Monocyte Expression of Toll-like Receptor-4 in Patients with Stable Angina Undergoing Percutanoeus Coronary Intervention

Bahador Bagheri[1], Bahram Sohrabi[2], Aliakbar Movassaghpur[3], Siminozar Mashayekhi[4], Afagh Garjani[5], Mehriar Shokri[2], Mohammad Noori[2], Alireza Garjani[1*]

[1]Department of Pharmacology, Faculty of Pharmacy, [2]Shahid Madani Heart Hospital, [3]Hematology Research Centre, [4]Department of Clinical Pharmacy, Faculty of Pharmacy, [5]School of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

Background: Toll like receptors (TLRs) are well recognized players in inflammatory conditions. Among them TLR-4 is involved in chronic inflammatory processes such as formation of atherosclerotic plaques. **Objective:** The present study was aimed to examine the effects of percutanoeus coronary intervention (PCI) as a revascularization method on monocyte expression of hTLR-4 and on the serum levels of two proinflammatory cytokines (TNF-α and IL-1β). **Methods**: Blood samples were obtained from 41 patients with stable angina who were candidates for PCI. The samples were collected immediately before and 2h and 4h after PCI. The expression of hTLR-4 on $CD14^+$ monocytes and the serum levels of TNF-α and IL-1β were measured using flowcytometry and ELISA techniques, respectively. **Results:** By comparing the frequency of circulating hTLR-4^+/$CD14^+$ monocytes at different time points, it was observed that PCI procedure up regulates the monocyte expression of hTLR-4 ($p<0.05$). The increase in expression was associated with the elevation of the serum levels of proinflammatory cytokines ($p<0.05$). There was a significant correlation between monocyte expression of hTLR-4 and serum levels of TNF-α and IL-1β only before PCI. In spite of parallel increase in the serum levels of proinflammatory cytokines and the monocyte expression of hTLR-4, the correlation did not attain a significant level after PCI intervals. **Conclusion:** PCI is positively associated with an increase in the monocyte expression of hTLR-4. It is also associated with the elevation in the serum levels of proinflmmatory cytokines. These findings suggest that hTLR-4 monocyte expression may be used as a potential prognostic tool in patients with stable angina undergoing PCI.

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Keywords: Inflammation, Percutanoeus Coronary Intervention, Stable Angina, Toll Like Receptor-4

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*Corresponding author: Dr. Alireza Garjani, Department of Pharmacology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran, Tel: (+) 98 411 3341315; (+) 98 914 3014818, Fax: (+) 98 411 334479, e-mail: garjania@tbzmed.ac.ir

INTRODUCTION

Atherosclerosis is considered as a chronic inflammatory process that results in heart attacks. In line with other crucial components of atherosclerosis, immune cells such as monocytes and T cells are believed to play a pivotal role in formation, progression and deterioration of an atherosclerotic plaque (1). Monocyte-derived macrophages are scavenging and antigen-presenting cells and can secrete cytokines, growth-regulating molecules, and metalloproteinases and other hydrolytic enzymes. Interactions between monocytes, Th1 and Th2 cells cause a chronic inflammatory process (1-5). Toll-like receptors (TLRs) are a family of pattern recognition receptors that are important in the regulation of immune responses (6,7). Eleven human TLRs have been identified so far (7). Among them, human TLR-4 (hTLR-4) has prominent role in the development of cardiovascular diseases. hTLR-4 is mainly expressed on the surface of $CD14^+$ monocytes and is frequently observed in lipid-rich area of atherosclerotic lesions (8,9). CD14 is a glycoprotein marker that is not able to transducer a signal. It is chiefly expressed on the surface of monocytes/macrophages and loads ligands on hTLR-4 (10). In addition to exogenous compounds such as the lipopolysaccharide of bacteria, fungi and even bodies of bacteria, there are a number of endogenous ligands that could activate hTLR-4 (11,12). Heat shock protein 60 (13), minimally modified LDL (14) and oxidized LDL (15) have been identified as ligands for hTLR-4. These ligands are abundant in atherosclerotic lesions and can upregulate the expression of hTLR-4. The hTLR-4 sensitization is followed by interaction with an adaptor protein, myeloid differentiation factor (MyD88), and subsequent activation of nuclear factor kappa B (NF-κB). The eventual result is the synthesis of proinflammatory cytokines such as TNF-α, IL1-β and IL-6 (16-18). The enhanced monocyte expression of hTLR-4 in patients with acute coronary syndromes has been shown by a number of investigations (9,19). There is an elevated level of TNF-α in myocardial ischemia and reperfusion which may result in adverse coronary events (20). During acute myocardial infarction, intrinsic IL-1 receptor antagonist (IL1-Ra) is markedly increased and its level correlates with the extent of the infarct size (21). Revascularization with either percutaneous coronary intervention (PCI) or coronary artery bypass surgery (CABG) is indicated for patients with stable angina who are refractory to medical therapy or have high-risk anatomic features (22,23). The present study was designed to investigate the expression of human TLR-4 (hTLR-4) on circulating monocytes in patients with stable angina who underwent PCI. Our goal was to find out an association between hTLR-4 monocyte expression and the serum levels of proinflammatory cytokines.

