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## The CD154-CD40 T-Cell Co-stimulation Pathway in Liver Ischemia and Reperfusion Inflammatory Responses

Bibo Ke<sup>1</sup>, Xiu-Da Shen<sup>1</sup>, Feng Gao<sup>1</sup>, Seiichiro Tsuchihashi<sup>1</sup>, Douglas G. Farmer<sup>1</sup>, David Briscoe<sup>2</sup>, Ronald W. Busuttil<sup>1</sup>, and Jerzy W. Kupiec-Weglinski<sup>1,3</sup>

<sup>1</sup>The Dumont-University of California, Los Angeles Transplant Center, Division of Liver and Pancreas Transplantation, Department of Surgery, David Geffen School of Medicine at University of California, Los Angeles, CA

<sup>2</sup>Division of Nephrology, Children's Hospital, Harvard Medical School, Boston, MA

### Abstract

**Background**—Ischemia-reperfusion (I/R) injury is a prime antigen-independent inflammatory factor in the dysfunction of liver transplants. The precise contribution of T cells in the mechanism of I/R injury remains to be elucidated. As the CD154-CD40 co-stimulation pathway provides essential second signal in the initiation and maintenance of T-cell-dependent immune responses, this study was designed to assess the role of CD154 signaling in the pathophysiology of liver I/R injury.

**Methods**—A mouse model of partial 90-min warm hepatic ischemia followed by 6 hr of reperfusion was used. Three animal groups were studied: (1) wild-type (WT) mice treated with Ad(-gal versus Ad-CD40 immunoglobulin; (2) untreated WT versus CD154 (MR1) monoclonal antibody-treated WT mice; and (3) untreated WT versus CD154 knockout mice.

**Results**—The disruption of CD154 signaling in all three animal groups ameliorated otherwise fulminant liver injury, as evidenced by depressed serum glutamic oxaloacetic transaminase levels, compared with controls. These beneficial effects were accompanied by depressed hepatic T-cell sequestration, local decrease of vascular endothelial growth factor expression, inhibition of tumor necrosis factor-( and T-helper type 1 cytokine production, and induction of antiapoptotic (Bcl-2/ Bcl-x1) but depression of proapoptotic (caspase-3) proteins.

**Conclusions**—By using in parallel a gene therapy approach, pharmacologic blockade, and genetically targeted mice, these findings document the benefits of disrupting CD154 to selectively modulate inflammatory responses in liver I/R injury. This study reinforces the key role of CD154-CD40 T-cell co-stimulation in the pathophysiology of liver I/R injury.

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<sup>3</sup>Address correspondence to: Jerzy W. Kupiec-Weglinski, M.D., Ph.D., The Dumont-University of California, Los Angeles Transplant Center, Room 77-120CHS, 10833 Le Conte Avenue, Los Angeles, CA 90095., jkupiec@mednet.ucla.edu.

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## Keywords

Ischemia-reperfusion injury; Vascular endothelial growth factor; CD154-CD40; Gene transfer

Ischemia-reperfusion (I/R) injury, an antigen-independent inflammatory component of organ “harvesting,” remains an important problem in clinical transplantation. In the case of liver, primary and delayed hepatic nonfunction caused by I/R injury may continue to both early and late liver transplant outcomes (1). Although studies on both “warm” and “cold” components of hepatic I/R injury have provided some etiologic factors, including activation of Kupffer cells, release of proinflammatory cytokines, increased expression of vascular cell adhesion molecules, and neutrophil accumulation (2), the exact mechanisms and mediators involved remain unclear.

It has been postulated that prolonged ischemia results in an allograft becoming more susceptible to T-cell immune responses (3). Indeed, evidence is mounting on the importance of T cells in mediating both short- and long-term organ ischemic insult (4, 5). The contributory role of lymphocytes in hepatic I/R injury is likely a multifactorial one. The demonstration that systemic immunosuppression (cyclosporine A/FK506) attenuates hepatocellular damage implies a pathophysiologic role of T lymphocytes (6), data supported by our own findings in T-cell-deficient mice (7) and in rats in which treatment with FTY720 prevented I/R injury in parallel with redistribution of T cells from host peripheral blood into lymph nodes (8).

