Crucial Role of Endogenous Interleukin-10 Production in Myocardial Ischemia/Reperfusion Injury

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***Background*—**The anti-inflammatory cytokine interleukin-10 (IL-10) has been detected in the plasma of patients with myocardial ischemia/reperfusion. The aim of our study was to investigate the role of endogenously produced IL-10 in myocardial ischemia/reperfusion.

***Methods and Results*—**In the present study, we used wild-type and IL-10–deficient mice subjected to myocardial ischemia/reperfusion. Significant levels of IL-10 were produced in wild-type mice at 2 to 6 hours after myocardial reperfusion. The genetic deletion of IL-10 enhanced neutrophil infiltration into the reperfused tissues at 6 hours after reperfusion and increased infarct size and myocardial necrosis. Furthermore, in the absence of IL-10, an enhancement of the inflammatory response was seen, as demonstrated by increased plasma levels of tumor necrosis factor-α, nitrite/nitrate (breakdown products of NO), and increased tissue expression of intercellular adhesion molecule-1. Reperfusion for 24 hours was associated with a 75% mortality rate in IL-10–deficient mice, whereas no deaths occurred in the wild-type animals.

***Conclusions*—**The present findings provide the first direct evidence that endogenous IL-10 inhibits the production of tumor necrosis factor-α and NO and serves to protect the ischemic and reperfused myocardium through the suppression of neutrophil recruitment. **(*Circulation*. 2000;101:1019-1026.)**

Key Words: reperfusion ■ interleukins ■ nitric oxide ■ cell adhesion molecules

The prominent anti-inflammatory cytokine interleukin-10 (IL-10) is released into the plasma of patients with myocardial ischemia/reperfusion.[1–4] The production of IL-10 in many forms of shock and inflammation serves to limit the burst of proinflammatory mediators.[5–9] With the use of IL-10–deficient mice, here we defined the role of endogenous IL-10 in the modulation of myocardial ischemia/reperfusion injury.

Methods

Myocardial Ischemia/Reperfusion

The present investigation conforms with the "Guide for the Care and Use of Laboratory Animals" published by National Institutes of Health and was performed with the approval of the Institutional Animal Care and Use Committee. Male C57BL/6 IL-10 wild-type (IL-10 WT) and C57BL/6 IL-10 knockout mice (4 weeks old) were obtained from Jackson Laboratories. These mice were originally generated by Kuhn and colleagues,[10] as previously described. The mice used in the present study have been back-crossed for 10 generations to C57/BL6 mice for 10 generations at Jackson Laboratories. By definition, these mice are considered congenic, with >99.8% of the genome belonging to BL6 strain, and the only genetic region derived from the other strain is the region immediately surrounding the IL-10 gene; therefore, the difference between the two experimental groups is due to the presence or absence of the functional IL-10 gene and not to other factors. Animals received food and water on an ad libitum basis, and lighting was maintained on a 12-hour cycle.

After premedication with 0.04 mg/kg atropine sulfate IM, animals were anesthetized with 100 mg/kg sodium pentobarbital IP. The animals were placed in a supine position with their paws and tails taped to the operating table. The head was retracted with a thin rubber band fasten to the upper incisors. The upper portion of the trachea was exposed through a middle incision in the neck, and the pretracheal muscles were bluntly dissected. A black-tipped endotracheal tube, made with PE-60 tubing, was inserted by way of the mouth into the trachea with the black tip placed 5 to 8 mm below the thyroid cartilage.[11] Artificial respiration was maintained through the use of a respirator with an F_{IO_2} of 0.80, a frequency of 100 strokes/min, and a tidal volume of 0.8 to 1.2 mL to maintain normal arterial Pa_{O_2}, Pa_{CO_2}, and pH. The middle skin incision in the neck was extended down to the xiphoid. The left pectoris major muscle and the muscle beneath it were dissected longitudinally, without cutting these muscles, to expose the left 3rd and 4th ribs. A parasternal incision was made to open the chest by cutting the left 3rd and 4th ribs and intercostal muscles with a cautery pen (General Medical Corporation). The animal was slightly rotated to the right through the release of the left upper paw to fully expose the left ventricle. Coronary artery ligation was achieved with a balloon occluder fixed onto the left anterior descending coronary artery (LAD) with a 7-0 silk suture passed with a tapered needle underneath the LAD and 2 to 3 mm inferior to the left auricle. Coronary artery occlusion and reperfusion were induced through inflation and deflation of the balloon. Significant ECG changes, including widening of the QRS complex (monitored with a Maclab ETH-255 Bridge/Bio Amplifier; CB Science Inc) and elevation of ST segment, and color changes of the area at risk were considered indicative of successful coronary occlusion and reperfusion. Once the reperfusion

