

Article

Myosin Specific-T Lymphocytes Mediated Myocardial Inflammation in Adoptive Transferred Rats

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Myosin specific-T lymphocytes might mediate myocardial inflammation and remodeling after AMI. Myosin-activated or unactivated T lymphocytes *in vitro* were transferred into naïve syngeneic rats, respectively. T lymphocyte infiltration and myocyte apoptosis were explored by the H&E and TUNNEL. Proteins and mRNA levels of cytokines (IL-1 β , IL-6 and TNF- α) in myocardium were determined by RT-PCR and immunohistochemistry. T lymphocyte infiltration was evidently observed after one week of activated T cell transfer. The expressions of cytokines were elevated markedly one week later. The myocyte apoptosis occurred after T lymphocyte infiltration in myocardium. Our findings suggest that cardiac myosin activated-T lymphocytes may mediate myocardial inflammation and remodeling. *Cellular & Molecular Immunology*. 2006;3(6):445-451.

Key Words: myocardial inflammation, myosin, apoptosis

Introduction

Acute myocardial infarction (AMI) is one of the main diseases harming the human health. AMI can cause the ischemia and necrosis of myocytes, in which normally larvaceous antigens or determinants were exposed, then induce the autoimmune response. Cardiac myosin is the major structural protein of the myocardium. In rodent models, the role of cardiac myosin as an autoantigen in the pathogenesis of autoimmune myocarditis has been well established (1, 2). Evidence has been given about the myocardial damage mediated by lymphocyte in the Vardaboom's experiment, in which he put the spleen lymphocytes from post-AMI rats together with normal cardiocytes to co-culture *in vitro*, and found that the spleen lymphocyte proliferation obviously rose, and their cytotoxicity to normal cardiocytes significantly reinforced as

well (3).

Myosin heavy chain fragments can be determined in serum and myocardial necrotic zone in patients and dogs with AMI by radiolabeled anti-myosin antibodies (AMA) (4-7). Our studies found that the mortality of the post-AMI patients, the attenuated extent of the action of ventricular wall and the formation of ventricular aneurysm were strikingly higher in the AMA positive group than those in the negative group (8). The detecting of AMA in the AMI patients' serum suggests the occurrence of the myosin-specific autoimmune response after AMI in the organism. It indicates that the myosin is a major autoantigen that triggers the autoimmune response after AMI.

The previous study showed that there were massive interdigitating dendritic cells (iDCs) accompanied by a small cluster of T-helper lymphocytes at the edge of the infarction zone in the experimental AMI rats. It suggests that iDCs migrate to the edge of infarction zone to participate in the activation of lymphocytes and the beginning of the immune response (9).

We therefore hypothesized that T lymphocytes were activated by cardiac myosin and dendritic cells (DCs) that might mediate myocardial inflammation and remodeling after AMI.

Materials and Methods

Rats and reagents

Lewis rat, syngeneic inbred (Wei Tong Li-hua, Beijing, China), between 6 and 8 weeks of age. For all experiments, Lewis rats were used by age-matched. The investigation conforms the *Guide for the Care and Use of Laboratory*

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Animals published by the US National Institutes of Health (NIH Publication, revised 1996). Nycodenz (AXIS-SHIELD PoC AS, Oslo, Norway) was used for purification of splenic DCs. Monoclonal antibodies were used for immunohistochemical analysis. Rat T cell enrichment column (R&D) was applied to enriched T lymphocytes. Monoclonal antibodies of anti-rat cytokines: anti-rat IL-6 and TNF- α antibodies were purchased from R&D, and anti-rat IL-1 β was purchased from Serotec (Oxford, UK).

Dendritic cells and T lymphocytes preparations

According to the method of Frans et al. (10), splenic DCs were enriched by using a 14.5% (w/v) Nycodenz density gradient ($800 \times g$ for 20 min). T lymphocytes from Lewis rats were enriched using T cell enrichment columns of rat. According to procedure for use of columns, CD3⁺ T cells were enriched and the purity was routinely over 95%.

