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IL-10 Is Induced in the Reperfused Myocardium and May Modulate the Reaction to Injury[1]

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Reperfusion of the ischemic myocardium is associated with a dramatic inflammatory response leading to TNF-α release, IL-6 induction, and subsequent neutrophil-mediated cytotoxic injury. Because inflammation is also an important factor in cardiac repair, we hypothesized the presence of components of the inflammatory reaction with a possible role in suppressing acute injury. Thus, we investigated the role of IL-10, an anti-inflammatory cytokine capable of modulating extracellular matrix biosynthesis, following an experimental canine myocardial infarction. Using our canine model of myocardial ischemia and reperfusion, we demonstrated significant up-regulation of IL-10 mRNA and protein in the ischemic and reperfused myocardium. IL-10 expression was first detected at 5 h and peaked following 96–120 h of reperfusion. In contrast, IL-4 and IL-13, also associated with suppression of acute inflammation and macrophage deactivation, were not expressed. In the ischemic canine heart, CD5-positive lymphocytes were the predominant source of IL-10 in the myocardial infarct. In the absence of reperfusion, no significant induction of IL-10 mRNA was noted. In addition, IL-12, a Th1-related cytokine associated with macrophage activation, was not detected in the ischemic myocardium. In vitro experiments demonstrated late postischemic cardiac-lymph-induced tissue inhibitor of metalloproteinases (TIMP)-1 mRNA expression in isolated canine mononuclear cells. This effect was inhibited when the incubation contained a neutralizing Ab to IL-10. Our findings suggest that lymphocytes infiltrating the ischemic and reperfused myocardium express IL-10 and may have a significant role in healing by modulating mononuclear cell phenotype and inducing TIMP-1 expression. *The Journal of Immunology,* **2000, 165: 2798–2808.**

Myocardial ischemia and reperfusion is associated with an intense inflammatory reaction, which may lead to myocyte injury (1). Studies using a variety of anti-inflammatory agents in animal models of coronary ischemia demonstrated significant reduction in infarct size. However, it soon became apparent that nonspecific inhibition of the inflammatory cascade may also result in inadequate healing (2). This was emphasized by the catastrophic results of the methylprednisolone trial, which significantly increased the incidence of myocardial rupture (3).

Previous studies from our laboratory have demonstrated that monocyte chemoattractant protein (MCP)[3]-1 is markedly induced in the ischemic and reperfused myocardium (4), suggesting a significant role for MCP-1 in mononuclear cell (monocyte and lymphocyte) trafficking in the reperfused myocardium (5). In the absence of reperfusion, no significant MCP-1 mRNA induction is seen (4). Monocytes and lymphocytes chemotactically attracted in the reperfused myocardium may have an important role in ventricular repair by modulating the inflammatory response.

It is generally accepted that T lymphocytes mediate immunologic responses by release of cytokines. Mossman et al. (6) identified two very distinct cytokine secretion patterns among a panel of mouse T cell clones, introducing the concept of Th1 and Th2 cells. Th1 cells produce macrophage-activating cytokines, whereas Th2 cells augment humoral responses and inhibit Th1 responses (7). Th1 and Th2 responses have been implicated as regulators of cell-mediated immunity, as important mediators in clearance of many infectious organisms (8), and in the immunopathology of organ-specific autoimmune diseases (9, 10). IL-10 (6, 11), a cytokine initially described as cytokine synthesis inhibitory factor (12), is primarily a product of activated Th2 cells and endotoxin-stimulated monocytes (11). Among the different cell types affected by IL-10, monocyte macrophages appear to be particularly modified in regard to their function, morphology, and phenotype (11). IL-10 inhibits the production of IL-1α, IL-1β, TNF-α, IL-6, and IL-8 by LPS-activated monocytes (13, 14), suppressing the inflammatory response. IL-10 also suppresses expression of IL-12 (11), a cytokine primarily produced by activated monocytes and a dominant factor in directing Th1-type responses (15, 16). Furthermore, IL-10 may have a significant role in extracellular matrix formation by modulating expression of metalloproteinases and tissue inhibitor of metalloproteinases (TIMP) (17, 18).

In this study, we investigated the regulation of T cell-derived lymphokines using a canine model of experimental myocardial ischemia and reperfusion. We found significant numbers of infiltrating CD5-positive T cells in the reperfused myocardium. We provide the first evidence that IL-10 is induced in the ischemic and reperfused myocardium; over the same time period, IL-6 mRNA expression is down-regulated. IL-10 induction was highly dependent upon reperfusion of the previously ischemic myocardium. In contrast, the Th1-related cytokine, IL-12, was not detected. We identified infiltrating T lymphocytes and a subset of macrophages

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[3] Abbreviations used in this paper: MCP, monocyte chemoattractant protein; TIMP, tissue inhibitor of metalloproteinase; SSPE, standard saline citrate phosphate/EDTA.

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as the predominant source of IL-10 in the healing myocardium. Furthermore, we demonstrated that IL-10 is responsible for cardiac-lymph-mediated TIMP-1 up-regulation in isolated canine mononuclear cells. Our findings suggest a potential role for lymphocyte-derived IL-10 in suppressing proinflammatory cytokine synthesis by macrophages infiltrating the ischemic myocardium and in modulating scar formation and maturation following experimental myocardial infarction. This may represent an important mechanism explaining the benefit associated with late reperfusion of the ischemic myocardium when significant myocardial salvage cannot be expected (19–21).

Materials and Methods

Ischemia/reperfusion protocols

Healthy mongrel dogs (15–25 kg) of either sex were surgically instrumented as previously described (22–24). Anesthesia was induced i.v. with 10 mg/kg methohexital sodium (Brevital, Eli Lilly, Indianapolis, IN) and maintained with the inhalational anesthetic isoflurane (Anaquest, Madison, WI). A midline thoracotomy provided access to the heart and mediastinum, and cannulation of the cardiac lymph duct was then performed. Subsequently, a hydraulically activated occluding device and a Doppler flow probe were secured around the circumflex coronary artery just proximal or just distal to the first branch. Choice of location depended on the proximity and anatomical arrangement. Indwelling catheters placed in the right atrium, left atrium, and femoral artery allowed blood sampling and pressure monitoring as needed. After surgery, the animals were allowed to recover for 72 h before occlusion. Coronary artery occlusion was achieved by inflating the coronary cuff occluder until mean flow in the coronary vessel was zero as determined by the Doppler flow probe. At the end of 1 h, the cuff was deflated and the myocardium was reperfused. Reperfusion intervals ranged from 1 h to 7 days. Circumflex blood flow, arterial blood pressure, heart rate, and electrocardiogram (standard limb II) were recorded continuously. Analgesia was accomplished with 0.1–0.2 mg/kg i.v. administered pentazocine (Talwin, Winthrop Pharmaceuticals, New York, NY). After the reperfusion periods, hearts were stopped by the rapid i.v. infusion of 30 milliequivalent KCl and removed from the chest for sectioning from apex to base into four transverse rings ~1 cm in thickness. The posterior papillary muscle and the posterior free wall were identified. Tissue samples were isolated from infarcted or normally perfused myocardium based on visual inspection. Myocardial segments were fixed in 10% buffered formalin, Carnoy's fixative, or B*5 fixative (25) for histological analysis or immediately frozen, homogenized, and processed for RNA extraction. Duplicate adjacent samples were also processed for blood flow determinations using radiolabeled microspheres as previously described (22). Six ischemia/reperfusion experiments were performed for each reperfusion interval.

The presence of a myocardial infarct was based on light-microscopic examination of hematoxylin-eosin-stained tissue sections by findings of contraction bands, "wavy fibers," interstitial edema, and neutrophil infiltration, all in segments displaying markedly reduced blood flow (<25% control) during the ischemic period. For experiments lasting 24 h or more after the start of the ischemic insult, the presence of histological elements characteristic of myocyte necrosis and fibrosis was added to the required criteria. Samples described as ischemic were all from areas where ischemic blood flow was <25%. Samples of control tissues were taken from the anterior septum and had normal blood flow during coronary occlusion.