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TABLE 1. Animal Groups Used

Groups	Protocols	Heart Tissue Purpose	No. of Mice	Comment
Wild-type	Normal control	iNOS activity	3	
Knockout	Normal control	iNOS activity	3	
		MPO	6	
Wild-type sham	Open chest for 35 min	ICAM-1 staining	3	
		Mortality and exclusion	1	Died of anesthesia overdose
Knockout sham	Open chest for 35 min	MPO	3	
		ICAM-1 staining	3	
Wild-type	I/R for 30 min/6 h	Infarction measurement	8	
	I/R for 30 min/6 h	MPO	6	
	I/R for 30 min/6 h	ICAM-1 staining	3	
	I/R for 30 min/6 h	iNOS activity	3	
	I/R for 30 min/2 h	Time course study	6	
	I/R for 30 min/4 h	Time course study	6	
		Hemodynamic study	3	
	I/R for 30 min/24 h	Mortality study	8	No mortality
Knockout	I/R for 30 min/6 h	Infarction measurement	9	
	I/R for 30 min/6 h	MPO	7	
	I/R for 30 min/6 h	ICAM-1 staining	3	
	I/R for 30 min/6 h	iNOS activity	3	
	I/R for 30 min/2 h	Time course study	6	
	I/R for 30 min/4 h	Time course study	6	
		Hemodynamic study	3	
	I/R for 30 min/24 h	Mortality study	8	6 died
Total			110	

I/R indicates ischemia/reperfusion

started, the chest was closed in layers. The respirator was weaned, and the endotracheal tube was removed when the animal recovered spontaneous breathing and began to move. Five percent dextrose and whole blood from animals with the same genotype were administered intraperitoneally or intravenously to replace the fluid and blood loss that occurred during surgery. Five percent dextrose (0.5 to 1.0 mL IP) was injected after the animal was ventilated. Blood was transfused through the left external jugular vein immediately after the chest was entered and after the chest was closed, with a total volume of 0.4 to 0.8 mL. Rectal temperature was monitored with a rectal probe and was maintained within 36.5° and 37.5°C. Mean arterial blood pressure (monitored with the Maclab ETH-255 Bridge/Bio Amplifier) was measured for hemodynamic study through cannulation of the right common carotid artery.

Experimental Groups

Animals were assigned to various groups. For the sham groups, in wild-type control ($IL\text{-}10^{+/+}$) animals and IL-10–deficient ($IL\text{-}10^{-/-}$) mice, the chest was opened for 35 minutes and the suture was placed around LAD but not ligated. The animals were sacrificed 2, 4, or 6 hours later. For the myocardial ischemia/reperfusion groups, wild-type control ($IL\text{-}10^{+/+}$) animals and IL-10–deficient ($IL\text{-}10^{-/-}$) mice were subjected to 30-minute LAD occlusion and 2-, 4-, or 6-hour reperfusion.

Before the hearts were harvested, the animals were reanesthetized, and the blood was drawn via cardiac puncture. The excised hearts were either stained with tetrazolium for measurement of myocardial infarction size or maintained at −70°C for the preparation of frozen sections for immunohistochemical determination of ICAM-1 or for tissue myeloperoxidase (MPO) activity measurements (Table 1). In subsequent experiments, 24-hour reperfusion was also performed; in these experiments, 6 of 8 $IL\text{-}10^{-/-}$ animals died within 12 hours of reperfusion, whereas all 8 $IL\text{-}10^{+/+}$ wild-type animals that were tested survived for 24 hours.

Analysis of Myocardial Infarction

After excision, the hearts were cannulated through the ascending aorta with a 23-gauge needle and perfused with 2 to 3 mL of 37°C 0.9% sodium chloride solution and then with 3 to 4 mL of 37°C 1.0% tetrazolium red in phosphate buffer (pH 7.4). After tetrazolium staining, the LAD was reoccluded. Then, the hearts were perfused with 2 mL of 2% Evans blue to delineate the nonischemic tissue. The hearts were then frozen, and the right ventricle and atria were trimmed off. The left ventricle was cut into 5 to 7 transverse slices, which were fixed in 10% neutral buffered formalin solution. Each slice was weighed and photographed under a dissecting microscope. The pictures of both sides of each slice were traced along the borders of infarction area, the ischemic area (area at risk), and the nonischemic area. The corresponding areas were calculated as previously described[12] through scanning of the images, followed by the determination of the respective areas with the use of Adobe Photoshop. The sizes in weight of nonischemic area, area at risk, and infarction area of each slice were then calculated as a percentage of corresponding area multiplied by the weight of the slice.[12]

MPO Activity

The left ventricles, which were harvested 2, 4, or 6 hours after reperfusion, were homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7) and centrifuged for 30 minutes at 20 000*g* at 4°C. An aliquot of the supernatant was allowed to react with a solution of tetramethylbenzidine (1.6 mmol/L) and

0.1 mmol/L H_2O_2. The rate of change in absorbance was measured with spectrophotometry at 650 nm.[13]

Serum Creatine Phosphokinase Activity

Serum levels of creatine phosphokinase (CPK) and its myocardium-specific isoform (CK-MB) were measured with the use of commercial kits (Sigma Chemical Co).[13]