MATERIALS AND METHODS

Study Subjects. A total of 54 patients with stable angina who were scheduled for a PCI were studied. The exclusion criteria were as follows: previous myocardial infarction within 6 months (5 patients), autoimmune diseases (2 patients), inflammatory conditions (3 patients), advanced hepatic or renal disease , malignant neoplastic disease (1 patient) and those receiving hydrocortisone during PCI (2 patients). Cardiovascular risk factors, medications, sex, age and previous medical history were obtained by a questionnaire and the medical records of the patients. All PCI procedures were carried out according to protocols of Shahid Madani heart hospital, Tabriz, Iran. White blood

cell count, cholesterol, glucose, PT, PTT, BUN, creatinine, sodium and potassium were measured according to routine protocols. The ethical board of Tabriz medical university approved the study and informed written consent was obtained from all participants. Iranian Registry of Clinical Trials (IRCT) officially declared that due to absence of any intervention in routine treatment protocols, it is not necessary to register this study at IRCT.

Blood Collection and Processing. A total 6 ml blood was drawn from patients with a 21-gauge needle by antecubital venipuncture. Two ml of the blood was kept in tubes containing EDTA anticoagulant for flowcytometry and the rest was used for the analysis of biomarkers. Blood collection was done in a time dependent manner at the time of admission (0h), two hours (2h) and four hours (4h) after PCI procedure. Four ml of each blood sample were centrifuged immediately (3000 × g for 5 min) to obtain serum. Serums were kept in -80°C for future analysis.

Measurement of TLR-4 Expression on the Surface of Monocytes. In short, cells were stained for 30 minutes with monoclonal antibodies for human CD14 (Abcam, UK) conjugated with fluorescein isothiocyanate (FITC), and hTLR-4 (Abcam, UK) conjugated with phycoerythrin (PE) at room temperature in the dark. FITC and PE-conjugated non-specific mouse IgG2a antibodies were used as isotype controls (Abcam, UK). Red blood cells were lysed with RBC lysis solution (IQ, the Netherlands) according to the manufacturers' instructions. Cells were washed and centrifuged twice with PBS and cell- associated fluorescence was measured using FACSCalibur flow cytometer (BD Biosciences, US). Data were analyzed with CellQuest software (BD Biosciences, USA).

Measurement of Serum Levels of TNF-α and IL-1β. A sandwich enzyme-linked immunosorbent assay (ELISA) was applied for measurement of TNF-α and IL-1β in serum. A sandwich enzyme-linked immunosorbent assay (ELISA) was performed (Ray Bio, US). In short, 100 µl of serum was added to each well in microtiter plates and incubated for 2.5 hours at room temperature. Then, 100 µl of prepared biotinylated antibody was added to each well and incubated for another hour at room temperature, followed by the addition of 100 µl streptavidin solution and incubation for 45 minutes at room temperature. This was followed by adding 100 µl TMB One-Step substrate and incubation for 30 minutes at room temperature. Finally 50 µl stop solution was added to each well and the color intensity was measured at 450 nm by Stat Fax 2600 (Awareness Technology, USA) plate reader. For washing steps we used Stat Fax 2100 (Awareness Technology, USA).

Statistical Analysis. Data are presented as mean ± SD. The Wilcoxon signed rank test was used to compare differences between hTLR-4 expression and response. The correlations between hTLR-4 and cytokines were determined using Pearson test. Statistical significance was considered as $p<0.05$. All of analyses were performed using SPSS software version 16.