Activated T lymphocytes and adoptive transfer

T lymphocytes and DCs were co-cultured at a ratio of 1 DC per 10 T cells (11, 12) in the presence or absence of cardiac myosin (20 $\mu g/ml$) in T-50 flasks. The dose of myosin was elicited by advance experiment. The flasks were placed in an incubator, 37°C, 5% CO₂ for 5-7 days. Then, DCs were separated from T cells by using a 14.5% (w/v) Nycodenz density gradient ($800 \times g$ for 20 min). Activated T cells (CD25⁺) were monitored by flow cytometry and their percentage was routinely > 90%. While the syngeneic rats were lightly anesthetized, about $1.5-2.0 \times 10^7$ activated and unactivated T cells were injected *via* a single tail vein. The rats were killed after adoptive transfer at day 3, week 1, week 4 and week 8, respectively.

Echocardiography and hemodynamics analysis

Two-dimensional echocardiography was performed and long-axis, short-axis, and subcostal views were obtained. Then the rats were weighed. After anesthesia, the rats were prepared for hemodynamics analysis. Midline incision of neck was adopted to expose right common carotid artery. The artery was dissociated for catheter intubation. Left ventricular catheter filled with heparin solution was inserted into left ventricle through the artery. Left femoral artery was prepared for another catheter intubation. The pressure data gathered from artery and left ventricle together with electrocardiogram were analyzed by a computer based bio-signal analysis system.

Histological preparations

After the thorax was opened, heart was excised and rinsed in several changes of 0.1 M PBS. The atria and artery were resected, and then the left and right ventricles were weighed. Ventricles were dissected at midpoint of length axis and divided into two sections. The section of base ventricular was stored in the liquid nitrogen. The section of cardiac apex was fixed in 4% neutral buffered formalin overnight and embedded in paraffin.

Histopathology

Table 1. Primer sequence of RT-PCR

	Primer	Size (bp)
IL-1 β	5'-CCCATACACACGGACAACCTAGA-3' 5'-ATCCCAAACAATACCCAAAGAA-3'	508
IL-6	5'-AGAGCAATACTGAAACCCTA-3' 5'-CAACATAATTTAGATACCCATC-3'	295
TNF- α	5'-TGGCCCAGACCCTCACA-3' 5'-TGCCCGACTCCGTGAT-3'	360
GAPDH	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCCTGTTGCTGTA-3'	450

The part of cardiac apex was embedded in paraffin and sectioned at a thickness of 5 μm . Sections were stained with H&E. Microscopic findings of the heart sections were graded by blind as follows: 0, normal, no infiltration; 1, mild, < 10% of the tissue section involved; 2, moderate, 10% to 25% of the tissue section involved; and 3, severe, > 25% of the tissue section involved.

Detection of apoptosis

The part of heart was embedded in paraffin and cut at a thickness of 4 μm . The sections were placed on silanized slides and baked at 58°C overnight. After that the sections were deparaffinized according to the method recommended by the In Situ Cell Death Detection Kit. Proteins were digested using 20 $\mu g/ml$ Proteinase K in 10 mM Tris/HCl, pH 7.4-8 for 15 min at 21-37°C. After digestion the slides were washed twice changes of PBS, and then terminal deoxynucleotidyl transferase-mediated digoxigenin deoxyuridine triphosphate end labelling (TUNEL) was performed according to the protocol outlined for the In Situ Cell Death Detection Kit. Apoptosis was discovered using an anti-fluorescein antibody Fab fragments, conjugated with alkaline phosphatase (AP) and converter-AP. Slides were added with substrate solution, incubated for 10 min at 15-25°C in dark, and rinsed 3 times with PBS, then cover-slipped for evaluation by light microscopy.