Immunohistochemical Staining for ICAM-1

ICAM-1 expression was evaluated in cardiac sections through immunohistochemistry.[13] Frozen sections (5 μm thick) were fixed in 4% paraformaldehyde and incubated in 2% hamster serum for 2 hours to minimize nonspecific adsorption. Sections were then incubated overnight at 4°C with monoclonal biotinylated antibodies directed at ICAM-1 (hamster anti-mouse CD54) at a dilution of 1:500. Control preparations included buffer alone or nonspecific purified IgG. Antibody-binding sites were visualized with an avidin-biotin peroxidase complex immunoperoxidase technique (Vector Laboratories) with the use of diaminobenzidine. A grading system was used in which 0 indicates no staining, 1 indicates constitutive presence of staining along the endothelial wall, 2 and 3 indicate increasing degrees of intermediate staining along the endothelial wall, and 4 indicates increased staining along the endothelial wall and the presence of staining on myocytes. In each group, 5 or 6 sections were evaluated by 2 independent observers who were blinded to the experimental protocol.

Measurement of Serum Levels of Tumor Necrosis Factor-α, IL-10, and Nitrite/Nitrate

Immunoreactive murine IL-10 and tumor necrosis factor-α (TNF-α) were quantified with the use of ELISA according to the manufacturer's protocol.[14] Serum concentrations of nitrite/nitrate, stable breakdown products of NO, were measured according to the modified Griess reaction.[14] First, nitrate in 50 μL serum was reduced to nitrite through incubation with 25 μL nitrate reductase (670 mU/mL) and 25 μL NADPH (160 mmol/L) at room temperature for 3 hours. Then, 100 μL Griess reagent (0.1% naphthalethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated H_3PO_4; vol 1:1) was added. The absorbance at 550 nm was then measured.

Materials

Primary monoclonal ICAM-1 (CD54) antibody for immunohistochemistry was purchased from PharMingen. Reagents and secondary and nonspecific IgG antibodies for immunohistochemical analysis were obtained from Vector Laboratories. Plasma levels of IL-10 and TNF-α were measured with the use of ELISA kits purchased from Genzyme Co. All other chemicals were obtained from Sigma/Aldrich.

Data Analysis

All values in the figures and text are expressed as mean±SEM. The results were examined with the use of ANOVA, followed by the Bonferroni correction post hoc *t* test. Differences in survival rates were analyzed with a χ^2 test. A value of $P<0.05$ was considered statistically significant.

Results

Hemodynamic Parameters

Hemodynamic measurements were recorded under baseline conditions, during ischemia, and up to 15 minutes after reperfusion. There were no significant baseline differences in hemodynamics between wild-type and IL-10–deficient animals, as assessed with measurements of mean arterial pressure (MAP) and heart rates. Cardiac function was depressed in both IL-$10^{+/+}$ and IL-$10^{-/-}$ groups during the period of the occlusion, characterized by a drop in MAP. There was no difference between wild-type and IL-10–deficient animals in the recovery of MAP during the early reperfusion period (Table 2).

TABLE 2. Hemodynamic Parameters

	Baseline	Occlusion 15 min	Occlusion 30 min	Reperfusion 15 min
HR (n=8), bpm				
Wild-type	455±7	464±13	447±17	439±12
Knockout	422±16	456±14	426±6	435±11
MAP (n=3), mm Hg				
Wild-type	97±4	75±5	78±4	94±5
Knockout	103±5	79±2	83±2	92±3

Values are mean±SEM.

Endogenous IL-10 Regulates Myocardial Infarct Size and Neutrophil Infiltration After Coronary Occlusion and Reperfusion

In wild-type mice, 1-hour occlusion of the left coronary artery followed by 6-hour reperfusion resulted in a marked myocardial injury. On histological examination of the reperfused hearts, a marked necrosis of the tissue with the development of contraction bands was observed, with an infarct size of 49±4% of the ischemic area (Figure 1). Serum CPK level, an index of myocyte injury, increased compared with baseline level at 2, 4, and 6 hours after the start of reperfusion ($P<0.01$; Figure 2). There was a significant degree of neutrophil infiltration, as measured by an increase in tissue MPO activity over baseline at 2, 4, and 6 hours after the start of reperfusion ($P<0.01$; Figure 3).

