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DONOR GRAFT INTERFERON REGULATORY FACTOR-1 (IRF-1) GENE TRANSFER WORSENS LIVER TRANSPLANT ISCHEMIA/ REPERFUSION INJURY

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Abstract

Background—Liver ischemia and reperfusion (IR) injury is a phenomenon that leads to graft dysfunction following liver transplantation. Understanding the molecular mechanisms behind this process is crucial to developing strategies to prevent short and long term graft dysfunction. The purpose of this study is to explore the role of the transcription factor, IRF-1, in a model of orthotopic rat liver transplantation.

Methods—Orthotopic syngeneic LEW rat liver transplantation (OLT) was performed after 18 or 3 hours preservation in cold UW solution. AdIRF-1 or control gene vector (Adnull) was delivered to the liver by donor intravenous pretreatment 4 days before graft harvesting. Uninfected grafts also served as controls. Recipients were sacrificed 1 to 24 hours post-transplantation.

Results—Rats that underwent OLT with long-term preserved graft (18 hours) displayed increased hepatic nuclear expression of IRF-1 protein at 1 and 3 hours. Rats pre-treated with AdIRF-1 prior to transplantation had increased ALT levels and increased expression of IFN-β, IFN-γ, IL-12, and iNOS in short-term period graft(3 hours) when compared with donor livers pre-treated with Adnull. AdIRF-1 pre-treated donor livers also exhibited increased susceptibility to early apoptosis in the transplanted grafts with increased TUNEL staining expression of cleaved caspase-3. Additionally, AdIRF-1 pre-treated donor livers had increased activation of the MAP kinase JNK as compared with Adnull pre-treated donor livers.

Conclusions—IRF-1 is an important regulator of IR injury after OLT in rats. Targeting of IRF-1 may be a potential strategy to ameliorate ischemic liver injury after transplantation in order to minimize organ dysfunction.

INTRODUCTION

Liver ischemia and reperfusion (IR) injury occurs in a variety of clinical settings such as shock, elective liver resection, and transplantation. The IR injury that occurs with liver transplantation

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can have a significant impact on patient outcome, resulting in early graft dysfunction, longer hospitalization, increased susceptibility to infection, and allograft rejection (1-4). Liver IR injury can also lead to late complications after transplantation, such as non-anastomotic biliary strictures that carry significant morbidity for the patient (5-9). The pathophysiology of liver IR injury includes direct cellular damage from the ischemic insult, as well as delayed dysfunction resulting from the activation of inflammatory pathways. Although all donor livers exhibit some degree of IR injury, the initiating events that account for local organ damage after reperfusion are only partially understood. Elucidating the molecular mechanisms responsible for ischemic injury would be crucial in developing strategies aimed at reducing organ damage.

Interferon regulatory factor-1 (IRF-1) is a ubiquitous, highly conserved transcription factor that regulates the expression of a number of genes involved in both innate and acquired immunity. Initially, IRF-1 was identified as a transcriptional activator for interferon (IFN) beta production (10,11). IRF-1 levels are regulated primarily at the transcriptional level and accumulate in response to various stimuli such as IFNs (type I and type II), double stranded RNA, cytokines, and hormones. Key IRF-1 promoter elements include IFN-γ activated sequences (GAS) and NF-κB binding sites which mediate transcriptional activation upon STAT1 and NF-κB binding, respectively (12-14). Although IRF-1 was initially discovered as an activator of IFNβ production, it has since been found to be important in the expression of many proinflammatory genes (15).