The CD154-CD40 co-stimulation provides the essential second signal in the initiation and maintenance of T-cell-dependent responses (9). Indeed, the efficacy of CD154-targeted therapy to prevent rejection and to induce tolerance in some transplant models has been established (10–12). Much less is known about the role of CD154 signaling in antigen-independent inflammatory responses triggered by I/R injury. Indeed, we have shown that disruption of CD154 co-stimulation or treatment with CD154 monoclonal antibody (mAb) prevented hepatic I/R injury in mice (7), whereas gene transfer of CD40 immunoglobulin (Ig) protected rat livers from I/R injury (13). This study was aimed at delineating mechanisms of cytoprotection against “warm” hepatic I/R injury in three distinct mouse models: (1) gene therapy-mediated local CD154 blockade (Ad-CD40Ig); (2) antibody-induced systemic CD154 blockade (MR1 mAb); and (3) genetically targeted CD154 absence (CD154 knockout [KO] mice).

## MATERIALS AND METHODS

### Animals

Male B6/129 wild-type (WT) mice and CD154 KO mice (8–12 weeks of age) were used (The Jackson Laboratory, Bar Harbor, ME). Animals were maintained under conditions approved by the University of Southern California, Los Angeles Chancellor’s Animal Research Committee. All animals were housed in microisolator cages in pathogen-free facilities and according to National Institutes of Health guidelines.

## Generation of Recombinant Adenovirus

Generation of recombinant adenovirus (Ad)-CD40Ig was previously described (13). Briefly, the mouse CD40Ig cDNA was placed under the transcriptional control of a murine cytomegalovirus promoter. Ad-CD40Ig was constructed by homologous recombination in 293 cells with dl324 viral DNA, which was Ad5-derived, E1- and E3-deleted adenovirus. Purified Ad-CD40Ig was amplified in 293 cells grown in SMEM suspension medium (GIBCO, Grand Island, NY) with 10% newborn calf serum (GIBCO). The titration of the virus was analyzed by plaque assay. Virus stocks of  $2.5 \times 10^{10}$  plaque-forming units/mL were stored at  $-80^{\circ}\text{C}$ . The generation of Ad-encoding *Escherichia coli* (-galactosidase (Ad(-gal) was described (14).

## Hepatic I/R injury Model

A warm hepatic I/R injury model was used, as described (7). Briefly, mice were injected with heparin (100 (g/kg) and an atraumatic clip was used to interrupt the arterial-portal venous blood supply to the cephalad liver lobes. After 90 min of partial hepatic ischemia, the clip was removed, initiating reperfusion. Animals were killed at 6 hr; liver tissue and blood samples were harvested for analysis. The extent and severity of hepatic I/R injury were assessed in groups (n=4–6) of (1) WT mice that were injected intravenously with Ad-CD40Ig or Ad(-gal ( $2.5 \times 10^9$  plaque-forming units) at day -2; (2) WT recipients that were pretreated with CD154 mAb (MR1, 0.25 mg/mouse intraperitoneally at day -2 and day -1); and (3): CD154 KO mice. Sham WT controls underwent the same procedures but without vascular occlusion.

## Hepatocyte Function Assay

Serum glutamic-oxaloacetic transaminase (SGOT) levels, an indicator of hepatocyte function, were measured in blood samples obtained at 6 hr postreperfusion. The SGOT measuring was performed using an autoanalyzer manufactured by ANTECH Diagnostics (Los Angeles, CA).

## Immunohistochemical Analysis

Liver tissue samples were embedded in Tissue Tec OCT compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ . Cryostat sections (5 (m) were fixed in acetone and then endogenous peroxidase activity was inhibited with 0.3%  $\text{H}_2\text{O}_2$  in phosphate-buffered saline. Primary rat antibody against mouse  $\text{CD}3^+$  T cells (17A2) was added at optimal dilutions (BD Biosciences Pharmingen). Bound primary antibody was detected using biotinylated anti-rat IgG and streptavidin peroxidase-conjugated complexes (DAKO, Carpinteria, CA). Negative controls included sections in which the primary antibody was replaced with dilution buffer or normal mouse serum. The sections were evaluated blindly by counting the labeled cells in triplicate within 10 high-power fields per section.