The absence of the IL-10 gene resulted in a significant exacerbation of reperfusion injury of previously ischemic hearts. This was demonstrated by a statistically significant increase in infarct size to 64±4% of the ischemic area ($P<0.05$ compared with wild-type; Figure 1). Serum CPK levels showed a marked and significant increase over the levels seen in the wild-type animals at 6 hours after reperfusion (Figure 2), whereas at 2 and 4 hours, a tendency toward increase was observed (Figure 2). These measurements in the changes in total CPK were followed by measurement of the myocardium-specific isoform (CK-MB). These measurements confirmed a significant difference between the control and wild-type animals at 6 hours after the start of reperfusion: in the wild-type mice, CK-MB levels increased from 43±5 to 412±33 U/L, whereas in the IL-10–deficient mice, a significantly ($P<0.05$) larger increase in CK-MB activity was found: from 51±8 to 687±48 U/L (8 or 9 animals per group). At earlier time points of reperfusion (2 and 4 hours), similar to the measurements of total plasma CPK levels, no significant differences in CK-MB plasma levels were found between wild-type and IL-10–deficient mice (not shown). There was a more pronounced increase in the MPO levels in the IL-10–deficient animals at 6 hours compared with the wild-type response (Figure 3).

Endogenous IL-10 Regulates TNF-α Production and ICAM-1 Expression After Coronary Occlusion and Reperfusion

Using specific ELISAs, we observed that there was a substantial increase in IL-10 production in myocardial ischemia/

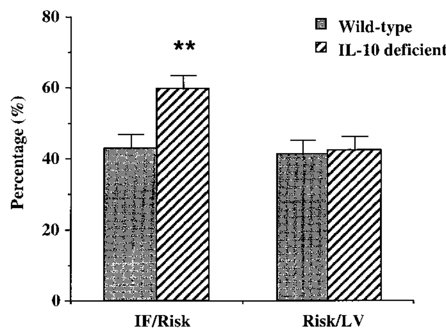

Figure 1. Myocardial infarct size after coronary occlusion and reperfusion is increased in IL-$10^{-/-}$ mice. Myocardial infarct size was measured in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice after 30-minute occlusion of LAD and 6-hour reperfusion. Infarct size is expressed as a ratio of weight of infarct tissue to weight of area at risk (IF/area at risk). No differences were found between these 2 groups with respect to ratio of area at risk to left ventricle (LV) ($P>0.05$). Infarct size is significantly increased in IL-$10^{-/-}$ mice compared with response in wild-type animals (** $P<0.01$). Data represent mean±SEM of 8 wild-type and 8 IL-10–deficient animals.

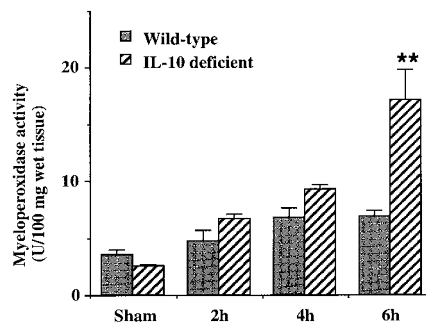

Figure 3. Neutrophil infiltration after coronary occlusion and reperfusion (30 minutes and 6 hours, respectively) is enhanced in IL-$10^{-/-}$ mice. MPO activity, an indicator of neutrophil infiltration, was measured in homogenates of left ventricle of mouse heart in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice subjected to 30-minute occlusion of LAD and 2-, 4-, or 6-hour reperfusion ($P<0.05$). MPO activity was significantly higher in IL-$10^{-/-}$ mice compared with wild-type (IL-$10^{+/+}$) control animals at 6 hours (** $P<0.01$). Data represent mean±SEM of 3 wild-type and 6 IL-10–deficient sham animals and 6 wild-type or IL-10–deficient animals for each time point of reperfusion.

reperfusion. IL-10 levels peaked at 4 hours and tended to return toward baseline at 6 hours after the start of the reperfusion (Figure 4). (In the IL-10–deficient animals, no IL-10 could be detected in the plasma.) TNF-α levels also increased at 2 to 6 hours after the start of the reperfusion in both groups of animals (Figure 5). At 6 hours after reperfusion, TNF-α levels were significantly higher in the IL-10–deficient animals than in the wild-type mice (Figure 5).

Endogenous IL-10 Regulates ICAM-1 Expression After Coronary Occlusion and Reperfusion

One of the early endothelial events in the process of neutrophil recruitment during myocardial ischemia is related to ICAM-1, which is constitutively expressed at low levels on the surface of endothelial cells but is upregulated and is responsible for the firm adhesion of neutrophils.[15,16] Stained myocardial tissue sections from sham-operated wild-type mice with anti–ICAM-1 antibody showed a specific staining along cardiac vessels that demonstrated ICAM-1 is constitutively expressed in endothelial cells (Figure 6). After ischemia followed by 6 hours of reperfusion, the staining intensity substantially increased in the area of early necrosis (Figures 6 and 7). Immunohistochemical staining was mainly localized in endothelial vascular wall, but a diffuse staining was also localized in myocytes within the necrotic lesion (Figures 6 and 7). Sections from IL-10–deficient mice revealed an increased upregulation of ICAM-1 (the degree of staining was scored 3.8±0.2 compared with 2.5±0.5 in the wild-type animals, $P<0.05$; Figure 7).

