

Improved Myocardial Ischemia/Reperfusion Injury in Mice Lacking Tumor Necrosis Factor- α

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OBJECTIVES	This study sought to assess the role of tumor necrosis factor- α (TNF- α) in myocardial ischemia/reperfusion (I/R) injury using TNF- α knockout (KO) mice.
BACKGROUND	Tumor necrosis factor- α is thought to be involved in the pathogenesis of myocardial I/R injury by promoting leukocyte infiltration of the myocardium. However, the precise role of TNF- α in I/R injury is still unknown.
METHODS	The hearts in TNF- α KO and wild-type (WT) mice were exposed by left lateral thoracotomy, and the left coronary artery was occluded for 30 min then reperfused for 120 min.
RESULTS	The infarct size in TNF- α KO mice was significantly reduced compared with WT mice. The frequency of arrhythmia was decreased, and cardiac function during reperfusion was significantly improved in TNF- α KO mice compared with WT mice. The activation of nuclear factor- κ B (NF- κ B), the expression of chemokines and adhesion molecules and the infiltration of leukocytes were also significantly reduced in TNF- α KO mice, compared with WT mice. These findings provide evidence that TNF- α aggravates I/R injury.
CONCLUSIONS	Tumor necrosis factor- α exacerbates myocardial I/R injury at an early stage of reperfusion by activating NF- κ B, thereby inducing chemokines and adhesion molecules and facilitating leukocyte infiltration. (J Am Coll Cardiol 2002;39:1229–35) © 2002 by the American College of Cardiology Foundation

Recanalization of occluded coronary arteries is a standard method of treatment for acute myocardial infarction. However, reperfusion itself can cause myocardial damage, which is designated as ischemia/reperfusion (I/R) injury. Numerous studies have demonstrated that leukocyte infiltration occurs in the reperfused myocardium (1). Leukocytes, especially polymorphonuclear neutrophils, mediate the damage to endothelial cells and cardiac myocytes during reperfusion.

Tumor necrosis factor- α (TNF- α) is known to be a proinflammatory cytokine. Recent studies have demonstrated that TNF- α is produced during I/R injury (2,3) and activates nuclear factor- κ B (NF- κ B), which initiates the cytokine cascade and facilitates the expression of chemokines and adhesion molecules. Treatment with anti-TNF- α antibody has been reported to improve myocardial recovery after I/R (4).

In contrast, beneficial effects of TNF- α on acute myocardial infarction (5) have also been reported, where TNF- α receptor knockout (KO) mice show accelerated myocardial apoptosis after ischemia, indicating that TNF- α has a protective action against ischemia. Accordingly, it is impor-

tant to reevaluate the effects of TNF- α on myocardial I/R injury using TNF- α KO.

METHODS

Animals. Tumor necrosis factor- α KO mice were generated by gene targeting, as described previously (6). C57BL/6J mice were used as wild-type (WT) controls. Male mice at 12 to 13 weeks of age were used for this experiment, which was performed according to the "Position of the American Heart Association on Research Animal Use," adopted by the American Heart Association November 11, 1984.

Surgical procedures. Mice were anesthetized with 2% halothane (Takeda Pharmaceutical, Osaka, Japan) and 40% oxygen, and maintained with 0.5% halothane and 40% oxygen. Tracheotomy was performed to provide artificial ventilation (0.3 ml tidal volume, 120 breaths/min), and the left coronary artery was ligated with an 8-0 nylon surgical suture 1.0 mm distal from tip of the left auricle.