Tissue samples from endotoxemic animals were used as positive controls for Northern hybridization and immunohistochemical experiments. Endotoxemia was achieved by i.v. injection of 500 μg/kg *Escherichia coli* endotoxin (Sigma, St. Louis, MO). Animals were sacrificed 3 h later, and the spleen, heart, lung, liver, and kidney were used for mRNA extraction and histological studies.

Immunohistochemistry and histology

For histological study of cardiac tissue, sections taken from endocardium to epicardium and sections taken parallel to the wall of the heart were fixed in 10% phosphate-buffered formalin or B*5 fixatives and embedded in paraffin. Immunohistochemistry for IL-10 was successful only with the use of the B*5 fixative. Sequential 3- to 5-μm sections were cut by microtomy. Immunostaining was performed using the Elite rabbit or mouse kit (Vector Laboratories, Burlingame, CA). Briefly, sections were pretreated with a solution of 3% hydrogen peroxide to inhibit endogenous peroxidase activity and incubated with 2% goat serum to block nonspecific protein binding. Subsequently, they were incubated with the primary Ab for 2 h at room temperature. After rinsing with PBS, the slides were incubated for 30 min with the secondary Ab. The slides were rinsed with PBS and incubated for 30 min in ABC reagent. Peroxidase activity was detected using diaminobenzidine with nickel. Slides were counterstained with eosin.

The following primary Abs were used for immunohistochemistry: rabbit anti-human polyclonal Ab to IL-10 (Genzyme, Cambridge, MA), rabbit anti-human Ab to TIMP-1 (Chemicon, Temecula, CA), monoclonal anti-bovine Ab to TIMP-1 (Chemicon), monoclonal anti-CD5 Ab (VMRD, Pullman, WA), rabbit anti-human CD3 Ab (Dako, Carpinteria, CA), mouse anti-human CD79a clone HM57 Ab (Dako), and monoclonal anti-human macrophage Ab AM-3K (26–28) (a generous gift from Dr. Takahashi, Kumamoto University, Japan). For T and B lymphocyte immunolabeling, staining with Abs to CD5 and CD3 with known cross-reactivity to canine species (29) was used. Sections from the spleen of an endotoxin-stimulated animal were used as positive controls. Fluorescent immunohistochemistry was achieved by using appropriate rhodamine- or Cy3-labeled secondary Abs (Sigma). Appropriate positive and negative controls were used for each Ab. Dual immunohistochemistry was performed by combining peroxidase-based immunostaining for CD5 with fluorescent immunohistochemistry for IL-10 rhodamine-labeled secondary Abs.

Quantitative analysis of immunohistochemical experiments

Stained sections were photographed with a Leaf MicroLumina digital camera (Leaf Systems, Southboro, MA) mounted on a Zeiss microscope (Oberkochen, Germany). Multiple digital images were taken and stored for each sample. Staining was analyzed by Zeiss image-analysis software.

Molecular cloning

Specific canine cDNA clones for IL-4, IL-10, IL-13, IL-12 p40, and TIMP-1 were prepared by reverse transcription using RNA extracted from Con A plus PMA-stimulated mononuclear cells (for IL-4 and IL-13), or the spleen of an endotoxin-stimulated animal (for IL-10, IL-12 p40, and TIMP-1). RT-PCR protocols were performed using the following primers: IL-10 sense primer, 5′-CTTGCTCGAGGACTTTAAGG-3′; IL-10 antisense primer, 5′-CTTGCTCTTGTTCTCACAGGGC-3′; IL-4 sense primer, 5′-ATCACCTTACAAGAGATCATCAA-3′; IL-4 antisense primer, 5′-CTT GGCTTCATTCACAGAACAG-3′; IL-13 sense primer, 5′-GCCCTCA GGGAGCTCATTGAGGAGCTG-3′; IL-13 antisense primer, 5′-ACA TTTTTGACATCAGAAAGAAAAATGA-3′; IL-12 p40 sense primer, 5′-CTCACCTGTGACACCCCTGAAG; IL-12 p40 antisense primer, 5′-GCA GAGAGTGTAGCAGCTCCAC; TIMP-1 sense primer, 5′-TCGTCAT CAGGGCCAAGTTCGTGGGGA-3′; and TIMP-1 antisense primer, 5′-AGCTGGTCCGTCCACAAGCAATGA-3′. The nucleotide sequence of the primers was based on areas of the published sequence for their human homologues that showed a high degree of interspecies conservation. Reverse transcription protocols were performed with 5 μg of total RNA. After first-strand synthesis was primed with the antisense primer, aliquots of the reverse-transcription reaction were amplified using 5 U *Taq* DNA polymerase (Promega, Madison, WI) for 30 cycles at 93°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The resulting fragments were purified, cloned in the PCR vector (Invitrogen, San Diego, CA), and sequenced.

RNA isolation

RNA isolation from myocardial tissue segments was performed using the acid-guanidinium-phenol-chloroform procedure. RNA (20 μg) was electrophoresed in 1% agarose gels containing formaldehyde and then transferred to a nylon membrane (Gene Screen Plus; New England Nuclear, Boston, MA) by standard procedures.

Northern hybridization

Membranes were hybridized in QuikHyb (Stratagene, La Jolla, CA) at 68°C for 2 h with 1×10^6 dpm random hexamer ^{32}P-labeled canine cDNA probes for IL-10, IL-4, IL-13, IL-12 p40, IL-6, and TIMP-1. Filters were washed with 2× standard saline citrate phosphate/EDTA (SSPE) at 68°C for 20 min, with 1× SSPE plus 1% SDS at 68°C for 15 min twice, and with 1× SSPE at 21°C for 15 min with constant shaking, and were exposed to Hyperfilm (Amersham, Arlington Heights, IL). Quantitation of the Northern hybridization results was performed using densitometry. Relative density was normalized to the intensity of the 28S ribosomal RNA as previously described (30, 31) and expressed as the following ratio: (IL-10 relative density: 28S relative density) × 100.

Mononuclear cell isolation and stimulation

Canine mononuclear cells were isolated by use of a Ficoll-Hypaque gradient and resuspended in PBS as previously described (32). For incubation experiments with cardiac lymph, aliquots of lymph were obtained before

coronary occlusion and during reperfusion. Mononuclear cells were incubated for 18 h at 37°C in the presence or absence of 50–100 ng/ml recombinant human IL-10 (R&D Systems, Minneapolis, MN). The postischemic lymph used for these experiments was collected following 24–48 h of reperfusion. Blocking studies were performed with the addition of 5 μl/ml polyclonal neutralizing Ab to human IL-10 (R&D Systems). After incubation, RNA was extracted from the mononuclear cells as previously described, and Northern hybridization for TIMP-1 was performed. Four experiments, using the cardiac lymph obtained from four different animals that demonstrated significant ischemia, were performed.

Statistical analysis

The statistical significance of the findings was assessed by ANOVA. This was followed by a Student's *t* test and corrected for multiple comparisons (Bonferroni). In histological studies, each experiment and time point was analyzed as a function of time of reperfusion after 1 h of occlusion. The findings described all that occurred in at least three consecutive experiments.

Results

Cloning of canine IL-4, IL-10, IL-13, IL-12 p40, and TIMP-1

Using RT-PCR techniques, we obtained partial clones for canine IL-4, IL-10, IL-12 p40, IL-13, and TIMP-1. The partial clones for IL-4 (245 bp), IL-10 (206 bp), IL-12 p40 (403 bp), and TIMP-1 (405 bp) showed excellent homology with the previously reported canine sequences (33–36). The percent identity with their respective human homologues was 75% for IL-4, 87% for IL-10, 87% for IL-12 p40, and 80% for TIMP-1. The partial clone for IL-13 (387 bp) showed a 79% identity with its human homologue (37).