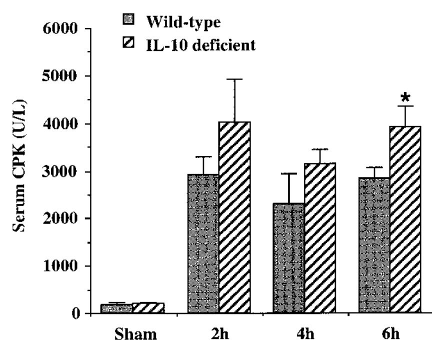

Figure 2. Myocardial necrosis after coronary occlusion and reperfusion (30 minutes and 6 hours, respectively) is enhanced in IL-$10^{-/-}$ mice. Serum CPK, an enzyme contained in viable myocytes and released into bloodstream during myocardial injury, was measured as an index of myocyte necrosis in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice subjected to myocardial ischemia/reperfusion. After 30-minute LAD occlusion and 2-, 4-, or 6-hour reperfusion, serum level of CPK was significantly increased in both groups of mice subjected to ischemia/reperfusion compared with baseline ($P<0.05$). At 6 hours after reperfusion, CPK levels in IL-$10^{-/-}$ mice were significantly higher than those in wild-type (IL-$10^{+/+}$) control animals (* $P<0.05$). Data represent mean±SEM of 3 wild-type and 6 IL-10–deficient sham animals and 6 to 10 wild-type or IL-10–deficient animals for each time point of reperfusion.

Endogenous IL-10 Regulates NO Production After Coronary Occlusion and Reperfusion

There are no differences in the baseline serum levels of nitrite/nitrate (breakdown products of NO) between wild-type

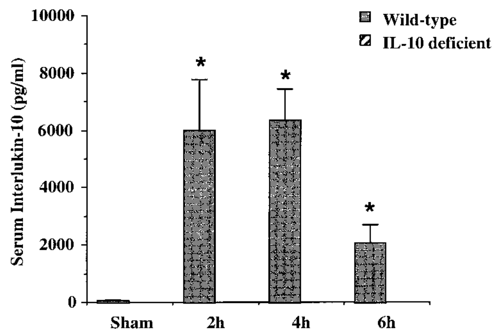

Figure 4. Myocardial ischemia/reperfusion in wild-type mice results in IL-10 production. Serum levels of IL-10 were measured with specific ELISA kits in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice subjected to 30-minute occlusion of LAD and 2-, 4-, or 6-hour reperfusion. Serum levels of IL-10 were significantly increased in IL-$10^{+/+}$ mice at 2, 4, and 6 hours compared with baseline levels (* $P<0.05$). In IL-$10^{-/-}$ mice, no IL-10 was detectable in plasma. Data represent mean±SEM of 3 wild-type and 3 IL-10–deficient animals at each time point.

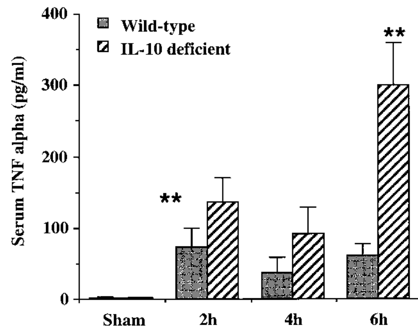

Figure 5. Upregulation of TNF-α after coronary occlusion and reperfusion (30 minutes and 6 hours, respectively) is enhanced in IL-$10^{-/-}$ mice. Serum levels of TNF-α were measured with specific ELISA kits in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice subjected to 30-minute occlusion of LAD and 2-, 4-, or 6-hour reperfusion. Serum levels of TNF-α were significantly increased in both groups of animals at 2, 4, and 6 hours compared with baseline levels ($P<0.05$). In IL-$10^{-/-}$ mice, significantly higher levels of TNF-α were measured compared with wild-type (IL-$10^{+/+}$) control animals at 6 hours (**$P<0.01$). Data represent mean±SEM of 3 wild-type and 3 IL-10–deficient animals at each time point.

and IL-10–deficient mice (Figure 8). By 6 hours of reperfusion (but not at 2 or 4 hours), serum nitrite/nitrate significantly increased in both IL-$10^{-/-}$ and IL-$10^{+/+}$ mice ($P<0.05$). The rise was more evident in IL-$10^{-/-}$ mice than in IL-$10^{+/+}$ mice at 6 hours of reperfusion ($P<0.05$; Figure 8).

Endogenous IL-10 Is Essential for Survival in Myocardial Ischemia/Reperfusion

Reperfusion for 6 hours did not result in high mortality rates in wild-type or IL-10–deficient animals (Table 1). In an additional set of experiments, we investigated the effect of 30-minute occlusion of LAD and 24-hour reperfusion on survival in wild-type and IL-10–deficient mice. Six of 8 IL-$10^{-/-}$ mice died within 12 hours of reperfusion after 30-minute occlusion of LAD, whereas all 8 IL-$10^{+/+}$ mice that were tested survived the 24-hour reperfusion, yielding a significant difference in the 24-hour survival rate between the two groups of animals ($P<0.01$).