## RNA Extraction-Competitive Template Reverse-Transcriptase Polymerase Chain Reaction

To study cytokine gene expression patterns, we used competitive template reverse-transcriptase polymerase chain reaction (PCR), as described (13). Briefly, total RNA was

extracted from frozen liver tissue samples using RNase Mini Kit (Qiagen, Inc., Chatsworth, CA), and RNA concentration was determined by a spectrophotometer. Five micrograms of RNA was reverse-transcribed using oligo(dT) primers and superscript reverse transcriptase (GIBCO). According to the varying contents of specific cDNA and amplification efficiencies, PCR was performed by different cycle numbers at the annealing temperature that was optimized empirically for each primer pair: 40, 60°C (interleukin [IL]-2); 35, 63°C (interferon [IFN]-); 37, 60°C (tumor necrosis factor [TNF]-); 35, 60°C (vascular endothelial growth factor [VEGF]); and 35, 63°C ( $\beta$ -actin), respectively. PCR products were analyzed in ethidium bromide-stained 2% agarose gel, and scanned for density using Kodak Digital Science 1D Analysis software (Version 2.0; Eastman Kodak, Rochester, NY). To compare the relative level of each gene in different samples, all samples were normalized against the respective  $\beta$ -actin wild-type cDNA to competitive template DNA ratio.

### Western Blot Analysis

Protein was extracted from tissues with protein lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, and 1% triton X-100; pH 7.2). Proteins (30 (g/sample) in SDS-loading buffer (50 mM Tris, 10% glycerol, 1% SDS) were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA). The gel was then stained with Coomassie blue to document protein loading. The membrane was blocked with 3% dry milk and 0.1% Tween 20 in phosphate-buffered saline. Polyclonal rabbit anti-mouse Bcl-2/Bcl-xl and anti-mouse caspase-3 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used. The membranes were incubated with antibodies and then developed according to the Amersham enhanced chemiluminescence protocol. Relative quantities of Bcl-2, Bcl-xl, and caspase-3 were determined by densitometer and expressed in absorbance units (AU) (Kodak Digital Science 1D Analysis Software; Eastman Kodak).

### Statistical Analysis

All data are expressed as mean $\pm$ SD. Statistical comparisons between groups were analyzed by Student's *t* test. All differences were considered statistically significant at a value of  $P<0.05$ .

## RESULTS

### CD154-CD40 T-Cell Signaling Is Required in the Mechanism of Hepatic I/R Injury

Ninety minutes of hepatic warm ischemia followed by 6 hr of reperfusion significantly increased SGOT levels from  $32\pm 10$  IU/L in sham-operated controls to  $2,283\pm 238$  IU/L in the WT group (Fig. 1) ( $P<0.0001$ ). However, treatment of WT mice with Ad-CD40Ig but not with Ad(-gal markedly decreased SGOT levels ( $465\pm 90$  and  $2,485\pm 478$  IU/L, respectively;  $P<0.005$ ). The disruption of CD154 signaling with CD154 (MR1) mAb in WT mice subjected to hepatic I/R injury or its absence in CD154 KO mice prevented hepatocellular damage, as evidenced by diminished SGOT (Fig. 1) ( $151\pm 21$  and  $181\pm 51$  IU/L, respectively;  $P<0.0005$ ) and preservation of liver histology (not shown). To analyze mechanisms by which CD154-CD40 co-stimulation promotes hepatic I/R injury, we

contrasted (1) WT mice treated with Ad(-gal versus Ad-CD40 Ig; (2) untreated WT versus CD154 (MR1) mAb-treated WT mice; and (3) untreated WT versus CD154 KO mice.

### Disruption of CD154-CD40 Signaling Prevents T-Cell Infiltration

As shown in Figure 2, the hepatic I/R injury was accompanied by significantly increased sequestration of T cells in untreated and Ad(-gal-treated WT mice, as compared with sham-operated controls ( $11.3\pm 2.1$  and  $11.6\pm 2.8$  vs.  $1.4\pm 0.8$ , respectively;  $P<0.0001$ ). In contrast, treatment of WT mice with Ad-CD40-Ig, CD154 mAb, or CD154 KO mice resulted in diminished T-cell levels ( $3.2\pm 1.3$ ,  $3.3\pm 1.9$ , and  $2.5\pm 1.6$ ;  $P<0.0001$ , as compared with WT and Ad(-gal-treated controls).

### Disruption of CD154-CD40 Signaling Depresses VEGF

As shown in Figure 3, warm hepatic ischemia followed by reperfusion significantly increased hepatic expression of mRNA coding for VEGF in WT and Ad(-gal-treated controls as compared with sham-operated mice ( $2.37\pm 0.17$  and  $2.45\pm 0.18$  vs.  $0.32\pm 0.11$ , respectively;  $P<0.005$ ). In marked contrast, WT mice treated with Ad-CD40 Ig or CD154 mAb and CD154 KO mice showed markedly decreased VEGF levels ( $0.82\pm 0.15$ ,  $0.85\pm 0.12$ , and  $0.71\pm 0.15$ , respectively) as compared with WT ( $P<0.01$ ) and Ad(-gal ( $P<0.01$ ) controls.