IL-10 and not IL-4 or IL-13 is induced following myocardial ischemia/reperfusion

The expression of IL-10 mRNA after experimental circumflex coronary artery occlusion and reperfusion was assessed using Northern hybridization with a ^{32}P-labeled canine IL-10 probe. IL-10 mRNA was not detected in normally perfused myocardial segments. In contrast, high levels of IL-10 mRNA expression were noted in experiments of 1 h of ischemia and 4 days of reperfusion, with the highest levels observed in the most ischemic myocardial segments (Fig. 1*A*). A trend to increased induction of IL-10 mRNA was first noted after 3 h of reperfusion (Figs. 2*A* and 3). IL-10 mRNA expression in ischemic segments peaked at 24–96 h (Figs. 2*B* and 3), with levels decreasing toward baseline after 7 days (Figs. 2*C* and 3). Northern hybridization was performed using the IL-4 and IL-13 cDNA clones. No induction of IL-4 and IL-13 was observed in the canine myocardium (data not shown).

Induction of IL-10 mRNA in the ischemic myocardium is reperfusion dependent

We assessed the role of reperfusion in IL-10 mRNA induction following myocardial ischemia by comparing experiments with and without reperfusion. In contrast to the relatively high levels of IL-10 mRNA expression noted in the ischemic and reperfused myocardium, in nonreperfused experiments undergoing 24 h and 96 h (Fig. 4) of coronary occlusion, we detected minimal levels of IL-10 mRNA, in the presence of comparable degrees of myocardial blood flow reductions.

Delayed and prolonged expression of IL-6 mRNA in the nonreperfused ischemic myocardium

Previous studies from our laboratory (24) have demonstrated early induction of IL-6 mRNA (peaking at 3 h of reperfusion, Ref. 24), which was markedly enhanced during the first 6 h by reperfusion of the previously ischemic myocardium; however, by 24 h, IL-6

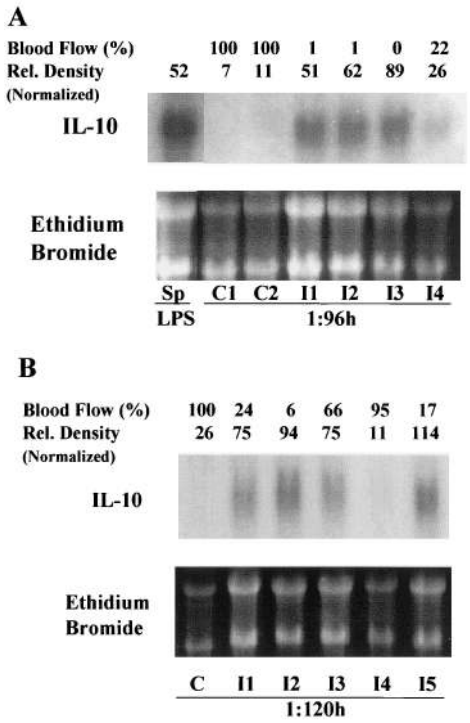

FIGURE 1. IL-10 mRNA induction in the ischemic and reperfused myocardium. mRNA extracted from representative experiments of 1-h ischemia/96-h reperfusion (*A*) and 1-h ischemia/120-h reperfusion (*B*) were probed with the cDNA clone for IL-10. Significant induction of IL-10 mRNA was noted in ischemic segments of the canine myocardium. Note that the highest levels of IL-10 mRNA expression are found in the most ischemic segments. mRNA extracted from the spleen of an endotoxin-stimulated animal served as the positive control. Blood flow (%) indicates the reduction in blood flow compared with a control segment from the same experiment. *Lane C*, Control myocardial sections from noninstrumented regions. *Lane I*, Myocardium from an instrumented, occluded, and ischemic region. The *lower panel* is stained with ethidium bromide to demonstrate comparable loading of all lanes. Sp, Spleen.

induction was also observed in nonreperfused infarcts. Our current experiments suggested that IL-6 mRNA expression, which is maximal at 3 h of reperfusion, is down-regulated after 24 h of reperfusion in the same ischemic segments in which IL-10 mRNA induction is found (Fig. 4). In contrast, animals with permanent occlusion showed minimal IL-10 mRNA up-regulation and high IL-6 mRNA expression, which persisted for 96 h.

IL-10 immunoreactivity in the ischemic and reperfused myocardium

To examine the localization of IL-10 immunoreactivity in the ischemic myocardium, we performed immunohistochemical studies using a rabbit anti-human polyclonal Ab to IL-10. Sections from the spleen of an endotoxin-stimulated animal were used as a positive control (Fig. 5*A*). In the ischemic myocardium, some cells with IL-10 immunoreactivity were identified first after 5 h of reperfusion (8.23 ± 1.89 cells/mm^2, Fig. 5*B*) and became more numerous with longer reperfusion intervals (72–120 h), peaking after 5 days of reperfusion (22.5 ± 2.7 cells/mm^2, Fig. 5, *C* and *D*).

Lymphocytes and macrophages infiltrate the ischemic and reperfused myocardium

Using immunohistochemical techniques, we demonstrated a striking infiltration of the ischemic and reperfused myocardium with

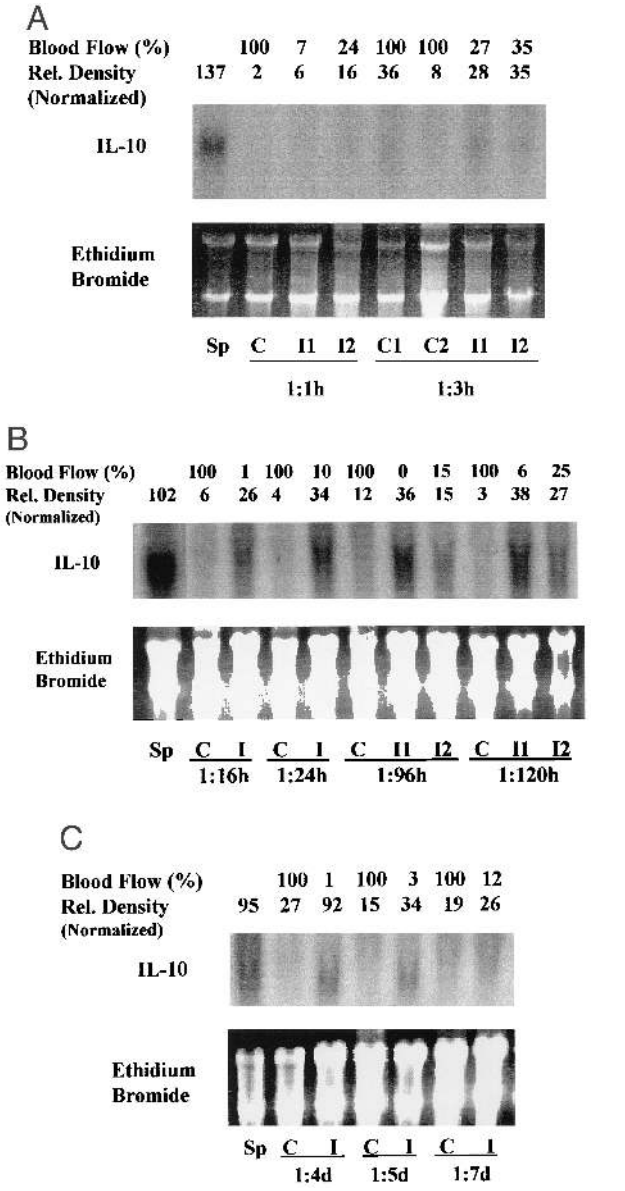

FIGURE 2. IL-10 mRNA regulation in the ischemic and reperfused canine myocardium. A series of representative experiments of various lengths of reperfusion are shown. *A*, Note that some up-regulation of IL-10 mRNA is first detected following 1 h of ischemia and 3 h of reperfusion. IL-10 mRNA levels peak after 96 h of reperfusion (*B*) and appear to decrease again after 7 days of reperfusion (*C*). mRNA extracted from the spleen of an endotoxin-stimulated animal serves as the positive control.