Discussion

IL-10 is a potent anti-inflammatory cytokine. Multiple studies have demonstrated the production of IL-10 during human myocardial ischemia/reperfusion injury and during cardiopulmonary bypass. In one of these settings in humans, an examination of the plasma levels of a large number of cytokines during human cardiopulmonary bypass revealed that IL-10 showed the least interindividual variations, suggesting that this cytokine may provide reliable information regarding modulation of the immune response after bypass and its consequences for patient outcome.[10] In the present study, we demonstrated the production of IL-10 in the plasma after myocardial ischemia/reperfusion in the mouse. The production of IL-10 has been previously demonstrated in various animal models of reperfusion injury (eg, in the reperfused liver, brain, kidney, or gut[17–19]). However, to our knowledge, our investigations are the first to demonstrate the production of IL-10 in the plasma after myocardial ischemia/reperfusion in an experimental animal. The present study did not identify the mechanism of IL-10 production or the cells responsible for it. In a study of IL-10 production from the reperfused liver, it was found that the inhibition of free radical generation by *N*-acetylcysteine or allopurinol decreased IL-10 levels in the effluents, as did an inhibitor of transcription factor nuclear factor-κB mobilization.[19] It is conceivable that free radical–mediated nuclear factor-κB activation was responsible for IL-10 production in the present study.

The protective effect of exogenously administered IL-10 in the outcome of myocardial ischemia/reperfusion injury is well established. For example, in a study in anesthetized rats, IL-10 (100 μg/rat) administered 15 minutes before reperfusion significantly attenuated myocardial injury, as indicated by a reduced loss of myocardial creatine kinase from the ischemic/reperfused myocardium.[20] Cardiac MPO activity was also significantly attenuated by IL-10. The authors concluded that IL-10 mediates its effects, at least in part, through the inhibition of leukocyte/endothelium interactions.[20] Similar conclusions were reached with IL-10 pretreatment in studies in animals of splanchnic artery, pulmonary, or hindlimb ischemia/reperfusion or of stroke.[21–23]

The present study provides the first direct evidence that the *endogenous* production of IL-10 during myocardial ischemia/reperfusion injury plays a key role in determination of the outcome. The crucial role of IL-10 is demonstrated by an increased infarct size, massively increased neutrophil infiltration, or increased plasma TNF-α and nitrite/nitrate and myocardial ICAM-1 levels in the IL-$10^{-/-}$ animals subjected to myocardial ischemia/reperfusion. There are a number of recent studies that demonstrate the crucial role of endogenously produced IL-10 in the modulation of the cytokine and chemokine response in various forms of systemic inflammation, induced by bacterial lipopolysaccharide, live bacteria, or staphylococcal enterotoxin B.[7–10,14] However, to our knowledge, the present report is the first to demonstrate the crucial role of endogenous IL-10 in modulation of the course of myocardial reperfusion injury or, in fact, any form of reperfusion injury. The present findings may be comparable to those of a recent study by Raisanen-Sokolowski et al,[24] in which the role of IL-10 in late graft outcome was investigated through the transplantation of BALB/c donor hearts into immunosuppressed wild-type or IL-10 gene–deficient recipients. In this study, similar to our findings, there was an increase in the leukocyte infiltration and parenchymal destruction, with more severe vascular occlusion in grafts from IL-$10^{-/-}$ recipients compared with wild-type responses. This was associated with an enhancement of the inflammatory response in the IL-10–deficient mice, as demonstrated by an increased expression in interferon-γ, Mac-1, inducible NO synthase (iNOS), and allograft inflammatory factor-1.[24]

Perhaps the most dramatic difference between wild-type and IL-10–deficient mice subjected to myocardial ischemia/reperfusion injury was the amount of neutrophils that infiltrated the reperfused myocardium. Based on the ability of exogenously administered IL-10 to suppress the expression of proinflammatory cytokines and adhesion molecules and the effect of exogenous IL-10 in limiting the neutrophil infiltration in various forms of reperfusion injury (see earlier), we