### Disruption of CD154-CD40 Signaling Decreases TNF-( and T-Helper Type 1 Cytokines

Next, we used competitive-template reverse-transcriptase PCR to analyze cytokine mRNA expression in the ischemic liver lobes. As shown in Figure 4, WT and Ad(-gal-treated mice revealed markedly increased levels of TNF-( ( $2.35\pm 0.19$  and  $2.24\pm 0.12$  vs.  $0.21\pm 0.03$ , respectively;  $P<0.005$ ), IL-2 ( $2.41\pm 0.16$  and  $2.51\pm 0.23$  vs.  $0.21\pm 0.03$ , respectively;  $P<0.005$ ), and IFN-( ( $2.08\pm 0.09$  and  $2.04\pm 0.09$  vs.  $0.21\pm 0.04$ , respectively;  $P<0.005$ ), as compared with sham-operated controls. In contrast, WT mice treated with Ad-CD40Ig or CD154 mAb and CD154 KO mice showed decreased hepatic expression of mRNA coding for TNF-( ( $0.86\pm 0.1$ ,  $0.88\pm 0.14$ , and  $0.79\pm 0.16$ , respectively), IL-2 ( $0.72\pm 0.15$ ,  $0.78\pm 0.16$ , and  $0.7\pm 0.14$ , respectively), and IFN-( ( $0.84\pm 0.14$ ,  $0.82\pm 0.15$ , and  $0.77\pm 0.16$ , respectively), as compared with WT ( $P<0.05$ ) and Ad(-gal ( $P<0.05$ ) controls.

### Disruption of CD154-CD40 Signaling Increases Antiapoptotic Molecules

We used Western blots to evaluate the expression of antiapoptotic Bcl-2 and Bcl-xl gene products in the ischemic liver lobes. As shown in Figure 5, the expression of antiapoptotic Bcl-2 and Bcl-xl (AU) was strongly increased in WT mice treated with Ad-CD40Ig ( $1.6\text{--}1.8$  AU and  $1.7\text{--}1.9$  AU, respectively) or CD154 mAb ( $1.8\text{--}2.0$  AU and  $1.7\text{--}1.9$  AU, respectively) and in CD154 KO mice ( $1.7\text{--}1.9$  AU and  $1.6\text{--}1.8$  AU, respectively), as compared with WT and Ad(-gal controls ( $0.1\text{--}0.3$  AU and  $0.1\text{--}0.2$  AU, respectively). In contrast, the expression of proapoptotic caspase-3 was inhibited in WT mice treated with Ad-CD40Ig ( $0.2\text{--}0.4$  AU) or CD154 mAb ( $0.3\text{--}0.4$  AU) and in CD154 KO mice ( $0.2\text{--}0.5$  AU), as compared with WT and Ad(-gal controls ( $1.7\text{--}1.9$  AU and  $1.9\text{--}2.1$  AU, respectively).

## DISCUSSION

The results of this study document the key role of the CD154-CD40 co-stimulation pathway in the mechanism of antigen-independent inflammatory responses triggered by hepatic I/R injury. Indeed, gene therapy-mediated prolonged local CD154 blockade (Ad-CD40 Ig), antibody-induced systemic CD154 blockade (MR1 mAb), and genetically targeted CD154 absence (CD154 KO mice) all ameliorated otherwise fulminant injury in a warm mouse liver ischemia model followed by reperfusion. These beneficial effects resulting from disruption of CD154-CD40 signaling were accompanied by (1) diminished intrahepatic T-cell sequestration; (2) decrease of VEGF expression; (3) inhibition of TNF- $\alpha$  and T-helper (Th) type 1 cytokine production; and (4) induction of anti-apoptotic (Bcl-2/Bcl-x1) and depression of proapoptotic (caspase-3) proteins. Thus, by using gene therapy, pharmacologic blockade, and genetically targeted mice in parallel, these findings document the benefits of disrupting the CD154 signaling that otherwise modulates inflammatory pathways in liver I/R injury.