Immunohistopathology

Myocardium specimens embedded with paraffin were serially sectioned to a thickness of 3 μm , dewaxed and rehydrated. Endogenous peroxidase was blocked with 3% H₂O₂ in PBS at room temperature for 10 min. The sections were heated to 95°C for 10 min to restore antigen activity. Non-specific binding sites were blocked with 5% goat serum in PBS at room temperature for 30 min. Excess serum was removed. The sections were incubated with primary antibodies (anti-rat IL-1 β , IL-6 and TNF- α) at 4°C overnight. After washing in PBS, the slides were incubated with biotinylated second antibody, pre-adsorbed with rat serum, added with strept-avidin-biotinylated horseradish peroxidase complex and then reacted with diaminobenzidine according to the protocol from the company (Santa Cruz). Tap water was used to end the reaction. Caesalpins were used to counterstain nucleus for

30 seconds, and then excess dyes rinsed off. Slices were analyzed under the microscope using the HPIAS-2000 software.

Determination of gene expression by RT-PCR

Total RNA was extracted using the Tri-Zol procedure. mRNA was then isolated using an mRNA isolation kit (TaKaRa Biotechnology) and was reversely transcribed into first-strand cDNA. Cytokine gene expression was analyzed by PCR. The primer sequences to rat IL-1 β , IL-6, TNF- α and GAPDH were displayed in Table 1.

PCR amplification was performed by adding 5 μ l aliquots of cDNA sample to 20 μ l of reaction mixture. Amplification was done in DNA Thermal Cycler under the following conditions: denaturation at 94°C for 30 s, annealing for 30 s (56°C for TNF- α and GAPDH, 60°C for IL-6; 51°C for IL-1 β), and extension at 72°C for 45 s, with final extension at 72°C for 5 min. Based on the results of preliminary studies, 32 cycles were used for and GAPDH, 35 cycles were used for TNF- α , IL-1 β and IL-6. Five microliters of each PCR product were subjected to electrophoresis on agarose gel in Tris acetic acid-EDTA buffer and stained with ethidium bromide. Gels were viewed, and the images were stored digitally by image analysis system (GSD8000, UVP, England). For each cDNA sample, the densitometric units of the amplified cDNA fragments were counted for semi-quantitatively evaluation by normalization with the GAPDH band.

Statistical analysis

All results for numerical variables are expressed as median \pm SD. Comparisons between groups were analyzed by paired-sample *t* test (2-sided). The comparisons among multi-groups were made using one way ANOVA of independent groups to determine the overall difference, and a post hoc Bonferroni/Dunn test was used to determine statistical

Table 2. Histological grades after receiving activated T cells

	Day 3	Week 1	Week 4	Week 8
Grade	0.67 \pm 0.36 [#]	1.87 \pm 0.49* [#]	0.75 \pm 0.52 [#]	0.14 \pm 0.06

* $p < 0.01$, compared with day 3 and week 4, respectively. [#] $p < 0.01$, compared with week 8.

significance between groups. Probability values of $p < 0.05$ were considered statistically significant. All results are managed by SPSS 10.0.

Results

Cardiac T lymphocyte infiltration and myocyte apoptosis in rats transferred by myosin-activated T cells

Configuration of heart did not show change. Histological changes were shown by light microscope, which included diffused T lymphocyte infiltration and occasionally myocardium necrosis. In all of the groups in recipient rats, these changes were more markedly observed after one week, a few on days 3 or after 4 weeks, but none after 8 weeks (Figure 1). The histological changes and the grade of heart sections were shown in Table 2. T lymphocyte infiltration was not observed in the myocardial sections of rats receiving unactivated T cells. In multiple sections of kidney, liver, lung, and brain no cellular infiltration was found. While few or none were detected by the TUNEL method on day 3 and day 7, apoptotic bodies were notably increased after 4 or 8 weeks. The apoptosis was not identified in age, and sex matched hearts of rats receiving unactivated T cells (Figure 1). The finding showed that the myocyte apoptosis occurred after T lymphocyte infiltration in myocardium and it might be mediated by inflammatory cytokines.