AM-3K-positive macrophages (Fig. 6*A*) and T lymphocytes labeled with Abs to CD3 and CD5 (Fig. 6*B*). A resident macrophage population was identified in the canine heart as previously demonstrated (28). A much smaller resident T lymphocyte population was also found. In contrast, CD79-positive B lymphocytes were very rare in control and ischemic canine myocardium. Macrophage and lymphocyte accumulation started as early as 5 h of reperfusion and was markedly increased after 72–120 h of reperfusion (Fig. 6, *C* and *D*). Following 5 days of reperfusion, there was an 8-fold increase of macrophage numbers and a similar increase of lymphocyte numbers in ischemic segments vs the respective controls (Fig. 6, *C* and *D*).

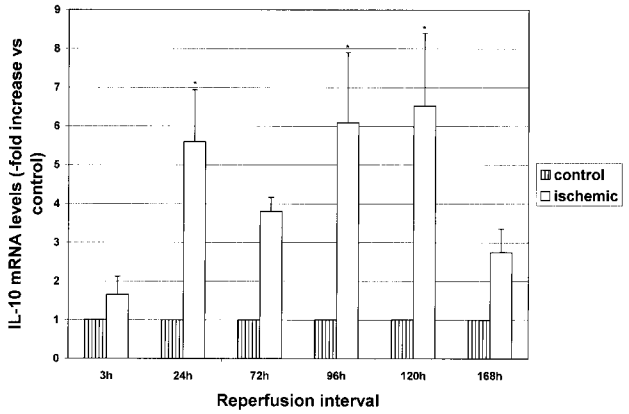

FIGURE 3. Quantitative analysis of Northern hybridization findings. Segments from experiments of coronary ischemia and reperfusion demonstrating significant ischemia (coronary flow <25%) were selected for statistical analysis. Five ischemic segments from consecutive experiments were used for each reperfusion interval, and the IL-10 mRNA levels were expressed as fold increases over their respective control segments. A marked up-regulation of IL-10 mRNA was noted in ischemic segments after 24 h of reperfusion. The IL-10 induction peaked after 120 h of reperfusion, when IL-10 levels in ischemic segments were 6.52 ± 1.88 ($p < 0.05$, $n = 5$) times higher than in the corresponding control segments from the same experiments.

Lymphocytes and a small subset of macrophages are the source of IL-10 following myocardial ischemia and reperfusion

IL-10-positive cells demonstrated characteristics of mononuclear cells. Staining of serial sections and dual immunohistochemistry localized IL-10 immunoreactivity in lymphocytes identified by their positive staining for the T cell markers CD3 (Fig. 7) and CD5

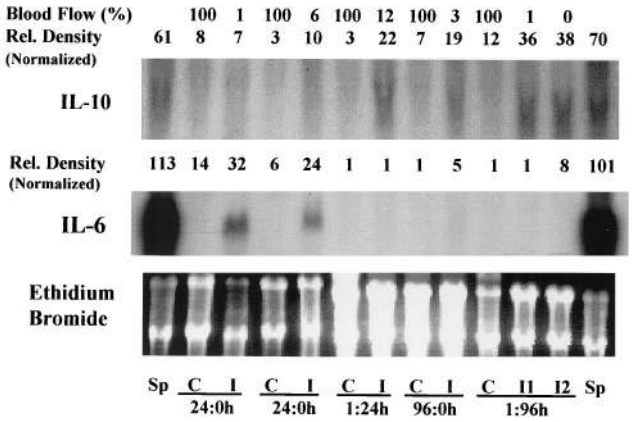

FIGURE 4. *Upper panel,* Requirement for reperfusion in induction of IL-10 mRNA. Representative experiments of 1-h ischemia/96-h reperfusion, 1-h ischemia/24-h reperfusion, 24-h ischemia without reperfusion, and 96-h ischemia without reperfusion are shown. Minimal expression of IL-10 mRNA is noted in the nonreperfused experiments. *Lower panel*, Induction of IL-6 mRNA in reperfused and nonreperfused canine myocardial infarcts. The same experiments were probed with the IL-6 probe. Previous experiments have demonstrated early IL-6 mRNA induction following myocardial ischemia/reperfusion peaking at 3 h of reperfusion (24). Note that following 1-h ischemia and 24-h reperfusion, minimal IL-6 expression is found. In contrast, experiments undergoing 24 h of coronary occlusion without reperfusion demonstrated delayed and prolonged induction of IL-6 mRNA. We suggest that IL-10 induction down-regulates IL-6 expression in reperfused myocardial infarcts. However, the animals undergoing coronary occlusion without reperfusion demonstrate a prolonged IL-6 mRNA induction, because of the absence of IL-10 expression.