Previously, we reported that cultured rat hepatocytes expressed IRF-1 in response to stimulation by IFNγ, IFNβ, and to a lesser extent TNFa and IL-1β (16). Recently, we have shown that IRF-1 plays a central role in orchestrating inflammatory gene expression and contributes to liver damage in a murine model of warm liver IR injury (17). Nuclear IRF-1 protein levels increase as early as 1 hour following reperfusion and are essential to the induction of inflammatory mediators including ICAM, iNOS, TNFα and IL-6. In addition, IRF-1 knockout mice are protected from injury in this model when compared to their wild type counterparts (17). The importance of IRF-1 in mediating inflammation was further confirmed when IRF-1 overexpression via adenoviral gene delivery induced hepatic damage even in the absence of IR injury. While this study points to the importance of IRF-1 in a model of warm liver IR injury, little is known about its importance in hypothermic IR injury during liver transplantation. Because liver graft preservation and reperfusion injury can lead to dysfunction after transplantation, we undertook the current study to better define the role that IRF-1 plays in the pathogenesis of hypothermic IR injury in a model of rat orthotopic liver transplantation (OLT). In this study we characterize hepatic IRF-1 expression in normal donor rat livers after transplantation, determine if over-expression of IRF-1 in donor livers prior to transplantation worsens IR injury, and identify inflammatory mediators and pathways mediated by IRF-1.

MATERIALS AND METHODS

Preparation of Adenoviral Vectors

An E1- and E3-deleted adenoviral vector carrying the human AdIRF-1 cDNA was constructed as previously described (18,19). Concentrations of AdIRF-1 and the control adenovirus with no construct, Adnull, were determined by plaque-forming assay, and expressed as plaque-forming units (pfu). The titers of AdIRF-1 and Adnull were 1×10^{10} pfu/mL, and both vectors were diluted to 3×10^{9} pfu/mL with saline for intravenous injection to the donor rat.

Orthotopic Liver Transplantation (OLT)

Male Lewis (LEW, RT1) rats weighing 200 to 300 g (Harlan Sprague Dawley, Inc., Indianapolis, IN) were maintained in a laminar-flow, specific-pathogen-free atmosphere at the University of Pittsburgh. All of the initial transplants were performed by a single surgeon

(KH.K.). The anhepatic and warm ischemia times were well controlled for with warm ischemic time of 18.±1.5 minutes. Basic techniques of liver harvesting and orthotopic transplantation without hepatic arterial reconstruction were according to the method previously described by Kamada and Calne (20). All procedures in this experiment were performed according to the guidelines of the Council on Animal Care at the University of Pittsburgh and the National Research Council's Guide for the Care and Use of Laboratory Animals.

Experimental Design for Transplant Experiments

The first series of experiments involved transplantation of untreated donor rat livers and sacrifice at 1, 3, 6, and 24 hours to assess nuclear IRF-1 expression in the liver. In these experiments, the donor liver was preserved for 18 hours to induce a severe IR injury. N=4 for each time-point.

The next series of experiments were undertaken to determine optimal dosing of AdIRF-1 and timing of organ procurement from the donors. AdIRF-1 or Adnull (1×10^8, 1×10^9, or 3×10^9 pfu/mL) was injected intravenously to prospective donor animals under brief isoflurane anesthesia. Rats were sacrificed 2 or 4 days later to assess nuclear IRF-1 expression in the livers. Blood was drawn at days 1- 4 for assessment of ALT levels. After the optimal viral dose (3×10^9 pfu/mL) and timing for harvest was determined (4 days), AdIRF-1 or Adnull was injected into donor rats.

The final series of experiments were undertaken to investigate the alteration of proinflammatory, apoptosis-related, and intracellular signaling molecules in transplanted livers from AdIRF-1 or Adnull pre-treated livers. Based on the results of the first set of experiments, recipient rats were sacrificed at 1, 6, and 24 hours after transplantation with either AdIRF-1 or Adnull pre-treated donors for serum and liver graft samples. Liver grafts were kept in a bath of UW solution at 4 °C for 3 hours of cold preservation period prior to orthotopic transplant into syngeneic LEW recipients to induce a mild IR injury. Endpoints examined were IRF-1, IFN-γ, IFN-β, JNK, p-JNK, iNOS, IL-12, cleaved Caspase-3 expression, liver enzyme release, histology, and apoptosis.

Liver Function Tests

Hepatic function and injury after rat liver transplantation was assessed by serum alanine aminotransferase (ALT) levels using the Opera Clinical Chemistry System (Bayer Co., Tarrytown, NY).