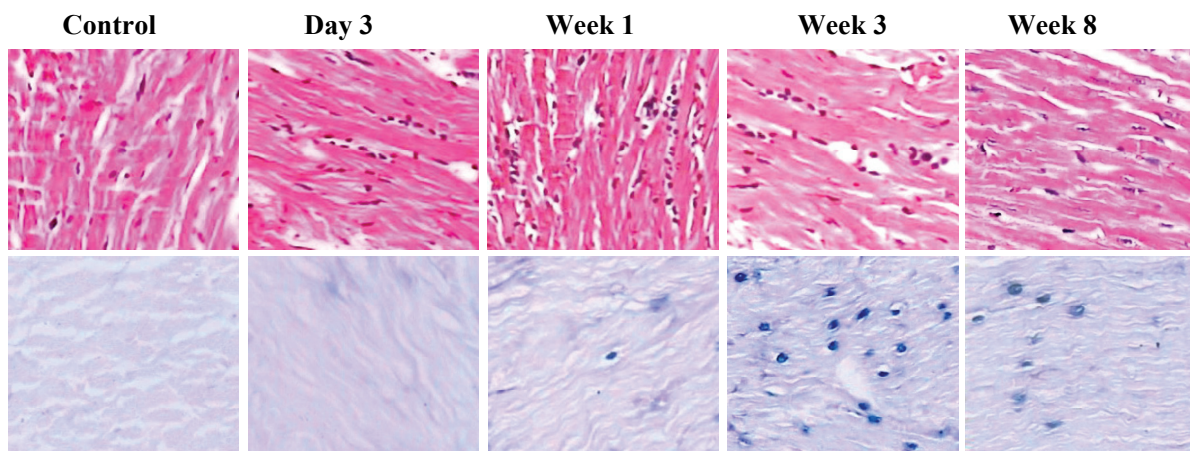


Figure 1. Histopathologic and apoptosis changes in myocardium of rats after adoptive transfer of myosin activated T cells. There were 12 rats in groups of adoptive transfer of activated T cells and unactivated, respectively. T lymphocyte infiltration and myocyte apoptosis were shown (10 \times). Lane up, myocardial section of HE; Lane down, myocardial sections stained to show apoptosis by the TUNNEL method.