Measurements of infarct area and area at risk. After 30-min ligation and 120-min reperfusion, the left coronary artery was reoccluded at the same point and Evans blue dye was perfused from the left ventricular (LV) cavity. The heart was removed and cut transversely into five sections, which were then incubated in a 1.0% solution of 2,3,5-triphenyl-tetrazolium chloride (TTC; Sigma, St. Louis, Missouri) for 20 min at 37°C. The area at risk (AAR) and the infarct area (IA) corresponded to the area unstained with Evans blue dye and the area unstained with TTC solution, respectively. Each slice of the ventricle was weighed, and the AAR to LV

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Abbreviations and Acronyms	
AAR	= area at risk
IA	= infarct area
ICAM	= intercellular adhesion molecule
IL	= interleukin
I/R	= ischemia/reperfusion
KO	= knockout
LV	= left ventricle
MCP	= monocyte chemoattractant protein
MIP	= macrophage inflammatory protein
NF- κ B	= nuclear factor- κ B
RT-PCR	= reverse transcription and polymerase chain reaction
TNF- α	= tumor necrosis factor- α
WT	= wild-type

ratio and IA to LV ratio of each slice were determined using National Institutes of Health Image version 1.62.

Neutralization of TNF- α with a selective antibody. To neutralize the action of TNF- α in WT mice, 10 μ g of goat anti-mouse TNF- α polyclonal antibody (AF-410-NA; R&D Systems Inc., Minneapolis, Minnesota) dissolved in 0.1 ml of saline was injected through the tail vein 30 min before left coronary artery ligation.

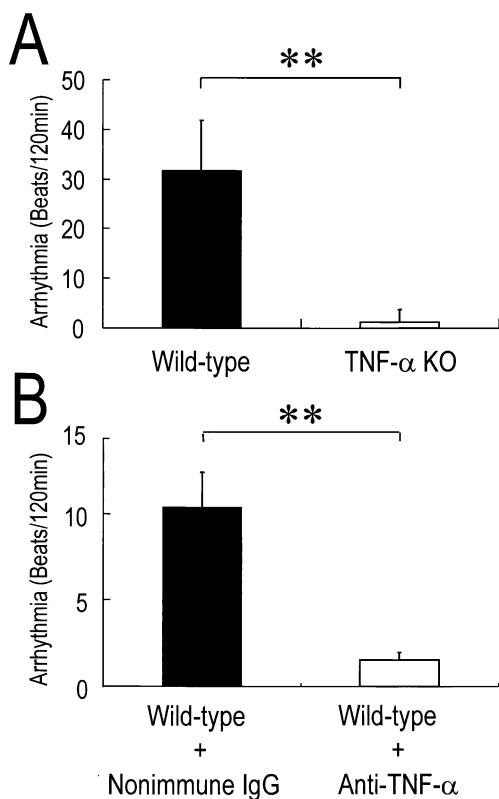


Figure 1. Arrhythmia observed during myocardial ischemia/reperfusion. (A) Summarized data on the arrhythmia during reperfusion in wild-type (n = 11) and tumor necrosis factor (TNF)- α knockout (KO) (n = 9) mice. (B) Summarized data on the arrhythmia during reperfusion in wild-type mice receiving nonimmune IgG (n = 5) and anti-TNF- α antibody (n = 5). **p < 0.01.

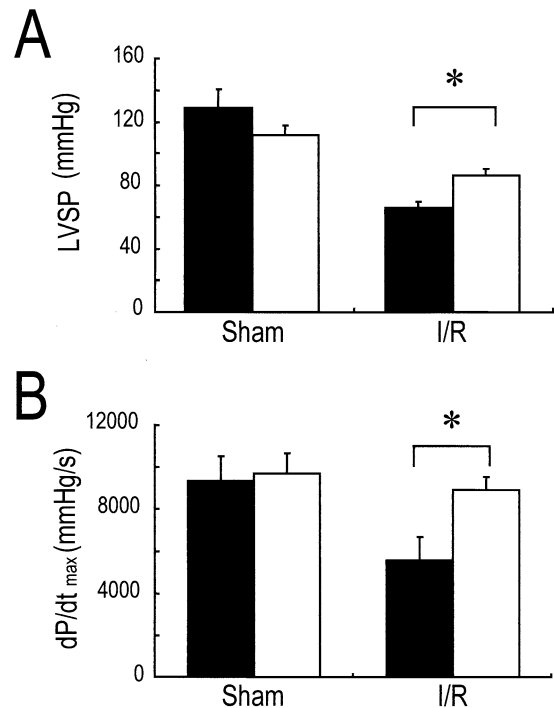


Figure 2. Hemodynamic parameters measured just before the end of reperfusion. Summarized data on peak left ventricular systolic pressure (LVSP) (A) and maximal positive rate of the first derivative of left ventricular pressure (dP/dt_{max}) (B) in the sham-operated wild-type mice (solid bars) (n = 5), tumor necrosis factor (TNF)- α knockout (KO) mice (light bars) (n = 5), reperused wild-type (n = 6) and TNF- α KO (n = 5) mice. *p < 0.05. I/R = ischemia/reperfusion.