Histopathology

Liver graft tissues were fixed in 10% formalin, embedded in paraffin, sectioned into 6-μm thickness, and stained with hematoxylin-eosin (H&E).

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Assay

Apoptosis was determined using the *in situ* end-labeling technique. Formalin-fixed paraffin-embedded sections (4 μm) of liver were investigated using the Apop Tag Peroxidase Kit (Intergen Co., Purchase, NY). The peroxidase activity was visualized with AEC substrate, yielding a brown-red oxidation product. Hematoxylin was used as counter stain.

RT-PCR analysis

Total RNA from liver specimens was isolated with Trizol solution (Invitrogen). RT-PCR was performed using the One Step RT-PCR Kit (Clontech Inc., Mountain View, CA) and a PCR machine from Perkin-Elmer according to the manufacturer's instructions. Primer pairs for

detecting IRF-1 level were as follows: 5'-CTAGCCCTGACACCTTATCTGACGG-3'(forward) and 5'-GTCCCCTCCAGGGCTGTCGATCTCT-3'(reverse), IFN-γ: 5'-AGTCTGAAGAACTATTTTAACTCAAGTAGCAT-3'(forward) and 5'-CTGGCTCTCAAGTATTTTCGTGTTAC-3'(reverse), iNOS: 5′-GGAGAGATTTTTCACGACACCC-3'(forward) and 5'-CCATGCATAATTTGGACTTGCA-3'(reverse), IFN-β: 5′-GAGGTGATGCACCCGTCACAG-3'(forward) and 5'-GATGTAGTTCATCCAAGAGAC-3'(reverse), IL-12p40: 5'-GCCAATACACCTGCCACAGAG-3'(forward) and -CTTGATGTTAAACTTCAAGTC-3'(reverse). Primers for β-Actin were purchased from Clontech Inc. (Mountain View, CA). The PCR products were run on 1.5% agarose gel. Bands were quantified using NIH Image 1.62 software and normalized with the loading controls. Quantities are expressed as fold increase over the untreated group.

Isolation of cytoplasmic and nuclear proteins

Frozen liver tissues were suspended in buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5% Nonidet P-40 and homogenized for 20 s with a Polytron homogenizer (Kinematica, Littau, Switzerland). Nuclei were recovered by microcentrifugation at 7,500 rpm for 5 minutes. The supernatant containing cytoplasmic protein was collected and stored at -80°C for Western blot analysis. Nuclear proteins were extracted at 4°C by gently resuspending the nuclei pellet in buffer, containing 20 mM HEPES (pH 7.9), 10% glycerol, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.2 mM EDTA followed by 30min of incubation at 4°C with occasional vortexing. After microcentrifugation at 13,000 rpm for 15 min at 4°C, the supernatant containing nuclear protein was collected and frozen at -80°C. All buffers contained the following additional ingredients: 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM ditiothreitol, and 0.1 mM Na-vanadate and protease inhibitors. Protein concentration was quantified with Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Western blot assay was performed using whole cell lysate from liver specimens (20-50 μg) as previously described (21). For IRF-1, 20μg of nuclear protein was used. Membranes were incubated with primary polyclonal antibody for IRF-1 (Santa Cruz Biotechnology, Santa Cruz, CA), cleaved caspase-3, phosphorylated-JNK and total-JNK (Cell Signaling Technology, Beverly, MA), iNOS (Transduction Laboratories, Lexington, KY), or actin (Sigma-Aldrich, St. Louis, MO). After incubation with secondary goat anti-rabbit antibody (Pierce Chemical, Rockford, IL), membranes were developed with the Super Signal detection systems (Pierce Chemical) and exposed to film. Bands were quantified using NIH Image 1.62 software. Quantities are expressed as fold increase over the untreated group.