RESULTS

Characteristics of patients. Demographic and baseline data for 41 patients are presented in Table 1. There was no significant difference among patients as to the use of medications (aspirin, beta blockers, statins, ACE inhibitors, and nitroglycerin); white blood cell count or the risk factors. In addition to home medications, all of the patients were treated with standard PCI medications including heparin, aspirin and clopidogrel.

Table 1. Baseline characteristics and laboratory parameters of fourty one study patients.

Characteristics	Value
Age, years	57.5 (58 ± 9.7)
Male	28 (68.3)
Hypertension	20 (48.8)
Diabetes	22 (53)
Hyperlipidemia	11 (26.8)
Familial history	9 (22)
Smoking	13 (31.7)
Medications	
Nitrates	21 (51.2)
β blockers	27 (65.9)
ACE inhibitors	19 (46.3)
A2 blockers	13 (24.07)
Calcium channel blockers	7 (17.1)
Statins	35 (85.4)
ASA	38 (92.7)
Warfarin	4 (7)
Laboratory parameters	
Cholesterol, mg/dl	190 ± 40
LDL, mg/dl	141 ± 31
HDL, mg/dl	42 ± 6
Glucose, mg/dl	111 ± 23
WBCs, U/L	7.1 ± 0.4

Data are shown in number (%) or mean ± SD.
ACE, angiotensin converting enzyme; ASA, acetyl salicylic acid.

hTLR-4 Monocyte Expression. The flowcytometry results showed that hTLR-4 is mainly expressed on the surface of $CD14^+$ monocytes and its expression on neutophils and lymphocytes was less than 5%. Before PCI, hTLR-4^+/$CD14^+$ was 23.3 ± 2.7%. Two h and four h after PCI, it changed to 37.2 ± 3.7% and 32.9 ± 3.3%, respectively. Differences in two peak levels reached statistical significance ($p<0.05$; Figure 1).

Serum levels of TNF-α and IL-1β. The Serum concentrations of TNF-α and IL-1β are shown in Figure 2. The serum concentration of TNF- α prior to PCI was 21.5 ± 2.2 (pg/ml).

