including IL-10 and TGF- β) secreted by Tregs can inhibit APC activity [52]. In our study, in AMI group the expressions of CD25, FoxP3, CD127, GITR and TGFB1 were lower, while CD25 and CD127 were significantly lower than SA group, suggesting the dysfunction of Tregs.

Nowadays several studies suggested the essential role of Th17/Treg imbalance in the destabilization of CAD, whereas the data from the patients with SA were controversial [11-15]. In our present study, in AMI group the both

down-regulated gene expressions of Th17 and Tregs, showing the imbalance of Th17/Tregs in AMI stage. No significant Th17 and Tregs related gene expression differences were observed between SA and control group, which matches with the results of Ammirati and Potekhina [53, 54]. Thus, the unbalance gene expressions of Th1/Th2 and Th17/Tregs suggested the different T cells activity in the AMI and SA stages of CAD, and an immune impairment only exist in AMI patients.

Conclusions

In AMI group the TCR antigen recognition, binding, as well as the co-stimulators and regulators of T cell activation related gene expressions were all significantly down-regulated, showing the T cell activation was inhibited in the AMI patients. The lower expression of genes related to CTLs showed the decreased killing ability of CTL in the stage of AMI. The significantly differential expression of cytokines, transcription factors and chemokine receptors related to T cell subsets suggested an enhanced immune response in Th1, whereas a weakened response in Th2, Th17 and Tregs. However, there was no statistical gene expression difference in T cell related genes between the SA and control group. Therefore, the significantly different T cell expressions in the AMI and SA patients indicated the different cellular immunity in these two stages of CAD. The reduced T cell activation and the dysfunction of T cell subsets were observed in the AMI stage in our study. As a consequence, improving T cell mediated cellular immunity may be considered as a potential target for medical interventions in the patients with AMI.

Limitations

It should be pointed out that the present study was subjected to several limitations. First is that the statistical difference of the age in control group. The aging may affect the immune status, but we just choose the young people as the group with normal immunity, and also between the SA and control groups the mRNA expressions of cellular immunity showed no significant difference. Secondly, we proposed that impaired cellular immunity was related with the AMI patients. However, the phenomenon requires further *in-vitro* studies to prove that T cell dysfunction is presented in AMI patients.

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Disclosure of conflict of interest

None.

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