Confocal Immunofluorescence Imaging

A segment of transplanted liver was fixed in 2% paraformaldehyde at 4°C overnight and cryoprotected in 30% sucrose in PBS for another 24 hours. Samples were embedded in optimal cutting temperature compound and frozen in liquid nitrogen cooled isopentane and stored at -80 degree until sectioned. Samples were cut into 6-μm sections, washed with PBS and blocked with 5% BSA in PBS for 45 minutes, followed by 3 washes 0.5% BSA in PBS. The slides were then incubated with anti-cleaved caspase-3 antibody (1:1000 in 0.5% BSA) for 60 minutes at room temperature. The slides further treated as previously described (22) and visualized with a confocal microscope (Fluoview 1000, Olympus).

Statistical Analysis

Data are represented as the mean ± SEM. Comparisons between the groups at different time points were performed by using the Student's *t* test or analysis of variance (ANOVA) using the Statview program (Abacus Concepts, Inc., Berkeley, CA). Differences were considered significant at a *P* value less than 0.05.

RESULTS

IRF-1 nuclear protein expression increases in donor rat livers after orthotopic liver transplantation

We have previously shown in warm liver IR injury that nuclear IRF-1 protein expression increases as early as 1 hour after reperfusion, with a return to baseline levels after 6 hours of reperfusion (17). To determine if IRF-1 is upregulated in donor livers after OLT, we performed liver transplantation in rats and analyzed endogenous hepatic IRF-1 expression at various time-points after liver transplant reperfusion. IRF-1 expression was found to be increased in nuclear extracts from graft livers after OLT as early as 1 hour after reperfusion and returned to near baseline levels by 6 - 24 hours in 18 hour cold preservation model (figure 1A), demonstrating a similar pattern of early IRF-1expression as seen in our warm liver IR model (17).

AdIRF-1 pre-treatment leads to overexpression of IRF-1 protein in donor livers

To further examine the role of IRF-1 in subsequent OLT experiments, we first sought to optimize our technique of over-expressing IRF1 in donor livers. Based on our previous work with adenoviral marker gene transfer in the liver transplant setting (23,24), we delivered an adenovirus expressing IRF-1 (AdIRF-1) to normal rats at various doses and examined hepatic IRF-1 protein expression. Hepatic nuclear IRF-1 was initially observed to be expressed at low levels in rats at doses of 1×10^8 pfu of AdIRF-1, and to be prominently expressed at doses of 1×10^9 or 3×10^9 pfu 4 days after injection (figure 1B). The dose of 3×10^9 pfu was able to over-express IRF-1 in donor livers as early as 2 days after injection (figure 1B). Although serum ALT levels mildly increased with overexpression of IRF-1 in the liver by day 2 and 3 after administration, they returned to baseline by 4 days after injection (figure 1C), and no histological damage was noted when compared to Adnull injected rats (figure 1D). Therefore, we chose to harvest donor livers after 4 days of injection of normal rats with 3×10^9 pfu of AdIRF-1 or Adnull for subsequent transplant experiments.

AdIRF-1 pre-treatment of donor livers results in worsened hepatic IR injury after transplant

We next sought to determine if expression of IRF-1 in donor liver grafts prior to transplantation would worsen a mild IR injury. OLT was performed using donor livers that were pre-treated with 3×10^9 pfu AdIRF-1 (or Adnull) and then cold-stored in UW solution for 3 hours prior to transplantation. Animals transplanted with donor livers pre-treated with AdIRF-1 displayed a marked worsening of liver injury 6 hours after reperfusion as assessed by serum ALT (3,175 ±905 IU/mL vs. 1,424±696 IU/mL, $p < 0.05$) when compared with animals receiving donor livers pre-treated with control Adnull virus (figure 2A). Donor livers treated with AdIRF-1 prior to transplantation also demonstrated worsened hepatic necrosis 24 hours after transplantation when compared to livers pre-treated with Adnull virus as assessed by H&E staining (figure 2B). To confirm that the worsened injury after OLT was due to the transduced grafts expressing AdIRF-1, and not due to endogenous hepatic IRF-1, we examined the levels of IRF-1 mRNA and nuclear protein in liver grafts. As expected, untreated or Adnull treated livers expressed low amounts of endogenous IRF-1 nuclear protein and mRNA 1 and 6 hours after transplantation (figure 3A, 3B). In contrast, animals receiving grafts transduced to express AdIRF-1 exhibited strong IRF-1 mRNA and protein expression as early as 1 hour after transplant and remained elevated even 24 hours after reperfusion (figure 3A, 3B). We

quantified the mRNA levels and found that prior to transplantation IRF-1 mRNA expression was 38 times higher than the untreated group, 2 fold higher at 1 and 6 hours after reperfusion, and 6 times higher 24 hours after transplantation (data not shown). Taken together, these results demonstrate that IRF-1 overexpression in donor livers results in worsened liver IR injury after transplantation.