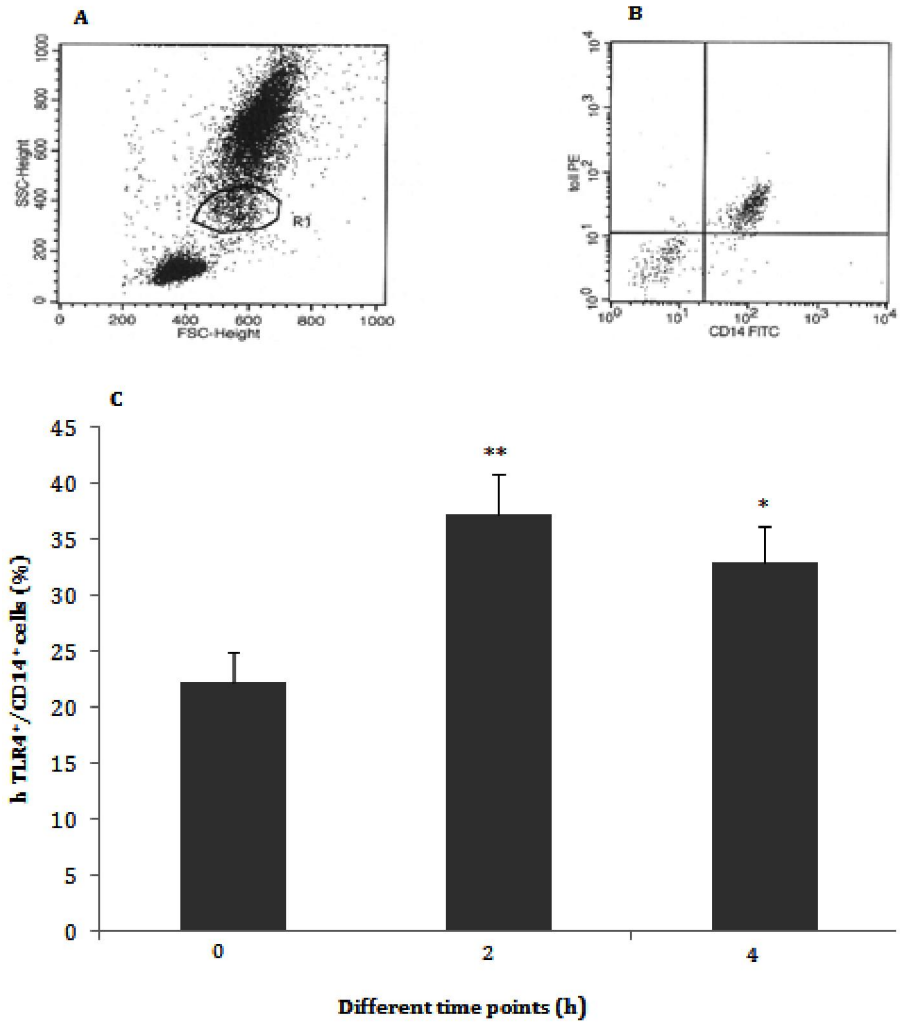

Figure 1. Gating monocyte population (A). Representative dot plot showing hTLR-4$^+$/CD14$^+$ monocytes (B). Frequency of hTLR-4$^+$/CD14$^+$ on circulating monocytes at different time points (%). Data are shown as mean ± SD (C). Ten thousand cells were analyzed by flowcytometry before PCI (0), 2h and 4h after PCI (2, 4), *p<0.05; **p<0.01 vs. 0h.

Two h after PCI, it reached the peak concentration of 35.4 ± 5.1 (pg/ml) and then declined to 34.2 ± 3.3 (pg/ml) four h after PCI. Serum level of IL-1β at PCI was 14.8 ± 1.8 (pg/ml) and it reached the peak level of 30.1± 4.0 (pg/ml) two h after PCI. Then 4 hours after PCI, the serum level of IL-1β declined to 18 ± 2.4 (pg/ml).

Pearson Correlations. A significant correlation was noted between the frequency of circulating hTLR-4$^+$/CD14$^+$ monocytes and serum level of TNF-α (r=0.60 and p=0.05) and also between hTLR-4$^+$/CD14$^+$ monocytes and serum level of IL-1β (r=0.40 and p=0.05) (Figure 3 and 4, respectively). These positive and significant correlations were observed only before PCI. However, two h and four h after PCI, the correlations were positive but failed to reach a significant level.

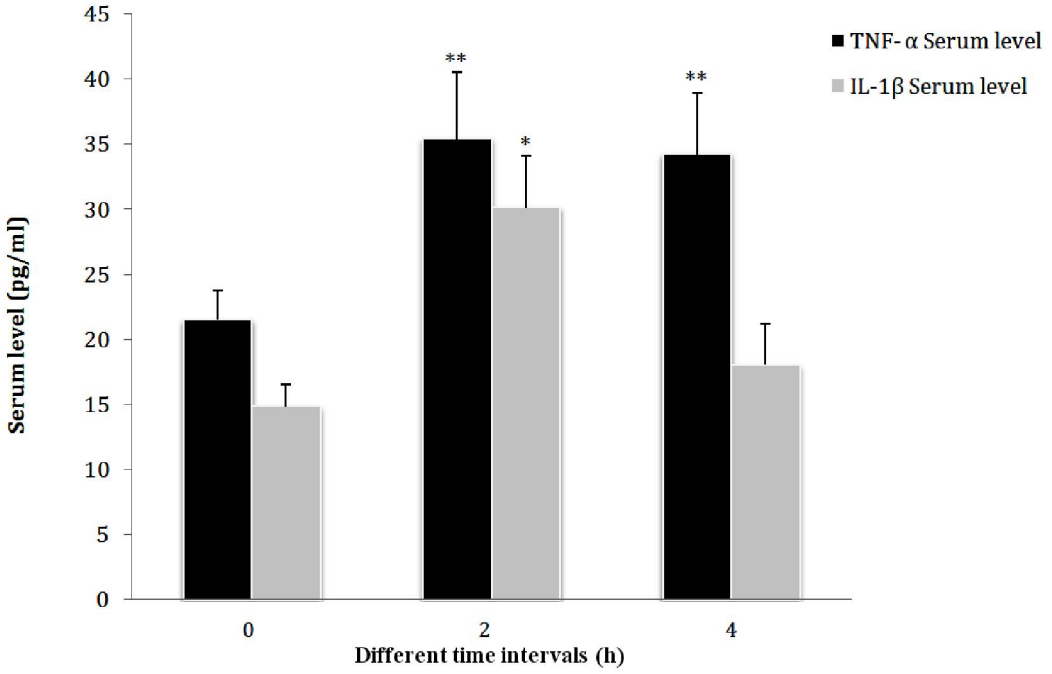

Figure 2. Serum levels of TNF-α and IL-1β at different time intervals (pg/ml), before PCI (0), 2h (2) and 4h (4) after PCI. *p<0.05; **p<0.01 vs. 0h.