VEGF, the most potent angiogenesis factor described to date, is a 45-kDa glycoprotein expressed by monocytes-macrophages, endothelial cells, activated T cells, and a variety of other cell types (15, 16). Indeed, VEGF is involved in an array of delayed-type hypersensitivity and chronic inflammatory disorders. Of interest are recent findings that CD154-CD40 interactions are the most potent for VEGF expression in endothelial cells and monocytes in vitro (17), and that the proangiogenic effect of CD154 in vivo is VEGF dependent (18). As the implication of VEGF, a hepatocyte product that acts on sinusoidal endothelial cells in the mechanism of I/R injury, remains to be elucidated, we asked whether 90 min of warm ischemia will affect its expression in our I/R injury model. Indeed, hepatic mRNA levels coding for VEGF increased sharply at 6 hr of reperfusion, a period of marked postischemic intrahepatic T-cell and macrophage sequestration (7). This is consistent with a robust and rapid increase of VEGF mRNA expression in rat hepatocyte cultures preexposed to periods of cold ischemia followed by warm reoxygenation (19). Interestingly, cold preservation alone did not trigger VEGF secretion. These in vitro findings support the role of VEGF in the mechanism of hepatic I/R injury, consistent with recent reports that in vivo myocardial ischemia significantly augmented VEGF levels in pig hearts (20), whereas anti-VEGF antibody treatment prevented ischemia-induced neovascularization in mice (21) and decreased the degree of I/R injury in cold-preserved rat liver grafts (22). Indeed, in our own studies, mice conditioned with anti-VEGF antibodies became resistant against otherwise fulminant warm hepatic I/R injury (J. W. Kupiec-Weglinski, 2005).

In agreement with the role of CD154-CD40 co-stimulation in T-cell function (9–12), the interruption of CD154 signaling not only depressed intrahepatic T-cell infiltration but also diminished activation of liver-infiltrating mononuclear cells, as measured by local Th1-dependent tissue inflammation. IFN- $\gamma$  can activate Kupffer cells and lead to a further recruitment and activation of neutrophils (23), consistent with our finding of decreased IFN- $\gamma$  mRNA and neutrophil infiltrate in Ad-CD40Ig-transfected cold-stored rat liver grafts (13). Moreover, we have shown that Th1-type inflammatory responses in mouse livers undergoing warm I/R injury are STAT4-dependent (24). Conversely, the benefits of inducing Th2-type responses in liver I/R injury models (25, 26) are in agreement with STAT4 disruption-mediated cytoprotection (24), or activation of STAT6, a key negative



regulator of the hepatic I/R injury response (27). In addition to depressing the Th1-type cytokine program, CD154 blockade also diminished local expression of TNF- $\alpha$ , the key effector molecule in liver I/R injury. Our studies in this murine model of hepatic I/R injury have documented that TNF- $\alpha$  induction is IRF-3 (but not MyD88) dependent (28), implying that multiple cell types might be involved in the pathogenesis of liver inflammation. One possible scenario of TNF- $\alpha$  induction is that IFN-inducible gene products (e.g., inducible protein-10) recruit or activate intrahepatic lymphocytes to produce the majority of TNF- $\alpha$ . This may provide a putative link between innate immune activation by liver I/R injury and adaptive immune responses in the absence of exogenous antigens.

The disruption of CD154-CD40 co-stimulation triggered increased expression of antiapoptotic (Bcl-2/Bcl-x1) yet diminished expression of proapoptotic (caspase 3) proteins. These “cytoprotective” molecules associate with the Th2-enriched microenvironment and remain increased exclusively in I/R injury-resistant livers (2, 29). Not surprisingly, gene therapy-induced antiapoptotic Bcl-2 (30) or Bag-1 (31) prevented hepatic I/R injury sequelae, whereas the blockade of apoptosis with a caspase inhibitor ameliorated I/R injury in liver grafts (32). Thus, disruption of CD154 signaling might interact with a number of pathways that mediate I/R injury cytotoxicity (1) by stabilizing antiapoptotic proteins and preventing cell death by reactive oxygen species; (2) by inhibiting postmitochondrial apoptosis and influencing apoptosome formation (33); and (3) by activating a positive feedback between cytoprotective genes and type-2 cytokines to amplify anti-inflammatory and antiapoptotic capacity, possibly by means of p38 MAPK (34) or STAT6-dependent signaling (24).

## CONCLUSION

By selectively modulating host inflammatory responses, the disruption of CD154-CD40 T-cell co-stimulation after gene therapy, pharmacologic blockade, or genetic engineering exerted potent cytoprotection against antigen-independent hepatic I/R injury. This study reinforces the key role of CD154 signaling in the pathophysiology of liver I/R injury.