Ad IRF-1 pre-treatment of donor livers is associated with exaggerated expression of inflammatory mediators after transplantation

We next sought to determine if the worsened IR injury after transplantation with AdIRF-1 pre-treatment of the donor liver was associated with increased expression of inflammatory mediators in the liver that are known to be regulated by IRF-1. Expressions of IFN-γ, iNOS, IFN-β and IL-12 mRNA were all greater in AdIRF-1 pre-treated livers at 1 hour after reperfusion compared to grafts pre-treated with Adnull (figure 3B). When quantified and compared to untreated animals, IFN-γ was 3 fold higher, iNOS was 1.3 fold higher, IFN-β and IL-12 1.6 fold higher (data not shown). iNOS protein expression was also most prominent in donor livers pre-treated with AdIRF-1, and was sustained in these livers up to 24 hours after transplantation (figure 3B, 3C). Quantification revealed 17 and 21 fold higher iNOS protein expression in the AdIRF-1 treated group compared to the untreated group at 6 and 24 hours, respectively (data not shown). These results indicate that increased expression of IRF-1 in transplanted livers is associated with an up-regulation of multiple downstream inflammatory mediators. It should be noted that livers pre-treated with Adnull did have a rise in inflammatory mediators greater than that seen with the untreated group, as would be expected after infection with Adenovirus.

Donor livers pre-treated with AdIRF-1 have increased apoptosis after transplantation

Mutations in IRF-1 have been associated with decreased apoptotic potential in tumor cell lines (25,26). In order to determine if the IR injury associated with increased IRF-1 expression was associated with increased apoptosis, we examined the expression of cleaved caspase-3 and performed TUNEL staining on the donor livers pre- and post-transplant. Donor livers treated with AdIRF-1 or Adnull showed no difference in TUNEL staining 4 days after injection (figure 4A). However, 6 hours after transplantation the donor livers pre-treated with AdIRF-1 showed increased TUNEL staining when compared with Adnull treated livers (figure 4A). Donor livers pre-treated with AdIRF-1 also showed increased cleaved caspase-3 by western blot and immunoflourescent staining six hours after transplantation as compared to livers pre-treated with Adnull or no treatment (figures 4B, 4C).

Increased expression of IRF-1 after transplantation modulates inflammatory signaling pathways

Activation of MAP kinases is a prominent intracellular signaling event during redox stress, and a role for JNK activation in liver I/R injury has been demonstrated (27,28). To determine if increased expression of IRF-1in transplanted livers has a role in JNK activation, we assessed the phosphorylation status of JNK in livers after transplantation. We found a prominent increase in p-JNK in AdIRF-1 pre-treated livers 1 hour after transplantation as compared to Adnull pre-treated livers (figure 5A, B).

DISCUSSION

Ischemia and reperfusion injury continues to be an important factor in early and late graft dysfunction after liver transplantation. Because of the relative shortage of available donor organs, understanding the molecular mechanisms involved in IR injury is crucial in order to develop strategies to prevent graft dysfunction and failure. Although the distal interacting elements in the cascade of inflammatory responses resulting in organ damage following hepatic

IR injury have been extensively studied, the initiating events are poorly understood. We have previously shown that transcription factor IRF-1 is an important early mediator in the pathogenesis of warm liver IR injury (17). Since there are significant differences described between the pathogenesis of warm and cold IR injury (29,30), we conducted this current study to determine if IRF-1 plays a significant role in the mechanism of organ damage in a model of rat orthotopic liver transplantation. The novel findings in this paper are (1) endogenous IRF-1 nuclear protein is up-regulated at early time-points after liver transplantation; (2) over-expression of IRF-1 in donor livers prior to transplantation worsens IR injury and results in increased production of inflammatory mediators; (3) over-expression of IRF-1 in transplanted liver grafts is associated with increased activation of JNK MAPkinase signaling pathway; and (4) hepatic IRF-1 expression in the liver transplant setting results in increased apoptosis in addition to enhanced hepatocellular necrosis.