Electrocardiogram monitoring of arrhythmia. Electrocardiogram recording started 10 min before occlusion and continued until the end of reperfusion (160 min total). Data were sampled at a rate of 10 kHz and analyzed using MacLab/8s (AD Instruments, Castle Hill, Australia).

Hemodynamic study. A 1.4 French high-fidelity catheter tip micromanometer (SPR-671; Millar Instruments, Houston, Texas) was inserted through the right carotid artery into the LV cavity to measure LV pressure. First derivative of LV pressure (dP/dt) was analyzed using MacLab/8s.

Electrophoretic gel mobility shift assay. Nuclear extract was prepared from the entire LV of the reperused heart according to the method described by Morishita et al. (7). Nuclear extract was incubated with 1×10^5 cpm 32 P-labeled oligonucleotide containing the NF- κ B consensus sequences (Promega Corp., Madison, Wisconsin). The protein content was measured, and 10 μ g of protein was applied to each lane.

RNA extraction, reverse transcription and polymerase chain reaction analysis. Total RNA was extracted from ischemic and nonischemic regions. Reverse transcription (RT) and real-time quantitative polymerase chain reaction (PCR) were performed using a Light Cycler (F. Hoffmann-LaRoche Ltd., Basel, Switzerland). The expression levels of TNF- α , interleukin (IL)-6, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule-1, macrophage