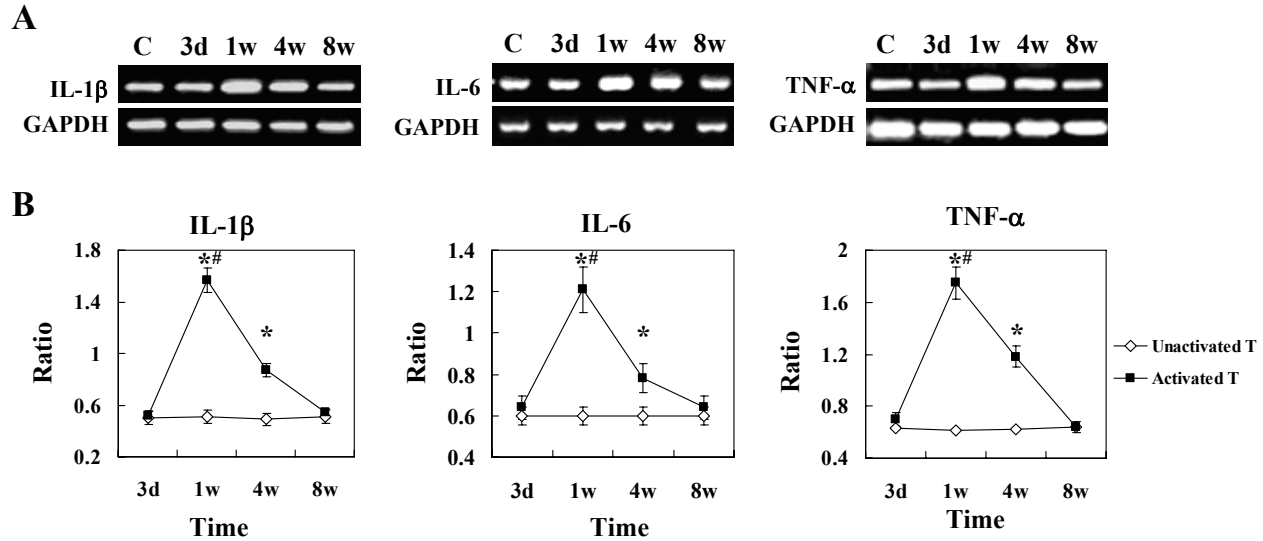


Figure 2. The expression and alterations of cytokine mRNA in myocardium of rats after adoptive transfer of myosin activated T cells. (A) The expression of cytokines was detected at the indicated time, and the relative ratios were shown in (B). * $p < 0.01$, compared with adoptive transfer of unactivated T cells; # $p < 0.01$, compared with adoptive transfer of activated T cells after 4 weeks. There were 12 rats in each group of adoptive transfer of activated or unactivated T cells, respectively.

Myocardial cytokines mRNA and protein expression in rats transferred by myosin-activated T cells

As shown in Figure 2, mRNA expressions of TNF- α , IL-1 β and IL-6 in myocardium were analyzed at day 3, week 1, week 4 and week 8 after receiving activated or unactivated T cells. All of these cytokines' mRNA expression in myocardium of rats receiving activated T cells began up-regulating on day 3, reached the top and gradually down-regulated after one week, but no significant differences were found in the rats receiving unactivated T cells. Just as mRNA expression results, the dynamic changes of protein expression of TNF- α , IL-1 β and IL-6 were detected by immunohistochemistry in myocardium of rats receiving activated T cells (Figure 3). The findings indicated that the development of cytokine expression in myocardium was consistent with T

lymphocyte infiltration and the expressions of myocardial cytokines might be induced by infiltrated T lymphocytes.

Hemodynamics analysis

No abnormality of cardiac structure and function was observed by echocardiography in the rats receiving unactivated T cells, despite of the histological infiltration in these rats receiving activated T lymphocytes. As shown in Table 3, hemodynamics data were analyzed at day 3, week 1, week 4 and week 8 after receiving activated and unactivated T cells. In the rats receiving activated T cells, LV pressure maximal rate of rise and fall (+/-dp/dtmax) were found decreased by the hemodynamics monitor ($p < 0.05$) after 4 and 8 weeks. The +/-dp/dtmax slightly alteration was consistent with myocyte apoptosis, both happened after 4 or

Table 3. Cardiac hemodynamics of rats after adoptive transfer of myosin activated T cells

Time	Transfer	HW/BW (mg/g)	LVEDP (mmHg)	LVSP (mmHg)	+dt/dp (mmHg/s)	-dt/dp (mmHg/s)
Day 3	Unactivated T	3.43 \pm 0.15	5.91 \pm 0.80	138.14 \pm 7.29	5886.1 \pm 143.2	4285.4 \pm 96.2
	Activated T	3.39 \pm 0.24	6.13 \pm 0.83	132.93 \pm 7.02	5884.7 \pm 143.2	4293.7 \pm 82.6
Week 1	Unactivated T	3.40 \pm 0.20	5.89 \pm 0.79	131.34 \pm 6.93	5893.6 \pm 151.4	4327.3 \pm 80.5
	Activated T	3.39 \pm 0.25	6.23 \pm 0.83	136.81 \pm 7.22	5846.2 \pm 143.1	4314.3 \pm 91.7
Week 4	Unactivated T	3.27 \pm 0.17	5.98 \pm 0.81	129.46 \pm 6.84	5769.3 \pm 146.3	4220.7 \pm 71.8
	Activated T	3.35 \pm 0.22	5.96 \pm 0.77	134.77 \pm 7.12	5432.1 \pm 139.1*	3961.3 \pm 71.8*
Week 8	Unactivated T	3.34 \pm 0.19	6.10 \pm 0.82	130.38 \pm 6.88	5770.5 \pm 133.5	4213.7 \pm 79.2
	Activated T	3.30 \pm 0.20	6.07 \pm 0.79	137.11 \pm 7.24	5413.4 \pm 135.7*	3958.3 \pm 87.1*

* $p < 0.05$, compared with adoptive transfer of unactivated T cells (n = 12).