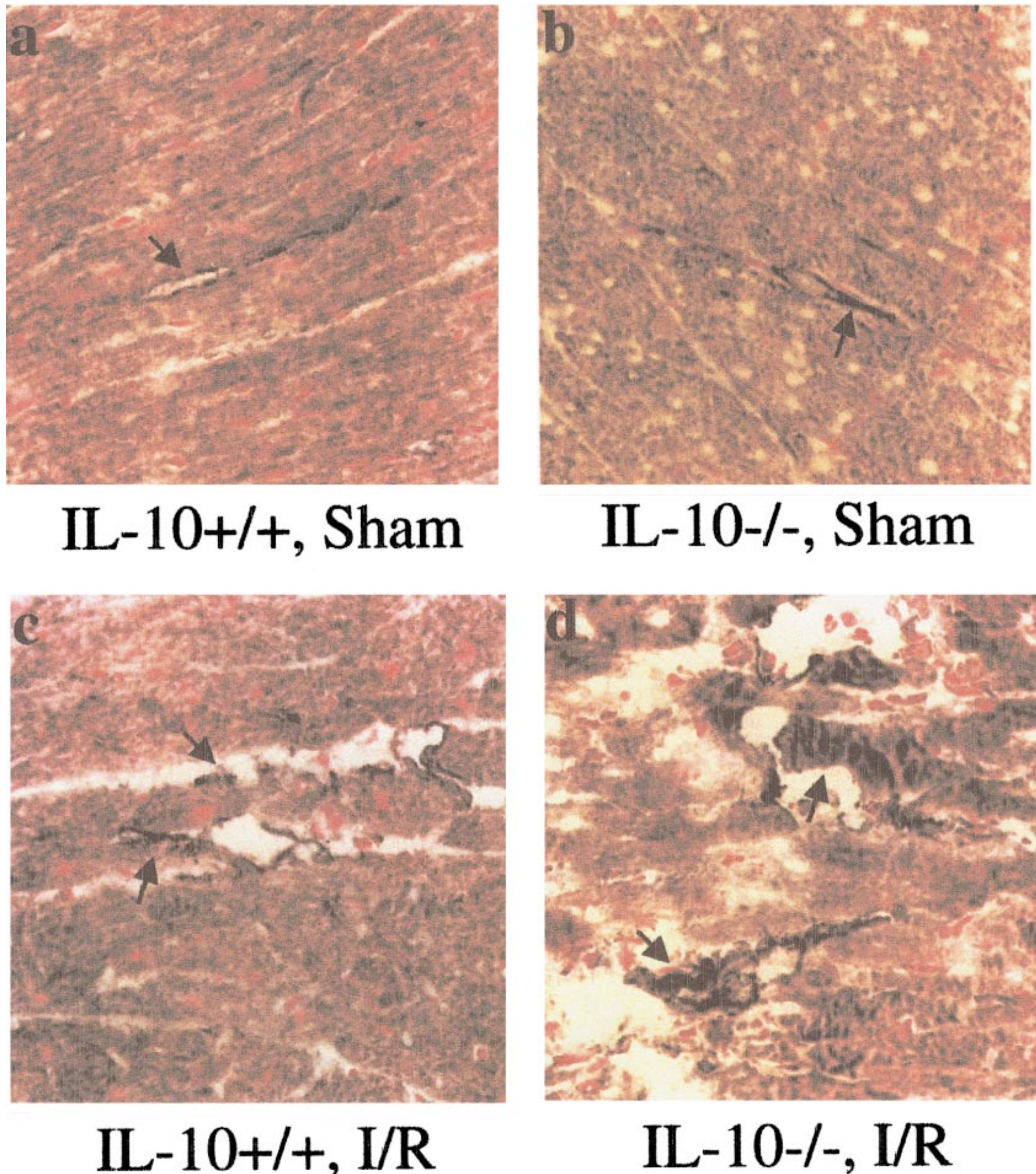

Figure 6. Upregulation of ICAM-1 after coronary occlusion and reperfusion (30 minutes and 6 hours, respectively) is enhanced in IL-10$^{-/-}$ mice. Control tissues from sham operated IL-10$^{+/+}$ (a) or IL-10$^{-/-}$ animals (b) showed dark brown staining (arrows) of endothelium of blood vessels, indicating presence of constitutive ICAM-1 protein. Myocardial ischemia/reperfusion (I/R, 30 minutes and 6 hours, respectively) induced an increase in positive staining for ICAM-1 along endothelial vascular wall and in injured myocytes (arrows) (c, d). In IL-10$^{-/-}$ mice subjected to ischemia/reperfusion, there was a more pronounced increase of immunostaining for ICAM-1 (arrows) (d) (magnification, 400×).

hypothesize that the primary actions of endogenous IL-10 are related to the modulation of the neutrophil activation and their tissue infiltration. These actions may be related to both the suppression by endogenous IL-10 of the activation of neutrophils[25] and the expression of endothelial adhesion molecules such as ICAM-1.[6,26,27] Interestingly, we observed most of the differences between wild-type and IL-10–deficient mice in terms of inflammatory mediator production, ICAM-1 expression, and myocardial necrosis at 6 hours after the beginning of reperfusion and not at the earlier time points studied. In comparison, plasma IL-10 levels peaked at 2 to 4 hours after the beginning of reperfusion. It is likely that a lag time is required for the endogenously produced IL-10 to exert its anti-inflammatory effects. It is conceivable that in experimental models in which IL-10 is administered exogenously before the beginning of reperfusion, the intervention bypasses this lag time, thereby ameliorating the proinflammatory response and suppressing the tissue recruitment of neutrophils.