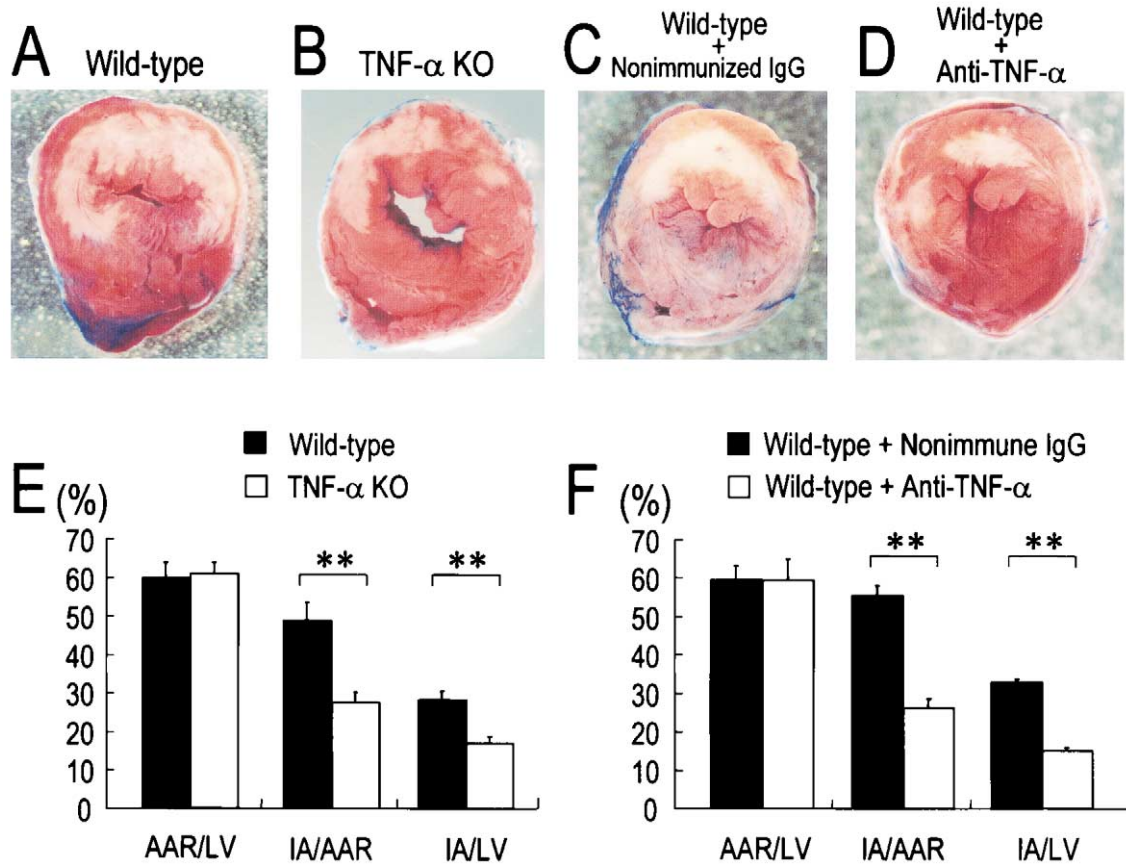


Figure 3. Infarct area (IA) and area at risk (AAR) of the left ventricle (LV) after 30-min ligation and 120-min reperfusion. Representative photographs of midventricular myocardium from wild-type mice (A), tumor necrosis factor (TNF)- α knockout (KO) mice (B), wild-type mice receiving nonimmune IgG (C) and wild-type mice receiving anti-TNF- α antibody (D). (E) Summarized data on infarct area to area at risk ratio (IA/AAR), IA to left ventricle ratio (IA/LV) and AAR to LV ratio (AAR/LV) in wild-type (n = 11) and TNF- α KO (n = 9) mice. (F) Summarized data on IA/AAR, IA/LV and AAR/LV in wild-type mice receiving nonimmune IgG (n = 5) and anti-TNF- α antibody (n = 5). **p < 0.01.

inflammatory protein (MIP)-1- α , MIP-2 and monocyte chemoattractant protein (MCP)-1 were investigated. The fluorescence intensity of each target gene was normalized with that of β -actin amplified under identical conditions.

Histopathological study. The heart embedded in -20°C cryostat compound (Miles Laboratory, Elkhart, Indiana) was snap frozen in 2-methylbutane prechilled with liquid nitrogen and cut into sections 5 μ m thick. Sections were stained with May-Giemsa (Muto Pure Chemicals, Tokyo, Japan). All fields of a midventricular section were observed at \times 1,000 magnification, and the number of neutrophils was counted in a blind fashion. Five sections obtained from five different mice were examined for each group.

Other sections were stained with goat anti-mouse TNF- α polyclonal antibody (SC-1348; Biotechnology Inc., Santa Cruz, California) followed by biotinylated donkey anti-goat IgG antibody (Biotechnology Inc.). The streptavidin-peroxidase complex (Dako, Glostrup, Denmark) and 3'-diaminobenzidine were used for visualization.

Statistical analysis. Results are expressed as mean \pm SEM. Comparisons between two groups were performed by Student *t* test. Comparisons in the RT-PCR analysis were performed by two-way analysis of variance, followed by

Scheffé's post-hoc test. Stat View 5.0 was used for statistical analyses. Results were considered statistically significant at p < 0.05.

RESULTS

Arrhythmia. The frequency of arrhythmia observed during reperfusion was significantly less in TNF- α KO mice (1.2 ± 0.5 beats/2 h) than in WT mice (31.8 ± 10.1 beats/2 h) (Fig. 1A). Wild-type mice receiving goat anti-TNF- α antibody showed a reduced frequency of arrhythmia (1.5 ± 0.4 beats/2 h) compared with those receiving nonimmune goat IgG (10.8 ± 2.2 beats/2 h) (Fig. 1B). These findings indicate that TNF- α has arrhythmogenic action during reperfusion.

Hemodynamic study. Peak LV systolic pressure and maximal positive rate of dP/dt in TNF- α KO mice were significantly higher than in WT mice during reperfusion (Fig. 2), whereas no significant differences were observed between the sham-operated WT and sham-operated TNF- α KO.