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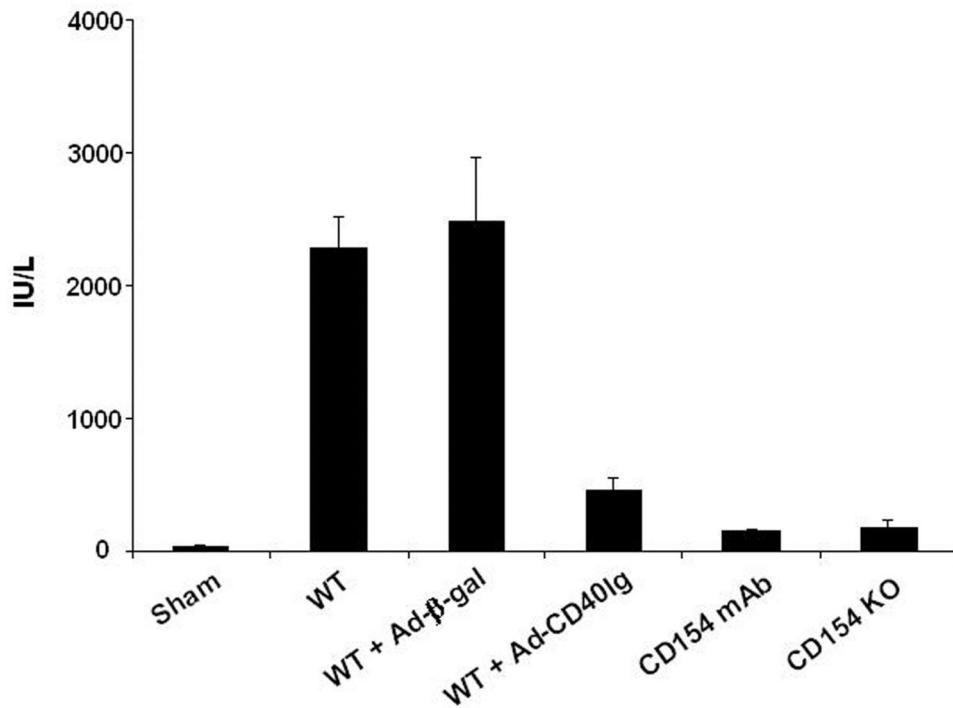
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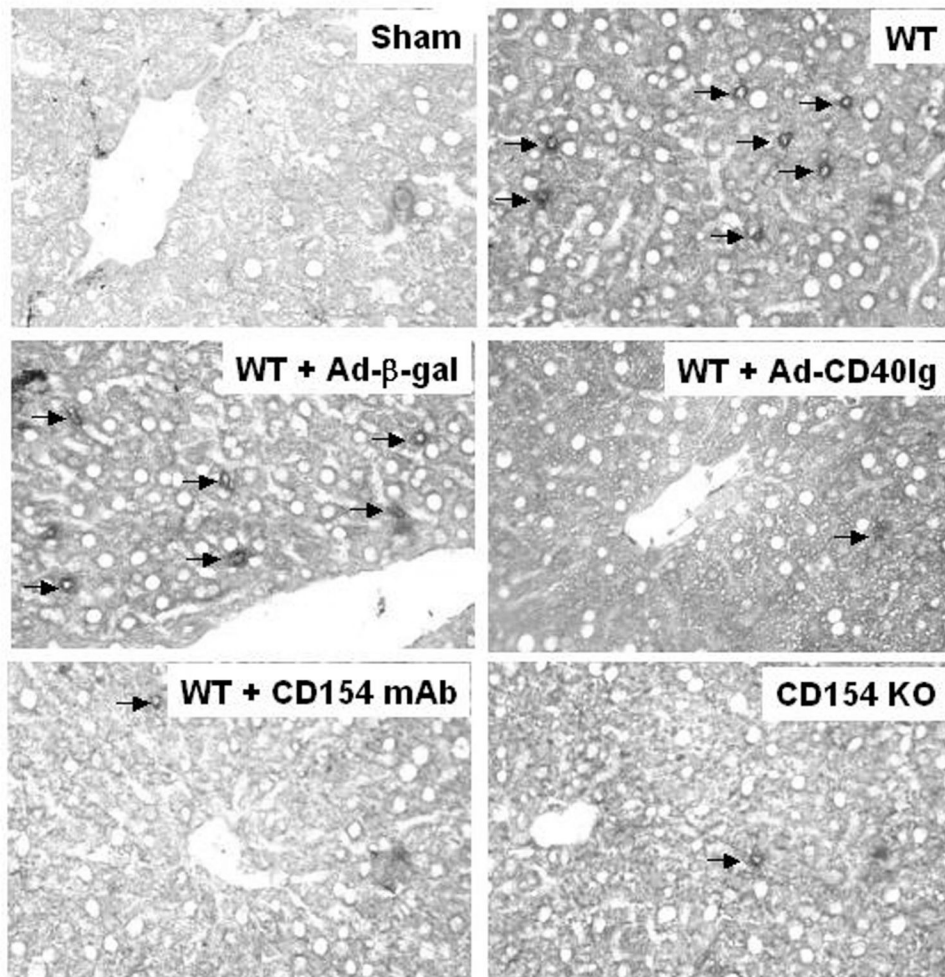