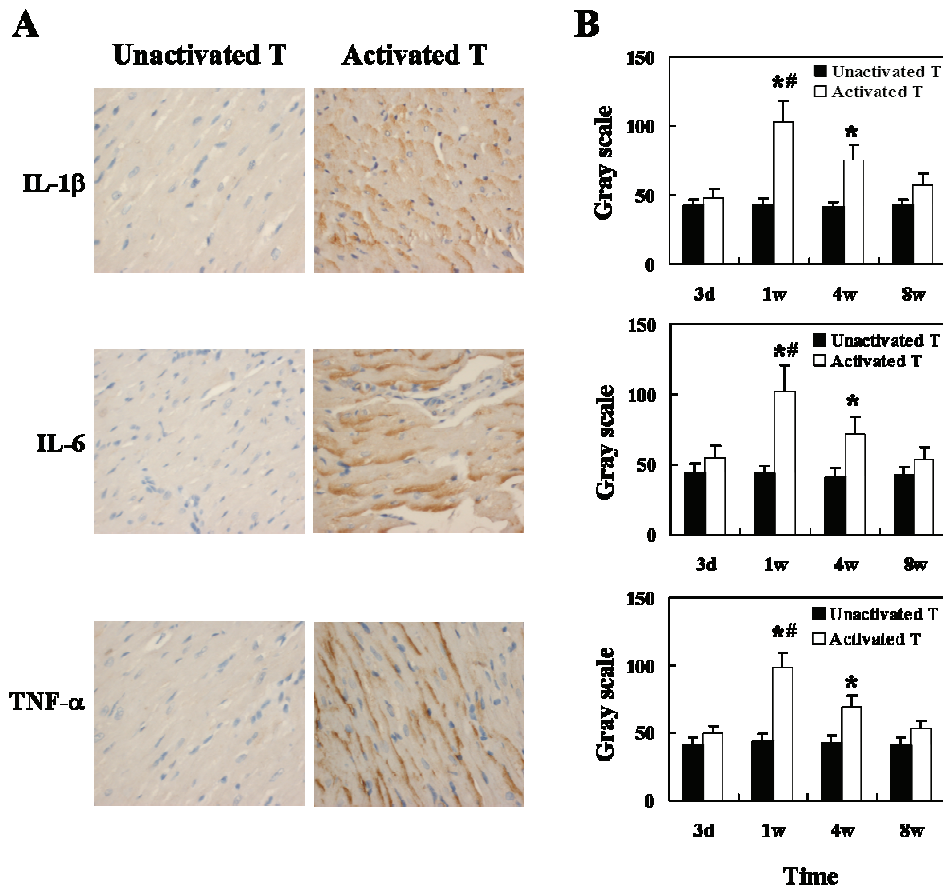


Figure 3. The expression of cytokines in myocardium of rats receiving myosin activated T cells. (A) TNF- α , IL-1 β and IL-6 were detected by immunohistochemistry in myocardium of rats receiving activated or unactivated T cells after one week. (B) The alterations of gray scale of cytokines in myocardium at the indicated time. * $p < 0.01$, compared with adoptive transfer of unactivated T cells; # $p < 0.01$, compared with adoptive transfer of activated T cells after 4 weeks. There were 12 rats in each groups of adoptive transfer of activated or unactivated T cells, respectively.

8 weeks, which means the myocardial remodeling might be mediated by inflammatory mechanism.