Infarct size. Left coronary artery occlusion caused a consistently large ischemic area (AAR). The AAR to LV ratio

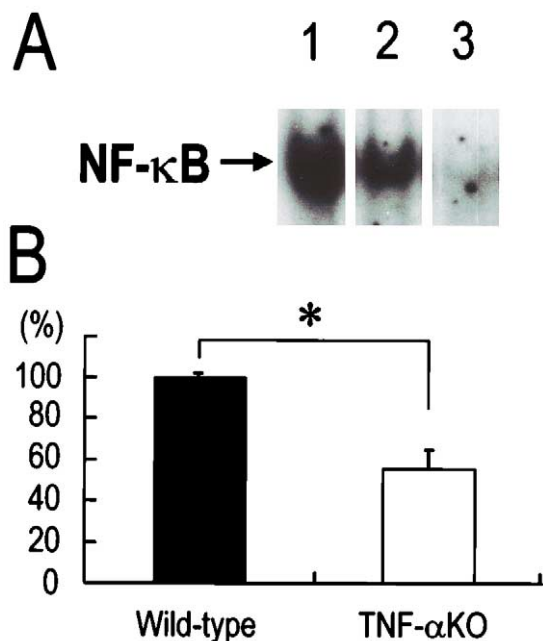


Figure 4. Electrophoretic gel mobility shift assay for activation of nuclear factor- κ B (NF- κ B). (A) Representative photographs. **Lane 1** = reperfusion myocardium from wild-type mice; **lane 2** = reperfusion myocardium from tumor necrosis factor (TNF)- α knockout (KO) mice; **lane 3** = reperfusion myocardium from wild-type mice with competitive oligonucleotides. (B) Summarized data on activation of NF- κ B in wild-type mice (n = 5) and TNF- α KO mice (n = 5). *p < 0.01.

in TNF- α KO mice ($60.8 \pm 3.0\%$) did not differ from that in WT mice ($59.6 \pm 4.2\%$) (Fig. 3E). The IA to AAR and the IA to LV ratios of TNF- α KO mice ($27.6 \pm 2.6\%$ and $17.0 \pm 1.8\%$, respectively) were significantly decreased compared with those of WT mice ($48.5 \pm 4.6\%$ and $28.2 \pm 23.4\%$, respectively) (Fig. 3E). Intravenous injection of anti-TNF- α antibody significantly improved the IA to AAR ratio ($55.4 \pm 2.6\%$ vs. $26.1 \pm 2.5\%$) and the IA to LV ratio ($32.8 \pm 2.1\%$ vs. $15.0 \pm 0.8\%$) (Fig. 3F).

Electrophoretic gel mobility shift assay. Figure 4A shows representative results of electrophoretic gel mobility shift assay for NF- κ B. Deoxyribonucleic acid binding activity of NF- κ B was significantly decreased in TNF- α KO mice ($65.1 \pm 9.5\%$), compared with WT mice ($100.4 \pm 3.5\%$) (Fig. 4B).

Measurements of mRNA expression by RT-PCR. In WT mice, levels of TNF- α and IL-6 expression in the ischemic area were significantly higher than those in the nonischemic area (Figs. 5A and 5B). Reperfusion-induced ICAM-1, MIP-2 and MCP-1 expressions were significantly suppressed in TNF- α KO mice (Figs. 5C to 5F), indicating that reperfusion-induced expression of these molecules was mediated at least partly by TNF- α .

Immunoreactivity to TNF- α . Tumor necrosis factor- α immunoreactivity was detected in the ischemic myocardium but hardly detected in the nonischemic myocardium (data not shown). Tumor necrosis factor- α immunoreactivity was also detected in infiltrating leukocytes and endothelial cells

(Fig. 6). Tumor necrosis factor- α staining was done on WT mice.

Counts of infiltrating neutrophils. The neutrophil infiltration was observed in the reperfused myocardium. Neutrophils were located mainly in the perivascular area of the border zone. The number of infiltrating neutrophils in WT mice (421 ± 11 , n = 5) was significantly (p = 0.029) greater than that in TNF- α KO mice (268 ± 56 , n = 5).