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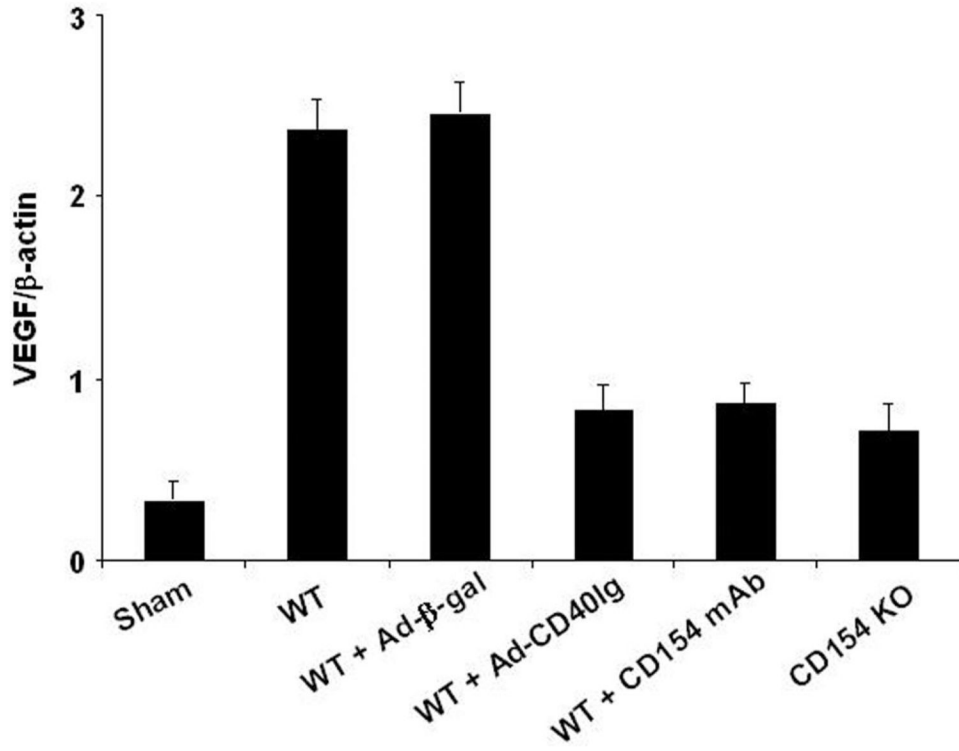


**FIGURE 1.**

SGOT levels in mice that underwent 90 min of hepatic warm ischemia followed by 6 hr of reperfusion. Groups included sham-operated controls; T-cell-competent untreated WT mice; WT mice that were pretreated with Ad-CD40Ig, Ad(-gal ( $2.5 \times 10^9$  plaque-forming units at day -2), or CD154 mAb (MR1; 0.25 mg at days -2 and -1); and CD154-deficient (KO) mice. There were four to six animals in each group. Means and SD are shown.