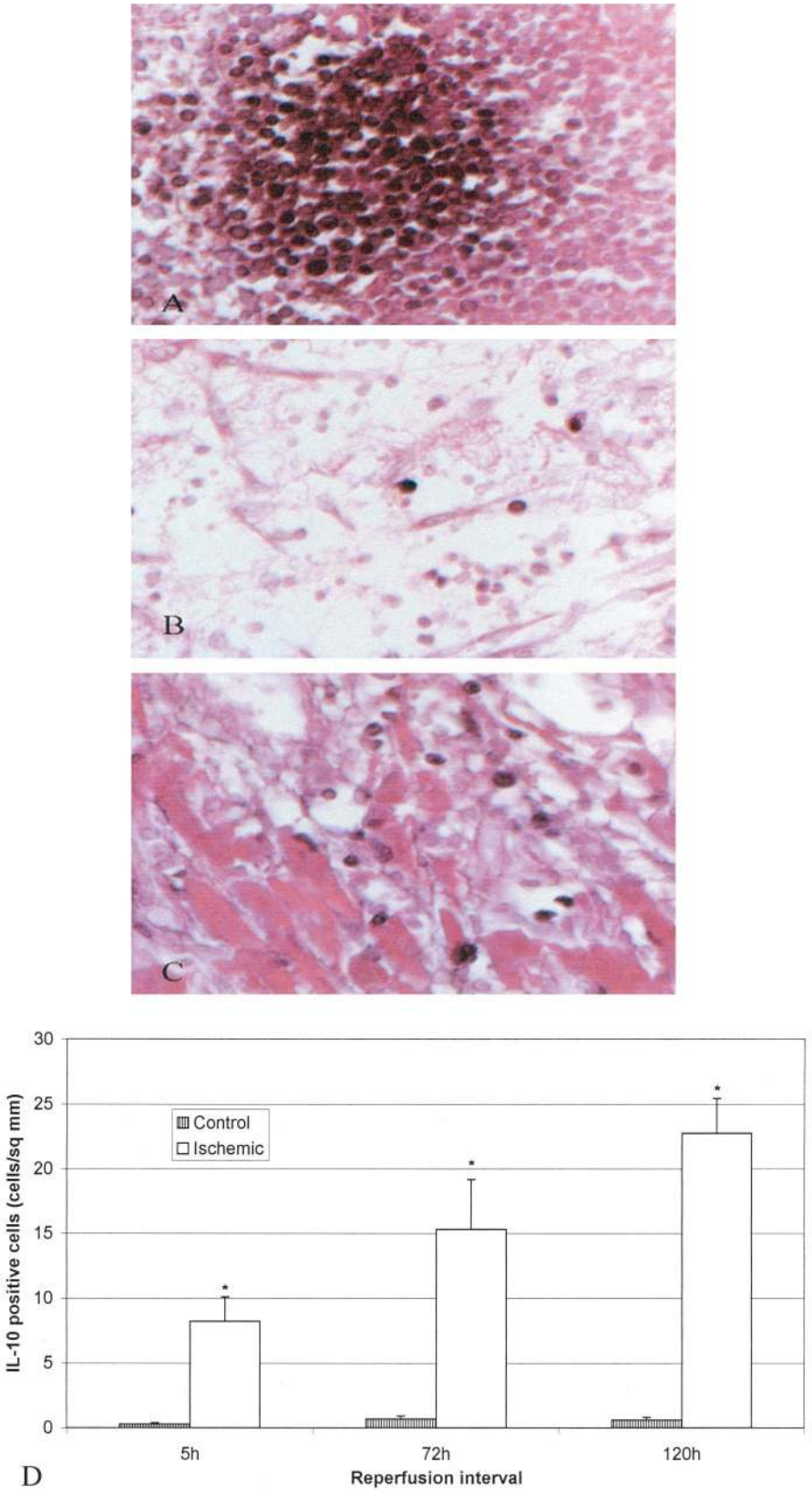

FIGURE 5. IL-10 immunoreactivity in the ischemic and reperfused myocardium. Immunohistochemical staining for IL-10 was performed in sections from the ischemic and reperfused myocardium. *A*, Section from the spleen of an endotoxin-stimulated dog served as a positive control, demonstrating intense staining especially in the white pulp. *B*, Rare IL-10-positive cells were seen as early as 5 h of reperfusion. *C*, Significant numbers of IL-10-positive cells were noted after 5 days of reperfusion. *D,* Numbers of IL-10-positive cells following myocardial ischemia and reperfusion. IL-10-positive cells were very rare in control areas of the canine myocardium. In ischemic sections 8.23 $\pm$ 1.89 IL-10-positive cells/mm^2 ($p < 0.05$, $n = 6$; compared with control sections from the same experiments) were counted after 1 h of ischemia and 5 h of reperfusion, and 22.8 $\pm$ 2.7 IL-10-positive cells/mm^2 following 1 h of ischemia and 5 days of reperfusion (*, $p < 0.05$, $n = 5$).

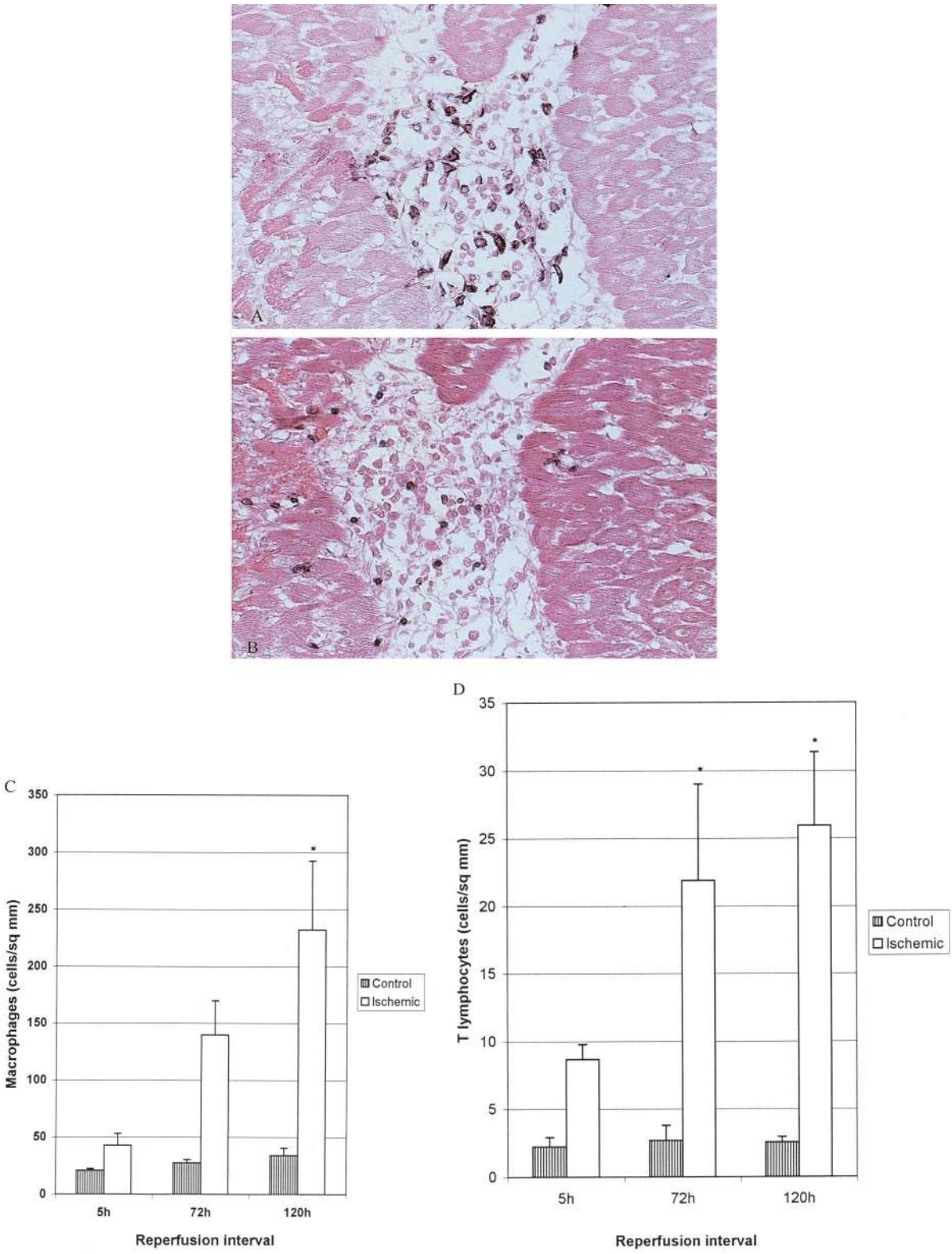

FIGURE 6. Macrophages and lymphocytes infiltrate the ischemic and reperfused myocardium. *A,* Staining for AM-3K identifies macrophages in the ischemic and reperfused heart following 1 h of ischemia and 72 h of reperfusion. *B,* Staining for CD3 labels T lymphocytes in a serial section of ischemic myocardium. *C,* Numbers of macrophages in the ischemic and reperfused myocardium. A resident macrophage population was noted in control areas of the canine heart. Significant accumulation of macrophages was noted in ischemic segments following myocardial ischemia and reperfusion, peaking at 5 days of reperfusion (ischemic 232 $\pm$ 60.4 cells/mm^2 vs control 34.1 $\pm$ 6.3 cells/mm^2; $*$, $p < 0.05$, $n = 5$). *D,* Numbers of T lymphocytes following myocardial ischemia and reperfusion. Small numbers of resident T cells were found in control segments of the canine myocardium. However, a 10-fold increase in lymphocyte numbers was noted following 1 h of ischemia and 5 days of reperfusion. Note that macrophages were 6.3 times more numerous in ischemic areas after 72 h of reperfusion and ~10 times more numerous following 5 days of reperfusion.

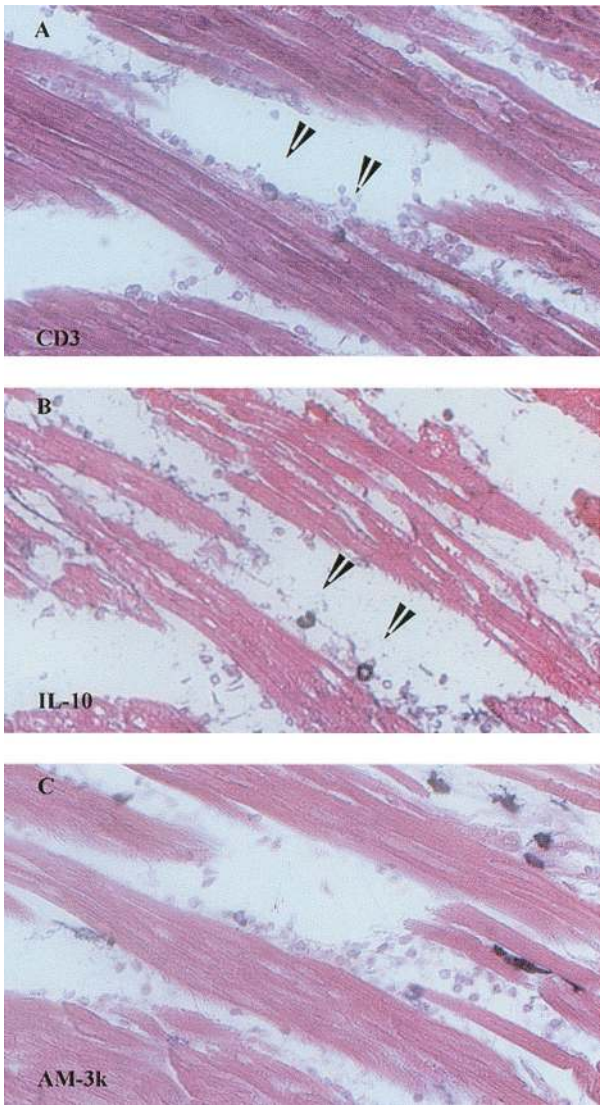

FIGURE 7. Lymphocytes are the predominant source of IL-10 in the ischemic and reperfused myocardium (1-h ischemia, 5-h reperfusion). Immunohistochemical staining of serial sections identified IL-10 immunoreactive cells (*B*) as CD3-positive lymphocytes (*A*). In contrast, most macrophages identified by their positive staining with the AM-3K Ab (*C*) did not stain for IL-10.