DISCUSSION

TNF- α and myocardial reperfusion. Numerous studies have demonstrated that reperfusion accelerates leukocyte infiltration of the infarcted myocardium (1). Neutrophil transendothelial migration and adhesion to cardiac myocytes are mediated mainly by the interaction between neutrophil Mac-1 (CD11b/CD18) and ICAM-1 expressed on endothelial cells and cardiac myocytes. This interaction induces a neutrophil respiratory burst, which causes myocyte injury via reactive oxygen species (8). In fact, anti-CD11b or -CD18 antibodies have been shown to have protective effects against I/R injury (9), and CD18 or ICAM-1 KO mice show reduced infarct size in I/R injury (10).

Postischemic cardiac lymph can induce ICAM-1 (11) and IL-6 (2) expressions, which are inhibited by anti-TNF- α antibody (2). Chemokines such as IL-8 and MCP-1, which are induced by TNF- α , mediate the chemotaxis of neutrophils (12) and monocytes (13) to the reperfused myocardium. Thus, TNF- α might be an upstream cytokine initiating the reperfusion-dependent cytokine cascade that induces the expression of adhesion molecules and chemokines (14).

In this study, the frequency of arrhythmia (Fig. 1), hemodynamic parameters (Fig. 2) and infarct size (Fig. 3) were significantly improved in TNF- α KO mice, compared with WT mice. Anti-TNF- α antibody also reduced the frequency of arrhythmia (Fig. 1) and infarct size (Fig. 3) significantly. These observations indicate that TNF- α has detrimental actions during reperfusion.

The role of TNF- α in reperfusion-induced NF- κ B activation. Nuclear factor- κ B is known to be a key transcription factor that regulates the expression of inflammatory cytokines, chemokines and adhesion molecules (15). Nuclear factor- κ B decoy oligonucleotides block the expression of IL-6 and ICAM-1 mRNA during reperfusion (3) and relieve myocardial I/R injury (7). Tumor necrosis factor- α has been reported to activate NF- κ B in endothelial cells and cardiac myocytes (7). As oxidative stress itself can activate NF- κ B (15), the role of TNF- α in reperfusion-induced NF- κ B activation is of interest. In the electrophoretic gel mobility shift assay for NF- κ B activation performed here, DNA binding activity of NF- κ B during reperfusion was significantly decreased in TNF- α KO mice, compared with WT mice (Fig. 4), suggesting that reperfusion-induced NF- κ B activation is mediated at least partly by TNF- α . The entire LV of the reperfused heart was