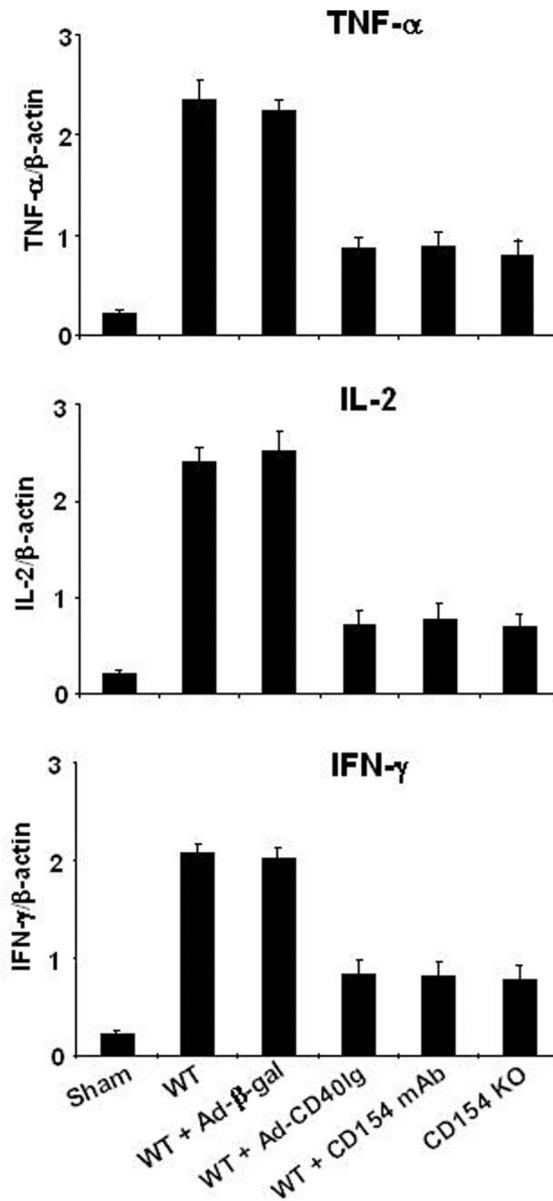


**FIGURE 2.** T-cell (CD3<sup>+</sup>) infiltration in murine livers undergoing I/R injury with or without CD154-CD40 co-stimulation blockade. Data are representative of three recipients per group (immunoperoxidase labeling of cryostat sections, hematoxylin counterstained; magnification  $\times 400$ ). For details, see *Materials and Methods* and Figure 1.



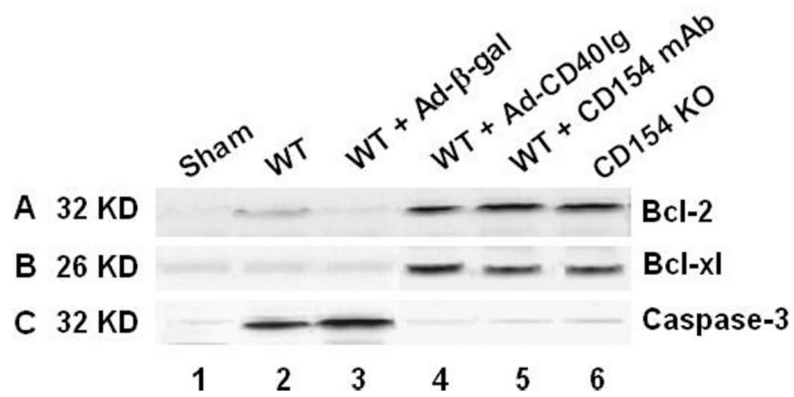
**FIGURE 3.**

Reverse-transcriptase PCR-assisted detection of mRNA coding for VEGF in hepatic ischemic lobes at 6 hr of reperfusion after 90 min of warm ischemia. For details, see *Materials and Methods* and Figure 1. There were four to six animals in each group. Means and SD are shown.



**FIGURE 4.**

Reverse-transcriptase PCR-assisted detection of mRNA coding for TNF-( and Th1 (IL-2 and IFN-() cytokines in hepatic ischemic lobes at 6 hr of reperfusion after 90 min of warm ischemia. For details, see *Materials and Methods* and Figure 1. There were four to six animals in each group. Means and SD are shown.



**FIGURE 5.**

Western blot analysis of antiapoptotic (Bcl-2/Bcl-xl) gene products in mouse livers after 90 min of warm ischemia followed by 6 hr of reperfusion. The protein expression was probed by using antibodies against mouse Bcl-2 (A), Bcl-xl (B), and caspase-3 (C). (lane 1) Sham-operated control; (lane 2) WT mice; (lane 3) WT mice treated with Ad(-gal); (lane 4) WT mice treated with Ad-CD40Ig; (lane 5) WT mice treated with CD154 mAb; (lane 6) CD154 KO mice. Data shown are representative of three separate experiments.