IRF-1 has previously been shown to be a transcription factor important in immunity, tumor suppression, and apoptosis. Additionally, work from our laboratory demonstrated that IRF-1 is an important mediator of liver damage in warm IR injury. The present study has confirmed that IRF-1 is also crucial to the pathogenesis of cold IR injury after transplantation. Our results suggest that IRF-1 contributes to IR injury by regulating the expression of multiple inflammatory mediators, such as IL-12, IFN-γ, and iNOS, which are important in IR injury (29). In addition, to further elucidate the mechanism of IRF-1 mediated injury after OLT, we studied apoptosis 24 hours after transplantation. Previous studies have demonstrated the presence of apoptotic cell death in cold IR injury (31,32). Indeed, caspase inhibitors have been shown to ameliorate damage in cold IR injury (33). Additionally, non-functional IRF-1 has been found in tumor cells, and over-expression of IRF-1 has been shown to increase apoptotic potential (25,26). We found increased activation of apoptotic pathways with higher levels of cleaved caspase-3 and stronger TUNEL staining 6 hours after reperfusion of donor livers strongly expressing IRF-1. Thus, IRF-1-mediated inflammation and organ injury after OLT appears to involve the coordination of a variety of inflammatory mediators as well as apoptotic pathways.

The MAP kinase family represents a group of proteins involved in signal transduction of a variety of cellular stimuli. The JNK subgroup of MAP kinases is activated in response to environmental stresses, including liver IR (27,28). In relation to IRF-1, our previous work showed a lack of JNK activation in IRF-1 KO mice after warm liver IR injury. In this study we found that liver grafts over-expressing IRF-1 prior to transplantation have strong activation of JNK one hour after transplantation. This highlights the importance of IRF-1 as an activator of MAP kinase mediated damage in liver IR injury.

In summary, we have shown that IRF-1 is up-regulated in cold IR injury and that over-expression of IRF-1 in donor livers worsens organ damage. IRF-1 mediated inflammation after OLT appear to involve multiple mechanisms that are vital to the cellular stress response, including production of inflammatory mediators, activation of apoptotic pathways, and activation of MAP kinase pathways. Strategies targeting IRF-1 may be considered to prevent both early and late graft dysfunction after transplantation.

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ABBREVIATIONS

(IR), Ischemia/reperfusion

(IRF-1), Interferon Regulatory Factor-1
(IFN), interferon
(pfu), plaque forming units
(ALT), alanine aminotransferase
(H&E), hematoxylin-eosin
(TUNEL), Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling
(JNK), c-Jun NH_2-terminal kinase
(p-JNK), phosphorylated-Jun NH_2-terminal kinase
(iNOS), inducible nitric oxide synthase
(IL-12), interluekin-12
(MAP), mitogen activated protein