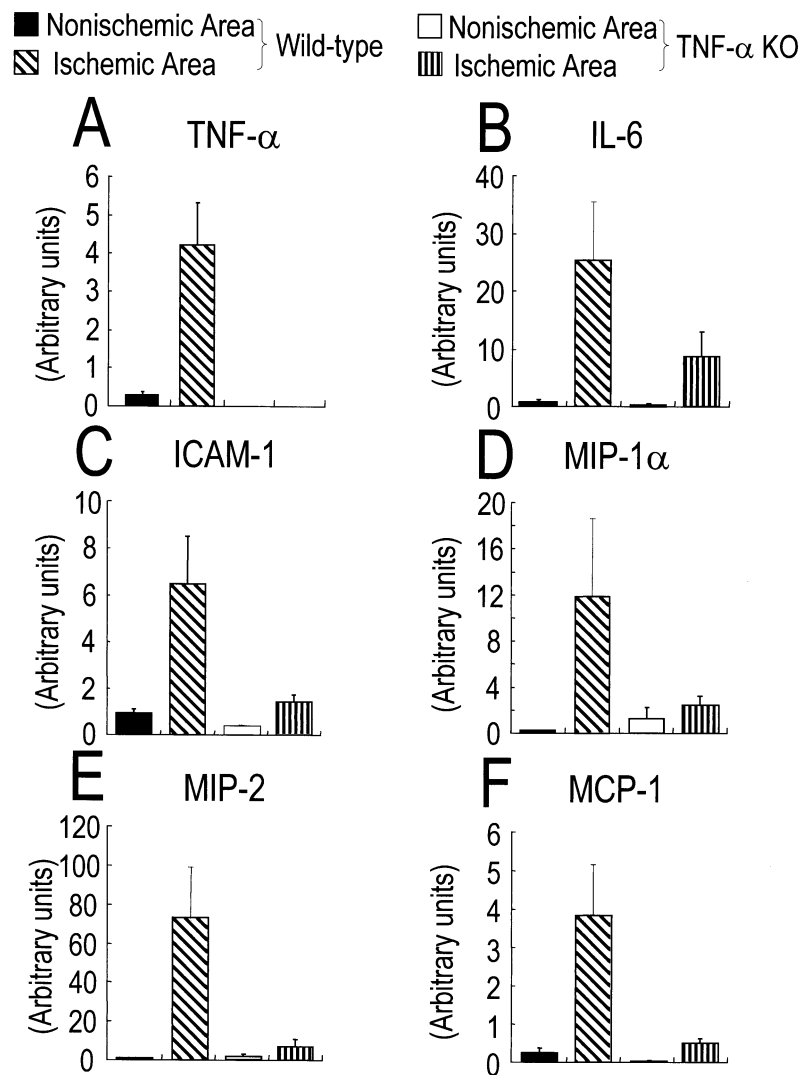


Figure 5. The results of messenger ribonucleic acid expression in wild-type mice (n = 5) and tumor necrosis factor (TNF)- α knockout (KO) mice (n = 6) determined by reverse transcription and polymerase chain reaction. (A) TNF- α (p = 0.007). (B) Interleukin (IL)-6 (p-values for the presence of TNF- α , ischemia and their interaction are 0.118, 0.005 and 0.129, respectively). (C) intercellular adhesion molecule (ICAM)-1 (p = 0.008, 0.002 and 0.026, respectively). (D) macrophage inflammatory protein (MIP)-1 α (p = 0.144, 0.037 and 0.139, respectively). (E) MIP-2 (p = 0.016, 0.006 and 0.017, respectively). (F) monocyte chemoattractant protein (MCP)-1 (p = 0.009, 0.004 and 0.019, respectively).

used instead of the reperfused region to obtain enough nuclear extract for NF- κ B analysis; this is considered to be one of the reasons the difference in NF- κ B activation between TNF- α KO mice and WT mice was relatively small.

The role of TNF- α in reperfusion-induced expression of ICAM-1 and chemokines. Tumor necrosis factor- α was expressed at higher levels in the ischemic area than in the nonischemic area (Fig. 5A), indicating that I/R induces TNF- α expression in WT mice. As the cytokine induction caused by acute surgical trauma may obscure the reperfusion-induced cytokine elevation (16), cytokine expression was compared between the ischemic and nonischemic areas. The expressions of IL-6, ICAM-1, MIP-1 α , MIP-2 and MCP-1 were induced by I/R in WT mice (Figs. 5B to 5F). Interestingly, the induction of MIP-2, which is a mouse homologue of IL-8, was the most prominent

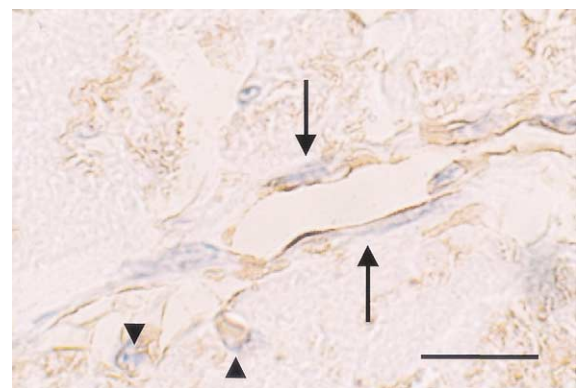


Figure 6. Immunostaining of tumor necrosis factor- α in the reperfused myocardium of wild-type mice. Tumor necrosis factor- α immunoreactivity was detected in not only myocytes but also infiltrating leukocytes (arrowheads) and endothelial cells (arrows) at $\times 1,000$ magnification. Bars = 20 μ m.