Discussion

We imitated that the autoantigen was released to activate immunocytes and cardiac myosin presented by DC *in vitro*, and then cause the activation of splenic T cells of the healthy Lewis rats, which are transfused to syngeneic healthy rats by a single tail vein infusion. After 3 days these transfused rats showed mild organ-specific myocardial inflammation, and the inflammation was the most obvious after 1 week, furthermore, the lymphocyte infiltration was only observed in the myocardium but not in other organs. After transfusion, activated T lymphocytes could identify the cardiac myosin of rat and bring about the immune response aiming at normal myocardium. It suggested that myosin activated T lymphocytes could generate autoimmune response aiming directly at the cardiac myosin.

Myocyte apoptosis was obviously observed in hearts of rats transfused activated T cells after 4 weeks. The process of

myocyte apoptosis occurred after T lymphocyte infiltration and expression of pro-inflammatory cytokines in myocardium. It is prompted that myocyte apoptosis might be induced by cytokines and lymphous toxins from T lymphocytes.

Maisel (13) and our previous study (14) indicated that transferred splenocytes of post-MI rats could lead to organ specific autoimmune myocarditis in recipient rats. When splenic T cells from AMI rats and cardiac myocytes from neonatal rat were co-cultured *in vitro*, T cell proliferation index was significantly higher and can recognize and kill normal cardiomyocytes (1, 15). Activated T cells can excrete IL-2, IL-6 and TNF- α (16), especially TNF- α and IL-6, could induce the apoptosis (17). TNF- α could cause the cultured rat cardiac myocyte apoptosis, and there was a direct relationship between the quantity of the apoptosis cell and the TNF- α concentration (18, 19).

In animal models of AMI, from the first hour to 1 day after infarction, the expressions of cytokines IL-1 β , IL-6 and TNF- α were significantly upregulated in infarction and non-infarction regions (20, 21). Jacobs et al. reported that mRNA expression of TNF- α was detected in the myocardium

of the infarction zone and the periphery in the AMI rat model (22).

On day 3 after the activated T cells was transfused to syngeneic healthy rats, the expression of mRNA and protein of pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α began rising, and the expressions of these cytokines rose significantly after one week, but down-regulated after 4 weeks. After 8 weeks, it was almost as high as the baseline level. It indicated that the expression of the pro-inflammatory cytokines predominates in the myocardial inflammatory reaction of post-transfer. The heart might synthesize TNF- α mRNA and protein and was a target organ for TNF- α (23). Immunohistochemical results also revealed that cytokines principally located in cardiac myocytes. It is suggested that the activated T cells not only mediated myocardial inflammation reaction and produced inflammatory cytokines, but also made myocytes synthesizing and excreting inflammatory factors, induced myocyte apoptosis, and aggravated myocardial damage.

Post-infarction myocardium remodeling corresponds with secretion of the cytokines in the focal heart. The post-MI remodeling process is most commonly associated with TNF- α , IL-1 β and IL-6. The cytokines could affect the ratio of the MMPs/TIMPs, upregulate MMPs, and MMPs could be counter-regulated by tissue inhibitors of MMPs (TIMPs) to certain extent (24-27).

We detected LV pressure maximal rate of rise and fall (+-dp/dt max) decreased mildly by hemodynamics 4 weeks after receiving myosin-specific T cells. There were slight pathological changes in the myocardium, and the myocyte apoptosis was correlative with hemodynamics that was insufficient to induce overt heart function lesion.

In conclusion, our findings suggested that cardiac myosin activated-T lymphocytes might mediate the inflammatory response and expression of pro-inflammatory cytokines in myocardium, and then induce the myocyte apoptosis, reduce the +-dp/dtmax slightly and lead to myocardial injury. The data confirmed that the cardiac myosin was a main trigger of myocardial inflammatory response after acute myocardial infarction. In our previous study, we found that the upregulation of Th1 cell functions might participate in the immune-mediated ventricular remodeling after AMI and atorvastatin could regulate the Th1/Th2 functional imbalance after AMI (28, 29). Further studies should be focused on the effects of cardiac myosin activated-Th1 lymphocytes on myocardial inflammation and remodeling and the establishment of more effective methods to intervene Th polarization after AMI.

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