(Fig. 8) at as early as 5 h of reperfusion (Fig. 7). A subset of macrophages also produced IL-10. However, the majority of the macrophages did not demonstrate IL-10 immunoreactivity (Fig. 7).

IL-12 is not induced in the ischemic myocardium

Northern hybridization studies demonstrated negligible amounts of IL-12 p40 mRNA (the inducible chain of IL-12) in control canine heart and in ischemic segments from experiments of canine ischemia and reperfusion (Fig. 9). RNA extracted from the spleen of an endotoxin-stimulated animal was used as a positive control and showed robust expression of IL-12.

IL-10 is responsible for TIMP-1 up-regulation in cardiac-lymph-stimulated canine mononuclear cells

Isolated canine mononuclear cells were stimulated with human recombinant IL-10 and pre- and postischemic cardiac lymph. IL-10 and late postischemic cardiac lymph collected from 24 to 48 h of reperfusion were capable of up-regulating TIMP-1 mRNA expression (Fig. 10). Furthermore, incubation with a neutralizing Ab to human IL-10 significantly inhibited the ability of postischemic cardiac lymph to induce TIMP-1 in isolated mononuclear cells (postischemic cardiac lymph plus Ab 1.36 $\pm$ 0.15 vs postischemic cardiac lymph 3.58 $\pm$ 0.88; $p < 0.05$, $n = 4$) (Fig. 10*B*).

Macrophages in healing myocardial infarcts express TIMP-1

Immunohistochemical experiments using two different Abs to TIMP-1 demonstrated significant TIMP-1 protein expression in healing myocardial infarcts after 72–120 h of reperfusion (Fig. 11). TIMP-1-expressing cells were rare after 5 h of reperfusion. Most TIMP-1-positive cells were identified as macrophages using serial section staining with the macrophage-specific Ab, AM-3K (Fig. 11, *D* and *E*). In addition, some spindle-shaped myofibroblast-like cells also expressed TIMP-1 (Fig. 11). TIMP-1 immunoreactive cells were predominantly located in the border zone of reperfused myocardial infarcts.

Discussion

The inflammatory response following myocardial ischemia and reperfusion is associated with activation of a cytokine cascade (1, 32, 38) involving mast cell TNF-α release (32) and IL-6 mRNA up-regulation (24). IL-6, predominantly induced in infiltrating mononuclear cells (32), may be a crucial factor in mediating myocyte ICAM-1 expression in the ischemic border zone of reperfused myocardial infarcts (39). IL-10 inhibits synthesis of various cytokines (including IL-1, TNF-α, IL-6, GM-CSF, and IL-8) by stimulated monocyte/macrophages, regulates matrix metalloproteinase expression by mononuclear cells (17) and fibroblasts (18), enhances monocyte growth and differentiation (40), modulates neutrophil function and apoptosis (41–44), and induces mast cell growth (45, 46). In the present work, we show the first evidence that IL-10 is induced following myocardial ischemia and reperfusion. We demonstrated that infiltrating lymphocytes are the predominant cell type that is positive for IL-10 in the healing myocardium. Our findings suggest that IL-10 may be an important factor in up-regulating TIMP-1 induction in infiltrating mononuclear cells, adding a new aspect to its potential beneficial effects in promoting healing of the injured myocardium.

IL-4, IL-10, and IL-13 have potent macrophage deactivating effects, markedly inhibiting cytokine expression (47–49). Endogenous IL-10 production has been described in models of endotoxemia (10, 11, 50), atherosclerotic plaques (51), myocarditis (52), and allograft rejection (53, 54). Furthermore, increased levels of plasma IL-10 were noted in patients with complicated acute myocardial infarction (Ref. 55, Killip class II–IV). Our experiments suggest endogenous induction of IL-10 in the ischemic heart following experimental canine myocardial ischemia and reperfusion. IL-10 mRNA (Figs. 1–3) and protein (Figs. 5 and 7) were expressed in the ischemic areas only as early as the first 5 h of reperfusion and could be detected for 7 days. Maximal IL-10 expression was seen from 24 to 120 h of reperfusion (Figs. 2 and 3). In contrast, no IL-4 and IL-13 expression was noted in the ischemic myocardium. IL-10 can be produced by monocytes/macrophages, lymphocytes, and keratinocytes (11). We demonstrated that IL-10 immunoreactivity is predominantly localized in lymphocytes infiltrating the reperfused myocardium (Figs. 7 and 8). In addition, a small subset of macrophages was stained for IL-10. IL-10 strongly inhibits its own synthesis by macrophages (14); in part, this may explain its limited expression in macrophages of the healing heart. Furthermore, IL-10 production by T cells and monocytes is differentially regulated; IL-6 can induce the expression of IL-10 by stimulated T cells, whereas TNF-α stimulates IL-10 synthesis by monocytes (56). Data from our laboratory have described

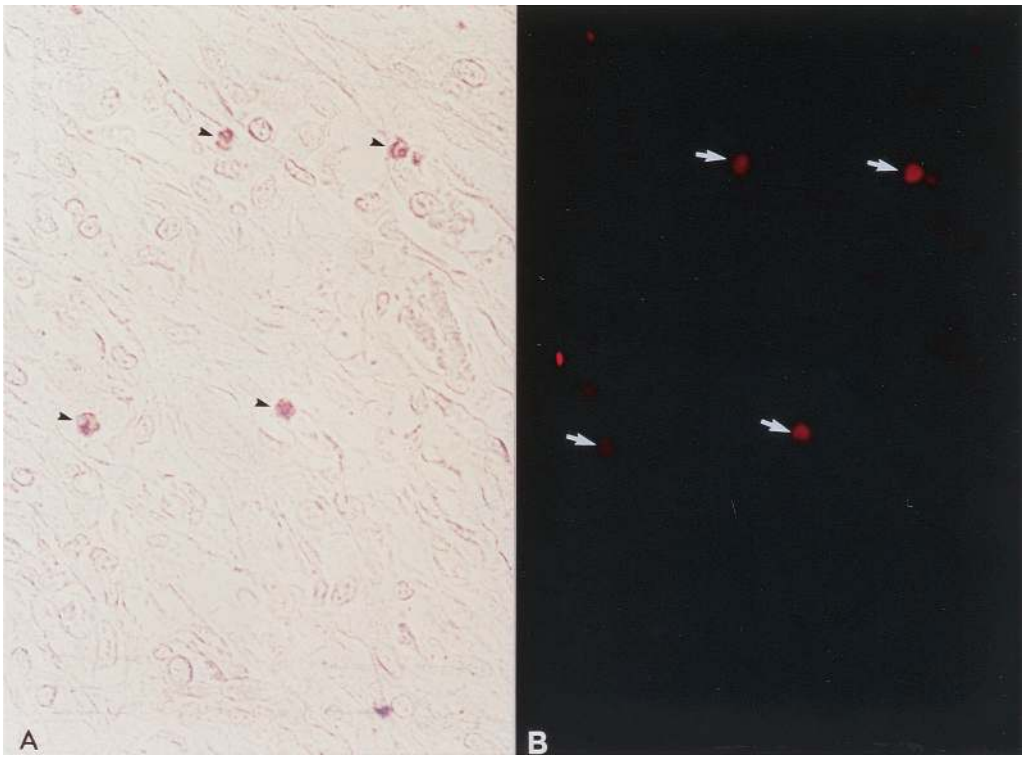

FIGURE 8. CD5-positive lymphocytes are the predominant source of IL-10 in the ischemic and reperfused heart (1-h ischemia, 5-day reperfusion). Dual immunohistochemical staining combining peroxidase-based staining for CD5 (*A*) and immunofluorescence for IL-10 (*B*) suggested that the majority of IL-10-positive cells were CD5-positive lymphocytes.

the sequential release of TNF-α from mast cells followed by induction of IL-6 in early reperfusion (32).