(82-fold higher than in the nonischemic area). However, the reperfusion-induced expression of these molecules was significantly suppressed in TNF- α KO mice, indicating that this expression was mediated at least partly by TNF- α . These molecules are thought to play important roles in leukocyte recruitment. In fact, neutrophil infiltration was significantly suppressed in TNF- α KO mice, compared with WT mice, as expected. Taken together, our findings indicate that TNF- α exacerbates myocardial I/R injury at an early stage of reperfusion by activating NF- κ B, thereby inducing chemokines and adhesion molecules and facilitating leukocyte infiltration.

Pleiotropic actions of TNF- α . Beneficial effects of TNF- α on acute myocardial infarction have been reported by Kurrelmeyer et al. (5), where TNF- α receptor KO mice showed accelerated myocardial apoptosis and increased infarct size within 24 h after coronary occlusion without reperfusion. Tumor necrosis factor- α has also been reported to confer resistance to hypoxic injury in cultured cardiac myocytes (17). The anti-apoptotic and cytoprotective actions of TNF- α may result from the activation of NF- κ B (18), which is mediated by TNF receptor-associated factor 2. Nuclear factor- κ B activation promotes the expression of cytoprotective genes, such as manganese superoxide dismutase. Tumor necrosis factor- α reduces cardiac contractility (19). Acute reduction in cardiac contractility does not necessarily lead to long-term cardiac dysfunction. Tumor necrosis factor- α -mediated contractile reduction during acute ischemia, therefore, may promote cardioprotection by attenuating the myocardial energy demand. Tumor necrosis factor- α expression is also induced directly by hemodynamic stress, such as pressure-overload, which elicits cardiac hypertrophy to maintain normal cardiac contractility. This expression, therefore, is thought to be an adaptive response to cardiac stress.

In contrast, TNF- α can induce apoptosis of cardiac myocytes and endothelial cells (20). Tumor necrosis factor receptor p55 and TNF receptor-associated death domain protein may mediate this signal. Although NF- κ B mediates the cardioprotective signals of TNF- α , NF- κ B activation also induces apoptosis of endothelial cells (7) and the expressions of chemokines and adhesion molecules (15), which facilitate leukocyte infiltration. In fact, cardiac-specific overexpression of TNF- α has been reported to cause severe myocarditis in mice (21). Taken together, it is likely that TNF- α itself has a protective role in cardiac myocytes during ischemia without reperfusion, but TNF- α during reperfusion facilitates leukocyte infiltration, resulting in myocardial damage. The difference between the role of TNF- α in ischemia with reperfusion and in ischemia without reperfusion might depend on the concentration of TNF- α produced by the myocardium (22). Although we did not compare the level of TNF- α during ischemia with that during reperfusion, the latter may induce a substantial amount of TNF- α . Tumor necrosis factor- α could have both short-term adaptive and long-term maladaptive roles

(23), and even in the acute phase, a high level of TNF- α could have a maladaptive role (22). However, to explore the mechanism by which reperfusion induces a high level of TNF- α is beyond the scope of this study. As the essential role of TNF- α is promoting innate immunity to eliminate infectious agents from the host by recruiting leukocytes, the expression of TNF- α during reperfusion is thought to be a maladaptive response.

Clinical implications and therapeutic potential. Because TNF- α is considered to be involved in the pathogenesis of various cardiovascular diseases, anti-TNF- α strategy may have therapeutic benefit. In fact, in patients with advanced heart failure, anti-TNF- α therapy using etanercept, a recombinant soluble p75 TNF- α receptor, showed a significant dose-dependent improvement in LV function and structure (24). As TNF- α could play a maladaptive role in chronic disorders such as heart failure, this outcome met the expectation. However, the efficacy of the anti-TNF- α therapy in ischemic heart disease is still unclear. Our findings show that deficiency of TNF- α reduced leukocyte infiltration and ameliorated myocardial I/R injury at an early stage of reperfusion, which suggests the potential advantage of anti-TNF- α therapy in I/R injury. However, because TNF- α has a cytoprotective action in cardiac myocytes, and the inflammatory reaction induced by TNF- α may facilitate tissue repair (1), the effects of TNF- α deficiency on I/R injury should be investigated, particularly at the healing stage.

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