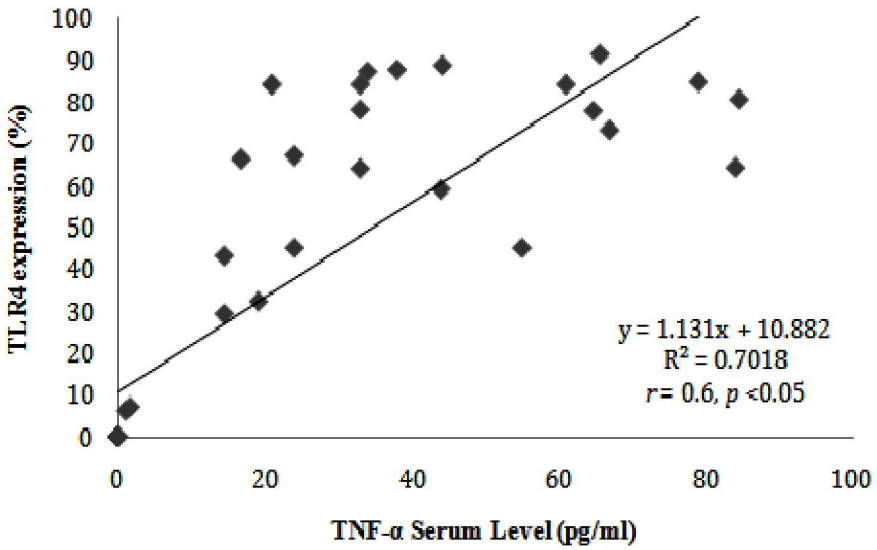

Figure 3. Correlation between hTLR-4 monocyte expression and serum levels of TNF–α prior to PCI (0h).

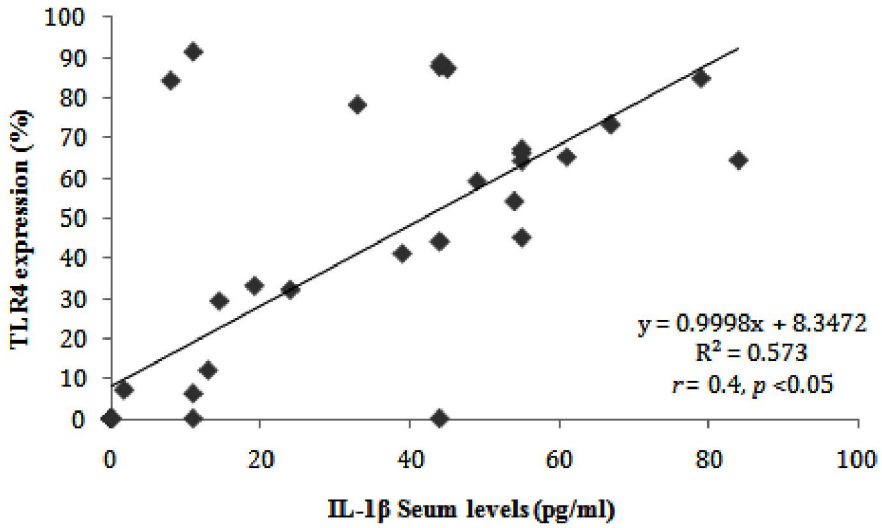

Figure 4. Correlation between hTLR-4 monocyte expression and serum levels of IL-1β prior to PCI (0h).

DISSCUSION

Toll like receptors are well defined barriers against microbial infections. Their sensitization mostly results in production of proinflammatory cytokines (24). However, large body of evidence suggests that TLRs (especially TLR-4) have additional roles in inflammatory processes in which there are no exogenous pathogens (25). One of such chronic inflammatory processes is atherosclerosis. TLR-4 is one of the well-recognized players in the formation of atherosclerotic plaques both in animal and human studies (26). Heat shock protein 60 (HSP60) is frequently observed in occluded coronary arteries. HSP60 is considered as a stimulatory ligand for TLR-4 (27). Yang and colleagues experimentally showed that during 30 min ischemia followed by reperfusion, there was enhanced mRNA expression of both TLR-4 and TNF-α and there was also a positive correlation between TLR-4 expression and the level of TNF-α (28). In the present study, we evaluated the monocyte expression of hTLR-4 using flowcytometry and measured the serum levels of main proinflammatory cytokines (TNF-α and IL-1β) by ELISA before and after PCI. As we expected, TLR-4 was chiefly expressed on the surface of monocytes and its expression on other leukocytes (neutrophils or lymphocytes) was nill. It has been shown that patients with acute myocardial infarction (AMI) can experience augmented monocyte expression of TLR-4 both in circulating blood and in the ruptured plaques (29). Yang Jun could show that AMI patients who were on reperfusion therapy had enhanced expression of hTLR-4 on monocytes. Furthermore, he confirmed that hTLR-4 expression was correlated with TNF-α level in serum. The author concluded that reperfusion therapy can increase hTLR-4 expression on monocytes (9). In this study, we chose patients with stable angina who underwent