IL-10 expression was markedly enhanced by reperfusion of the ischemic myocardium (Fig. 4). We postulate that this results from increased lymphocyte access to the ischemic region and from the reperfusion-dependent up-regulation of cytokines potentially responsible for IL-10 expression (such as IL-6, Ref. 56) and mononuclear cell chemotaxis (such as MCP-1, Ref. 4).

Macrophages infiltrating the ischemic myocardium can support a number of different responses depending on the local microenvironment. Monocyte/macrophages are the predominant source of IL-6 mRNA in the reperfused myocardium and may have a significant role in mediating the inflammatory response associated with myocardial ischemia/reperfusion. IL-10 is capable of modulating the phenotype and functional activity of monocyte/macrophages. The production of IL-1, TNF-α, IL-6, GM-CSF, IL-12 (11), IFN-γ-inducible protein-10, and macrophage-inflammatory protein-1α (57) by endotoxin-stimulated macrophages can be inhibited by IL-10. Kukielka et al. (24) have previously demonstrated marked IL-6 up-regulation in the ischemic and reperfused myocardium, peaking after 1–3 h of reperfusion. Furthermore, Chandrasekar et al. (58) have recently demonstrated a delayed induction of IL-6 following occlusion without reperfusion of rat myocardium. Our current experiments showed decreased expression of IL-6 mRNA following 24–96 h of reperfusion when IL-10 is maximally induced (Fig. 4). In addition, experiments of coronary occlusion without reperfusion demonstrated minimal induction of IL-10 mRNA. In contrast, IL-6 expression, which was delayed to 24 h in permanently occluded studies (Ref. 24, Fig. 4), persisted for 96 h. We speculate that reperfusion-dependent IL-10 expression modulates the IL-6 response and may be an important factor regulating proinflammatory cytokine synthesis in the ischemic myocardium.

Recently, Lacraz et al. (17) demonstrated that IL-10 inhibits metalloproteinase and stimulates TIMP-1 production in human macrophages. This alteration of the proteinase/antiproteinase balance in favor of matrix preservation may be important in suppressing the inflammatory response following myocardial ischemia. The importance of IL-10 in modulating collagen metabolism was suggested by a recent study demonstrating a very low percentage of collagen in atherosclerotic lesions of IL-10-deficient mice (59). Our experiments demonstrate that IL-10 is an important factor in enhancing TIMP-1 expression in mononuclear cells stimulated with late postischemic cardiac lymph (Fig. 10). Significant numbers of TIMP-1-positive macrophages are noted in the healing scar after 72–120 h (Fig. 11) of reperfusion when IL-10 expression is maximal. These findings suggest an important role for IL-10 in

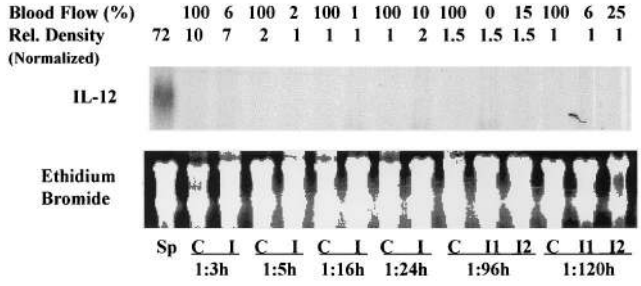

FIGURE 9. Absence of IL-12 expression in the ischemic and reperfused myocardium. Representative series of experiments from the ischemic and reperfused myocardium were probed with the IL-12 p40 probe. No significant induction of IL-12 p40 mRNA (the major inducible chain for IL-12) was noted. RNA extracted from the spleen of an endotoxin-stimulated animal was used as a positive control.