With respect to NO production and myocardial ischemia/reperfusion, it is well established that inhibition of the constitutive, endothelial isoform of NO synthase is detrimen-

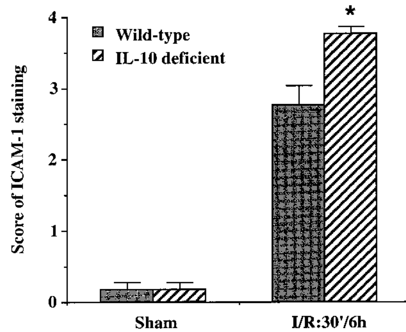

Figure 7. Upregulation of ICAM-1 after coronary occlusion and reperfusion (30 minutes and 6 hours, respectively) is enhanced in IL-$10^{-/-}$ mice. To quantify degree of ICAM-1 staining, a 0-to-4 grading system was used as described in Methods. ICAM-1 expression increased in response to myocardial ischemia/reperfusion in both groups of animals, and it was significantly (*$P<0.05$) higher in IL-$10^{-/-}$ mice compared with response in wild-type animals. Data represent mean±SEM of 6 randomly selected areas from 3 wild-type animals and of 6 randomly selected areas from 3 IL-10–deficient animals.

tal in myocardial reperfusion, especially in the early phase.[28–32] This is, in a significant part, due to the facts that inhibition of the endothelial NOS causes myocardial ischemia[28] and enhances intravascular platelet and neutrophil deposition.[30–32] On the other hand, expression of the proinflammatory iNOS has been demonstrated in the late phase of reperfusion, as shown in both experimental animals[33,34] and humans.[35] Selective inhibition of this particular isoform of NO synthase has been shown to have beneficial effects.[35–37] Based on these data, it is conceivable that by suppressing iNOS expression, endogenous IL-10 serves to maintain the patency of the reperfused hearts.

Taken together, the results of the present study demonstrate that (1) IL-10 is produced in a murine model of myocardial ischemia/reperfusion; (2) endogenously produced IL-10 serves to suppress the production of TNF-α and the expression of iNOS in the reperfusion phase; (3) endogenous IL-10, produced during myocardial reperfusion, serves to suppress the tissue infiltration of polymorphonuclear granulocytes; and (4) endogenous IL-10 is essential for survival during prolonged periods of myocardial ischemia/reperfusion. In the absence of endogenous IL-10, marked inflammatory response and neutrophil infiltration occur, which result in an enhancement of myocardial infarction, an increase in myocardial necrosis, and a marked increase in mortality rates. The increased production of TNF-α, NO, and expression of ICAM-1 may be examples of this inflammatory response. Based on prior studies, it appears likely that in addition to the above mediators/factors, a host of other inflammatory mediators (other types of cytokines, chemokines, adhesion receptors, oxygen-derived free radicals, and so on) are also upregulated in response to myocardial ischemia/reperfusion in the IL-10–deficient mice compared with the wild-type animals. As in many other forms of inflammation or reperfusion injury, it is likely that the massive injury is due to the synergistic action of many of these mediators.

There are a number of studies suggesting the potential use of IL-10 in experimental therapy for various forms of reperfusion injury.[17–23] Under systemic or local inflammatory conditions, a number of approaches have recently been identified that are able to boost endogenous IL-10 levels while simultaneously downregulating the production of proinflammatory cytokines and chemokines. Such approaches include, among others, cAMP phosphodiesterase inhibition and ligands of adenosine A_3 and other adenosine receptor subtypes.[38] Many of these approaches have been previously shown to exert protective effects in various models of myocardial reperfusion.[39–41] It remains to be determined whether the protection is related to the ability of these approaches to boost endogenous IL-10 levels.

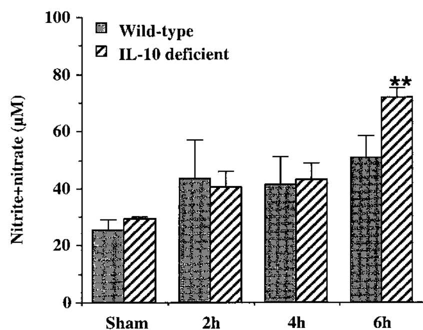

Figure 8. NO production after coronary occlusion and reperfusion (30 minutes and 6 hours, respectively) is enhanced in IL-$10^{-/-}$ mice. Nitrite and nitrate, stable breakdown products of NO in serum, were measured with use of modified Griess reaction in IL-$10^{+/+}$ and IL-$10^{-/-}$ mice subjected to 30-minute occlusion of LAD and 2-, 4-, or 6-hour reperfusion. By 6 hours of reperfusion (but not at 2 or 4 hours), serum nitrite/nitrate significantly increased in both IL-$10^{-/-}$ and IL-$10^{+/+}$ mice ($P<0.05$). Nitrite/nitrate levels were significantly higher in IL-$10^{-/-}$ mice than in IL-$10^{+/+}$ mice at 6 hours of reperfusion (*$P<0.05$). Data represent mean±SEM of 3 wild-type and 6 IL-10–deficient sham animals and 6 wild-type or IL-10–deficient animals for each time point of reperfusion.

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