PCI. We investigated monocyte expression of hTLR-4 at different time intervals. Our results showed that 2h after PCI there was a peak expression of hTLR-4. Moreover, the concentrations of TNF-α and IL-1β in plasma were also elevated after PCI. We can conclude that although hTLR-4 is involved in plaque formation and ischemia, it may have prominent roles in inflammation following PCI. Based on recent studies, we can find some documented hypotheses attributed to such an increase in the monocyte expression of hTLR-4. PCI is an invasive method that can potentially trigger inflammation and cause an increase in the expression of hTLR-4 (30). On the other hand, oxidized LDL which is abundantly seen in plaques, can up regulate hTLR-4 expression (15) and finally heparin as a drug of choice for most of PCI procedures has structural similarities with heparan sulfate, which is a documented agonist for hTLR-4 (31,32). However, heparin contribution to upregualtion of hTLR-4 is an emerging subject of debate and strong comparative clinical and experimental studies are needed to define its exact role in this setting. Several studies support the critical role of inflammation in re-stenosis after implantation of bare and drug-eluting stents (33-35). Versteeg et al (36) revealed that two hours after PCI procedure, hTLR-4 expression on and TNF-α level in monocytes were attenuated. In comparison to our study, they used a different method. They incubated the collected monocytes with LPS and then measured TLR-4 expression on the monocytes and the release of TNF-α from them. They attributed this attenuation to oxLDL paradoxical effects and nicotinic anti-inflammatory pathway of the vagus nerve which may attenuate cytokine release. They have also stated that flowcytometry showed a modest increase in intracellular TNF-α after PCI procedure. Generally, at the time of stent implantation, the overall inflammatory status is not equivalent in all patients and subsequently in all atherosclerotic plaques. In concert with several investigations, we have demonstrated that PCI causes an increase in inflammatory responses, although, we observed a modest decrease after 4 hours in hTLR-4 expression and also a decrease in the serum levels of TNF-α and IL-1β. Additionally our findings in link with other studies may partially explain the mechanism of restenosis after PCI in which inflammation plays a pivotal role. A number of studies showed that statins can regulate hTLR-4 (37,38). It should be noted that in such studies the collected monocytes were incubated with different doses of statins and a diminished expression of hTLR-4 was observed. Most of our patients were on statin therapy for a prolonged period; however, we failed to obtain any association between statins and hTLR-4 expression. In addition, we could not find any association between hTLR-4 and other routine drugs like nitroglycerin, beta blockers, aspirin, clopidogrel and calcium channel blockers. After successful PCI, related reperfusion injury is inevitable. Both reperfusion and ischemia trigger inflammation to myocardium. Several experimental evidences have proved that the beneficial effects of PCI outweigh its possible injuries during reperfusion. It can be proposed that direct hTLR-4 block or affecting its related downstream activities by novel methods may have beneficial effects for the myocardium. In this case we may encounter two challenges: first, until now two big clinical trials have been done to assess immunosuppressive therapies during heart failure and acute MI (39,40). Unfortunately both of them failed to improve clinical outcomes of the patients; therefore physicians are still unwilling to apply such therapies. Second, it is well proved that inflammation is a key contributor to arteriogenesis (41,42). Quite recently TLR-4 involvement in arteriogenesis has gained prominence (43). TLR-4 can trigger some special cascades that mainly contribute to arteriogenesis. Obviously TLR-4 as a single part of immunity system is a double-edged sword. More

and more studies are needed to provide deeper insight into TLR-4 roles in cardiovascular disorders. Our future studies will hopefully evaluate the prognostic value of TLR-4 in patients with stable angina who have undergone PCI.

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