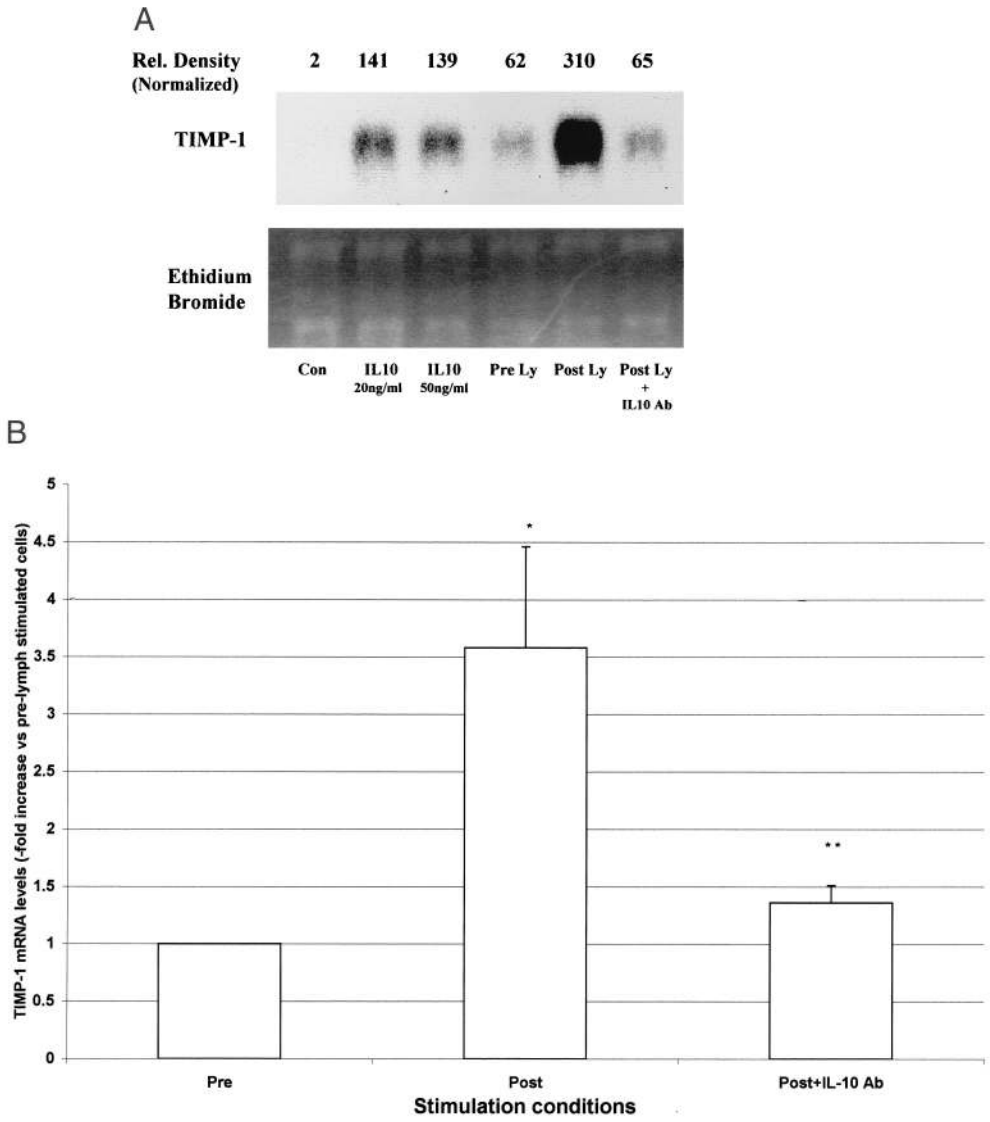

FIGURE 10. Postischemic cardiac lymph induces TIMP-1 mRNA induction in isolated canine mononuclear cells. *A,* A representative Northern hybridization experiment using RNA extracted from stimulated canine mononuclear cells is shown. Incubation with recombinant human IL-10 induced TIMP-1 mRNA in isolated canine mononuclear cells. Postischemic cardiac lymph at a 1:10 dilution caused marked up-regulation of TIMP-1 mRNA. This effect of postischemic cardiac lymph was inhibited in part with the addition of a neutralizing anti-IL-10 Ab. *B,* Quantitative analysis of the Northern hybridization experiments. TIMP-1 mRNA levels were expressed in comparison to the levels of TIMP-1 mRNA expression from mononuclear cells stimulated with preischemic cardiac lymph from the same experiment. Mononuclear cell incubation with postischemic cardiac lymph at the same dilution (1:10) caused a significant up-regulation of TIMP-1 mRNA levels compared with mononuclear cells stimulated with preischemic cardiac lymph (3.58 $\pm$ 0.88-fold increase; $*$, $p < 0.05$, $n = 4$). Addition of a neutralizing Ab to IL-10 significantly inhibited TIMP-1 mRNA expression in postischemic cardiac-lymph-stimulated isolated canine mononuclear cells (3.58 $\pm$ 0.88-fold vs 1.36 $\pm$ 0.15-fold; $**$, $p < 0.05$, $n = 4$). The cardiac lymph used for stimulation was collected following 24–48 h of reperfusion. Con, Control canine mononuclear cells; Pre Ly, mononuclear cells stimulated with preischemic cardiac lymph; Post Ly, mononuclear cells stimulated with postischemic cardiac lymph; Post Ly + IL10 Ab, postischemic cardiac-lymph-stimulated mononuclear cells incubated with a neutralizing Ab to IL-10.

scar formation and maturation following an experimental myocardial infarction. Other studies (40) suggest that IL-10 may act as a cytokine, enhancing monocyte survival, growth, and differentiation into macrophages by cooperating with M-CSF. Recent experiments from our laboratory demonstrated induction of M-CSF following myocardial ischemia and reperfusion (60). These effects may be of significance in the maturation of monocytes chemotactically attracted in the ischemic myocardium by MCP-1.

It is generally accepted that activated T cells mediate immunologic responses by differentiating into one of two subsets, Th1 and Th2, characterized by the release of distinct types of cytokines; Th1 cells activate proinflammatory effector mechanisms and are associated with macrophage activation, whereas Th2 cells inhibit Th1 responses and down-regulate local inflammation. IL-12 and IL-10 are two important cytokines regulating the Th1 and Th2 responses. IL-12 is a 70-kDa protein heterodimer (termed p70), of which the p40 component is the major inducible chain. It is primarily produced by activated monocytes and selectively induces the cytolytic activity of a number of effector cells, stimulating the Th1-cytokine pattern, which is associated with macrophage activation. In contrast, IL-10 facilitates Th2 responses and down-regulates IL-12 production. Our studies demonstrated marked IL-10

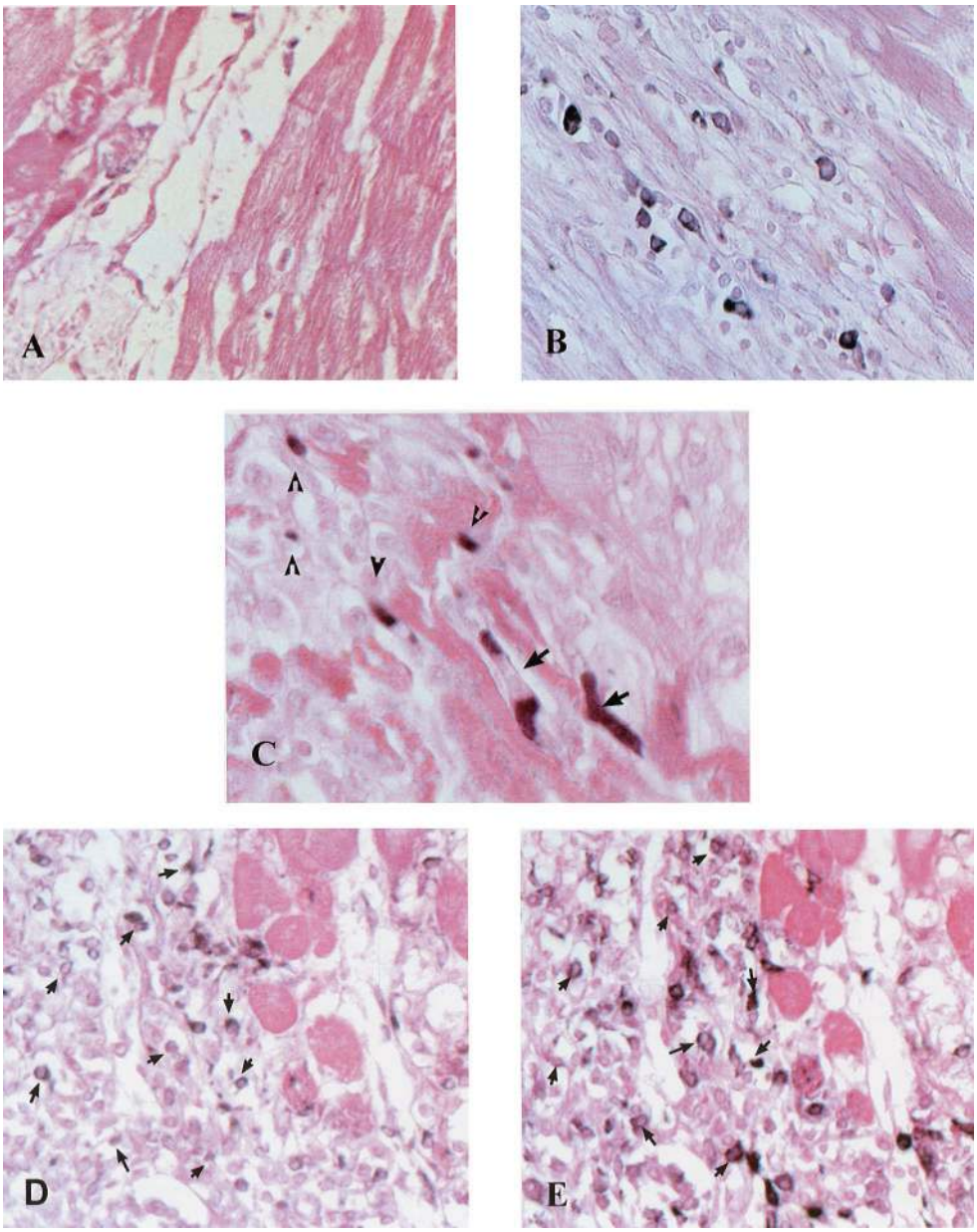

FIGURE 11. TIMP-1 expression in macrophages infiltrating the ischemic canine myocardium. *A,* Immunohistochemistry for TIMP-1 in the ischemic canine myocardium demonstrated no significant TIMP-1 expression after 5 h of reperfusion. *B,* In contrast, significant numbers of TIMP-1-positive cells with morphological characteristics of mononuclear cells were found in the healing scar after 5 days of reperfusion. *C,* Some spindle-shaped cells, resembling fibroblasts also expressed TIMP-1 (1-h occlusion/72-h reperfusion). Serial section staining with TIMP-1 (*D*) and the macrophage-specific Ab (*E*) suggested that a subset of infiltrating macrophages, often located in the border zone of the reperfused infarct were positive for TIMP-1 (arrows).

mRNA and protein induction but no significant IL-12 p40-mRNA (Fig. 9) expression in the reperfused myocardium. Following myocardial ischemia and reperfusion, IL-10 may be important in down-regulating IL-12 expression and in shifting the T cell response toward a Th2 direction.

In summary, our studies demonstrated a marked reperfusion-dependent induction of IL-10 following myocardial ischemia. IL-10 was predominantly localized in lymphocytes infiltrating the reperfused myocardium. We suggest a potential role for infiltrating lymphocytes in suppressing the acute inflammatory response by modulating macrophage-cytokine synthetic activity and function, and in promoting metabolic steps associated with tissue repair. This may represent an important aspect of the beneficial effect of reperfusion in healing of the injured myocardium.

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