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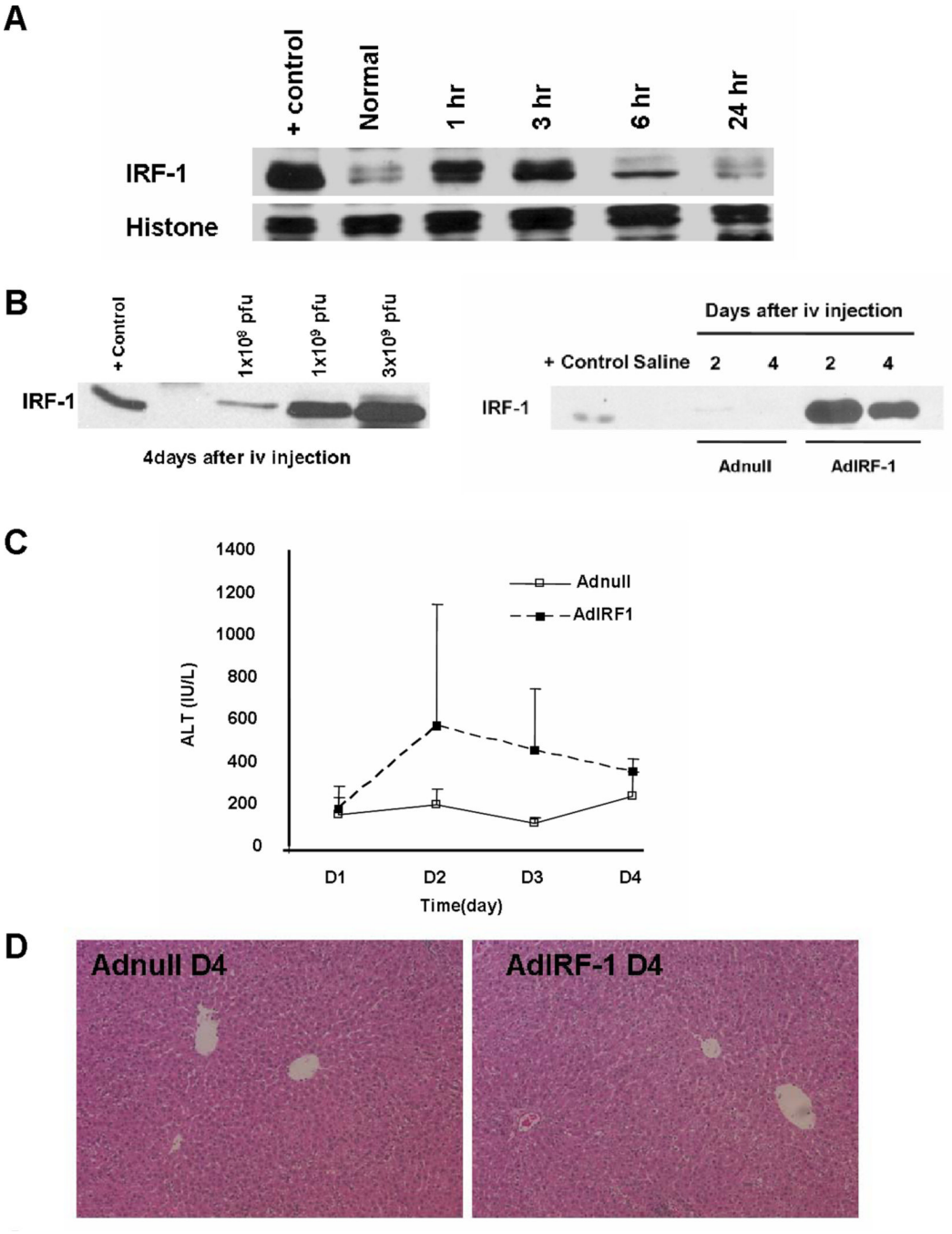

Figure 1. Ad-IRF-1 pre-treatment leads to over-expression of IRF-1 in donor livers

(A) Western blot of liver nuclear IRF-1 protein at different time-points following liver transplantation. After orthotopic liver transplantation with 18 hour cold preservation, IRF-1 is expressed strongly at 1 and 3 hours with a return to near baseline levels by 6 hours (B) Western blot analysis for nuclear IRF-1 shows a dose dependent increase in IRF-1 expression after AdIRF-1injection. IRF-1 expression is minimal at 1×10^8 pfu, but is strongly expressed at 1×10^9 and 3×10^9 pfu. IRF-1 expression is sustained 4 days after treatment with a dose of 3×10^9 pfu. (C) Time course of serum ALT levels after AdIRF-1 injection (3×10^9 pfu) shows a mild increase in ALT levels 2 days after injection with normalization by 4 days. (D) H&E

staining of liver sections from rats treated with AdIRF-1 or Adnull (3×10^9 pfu) show no necrosis after 4 days.

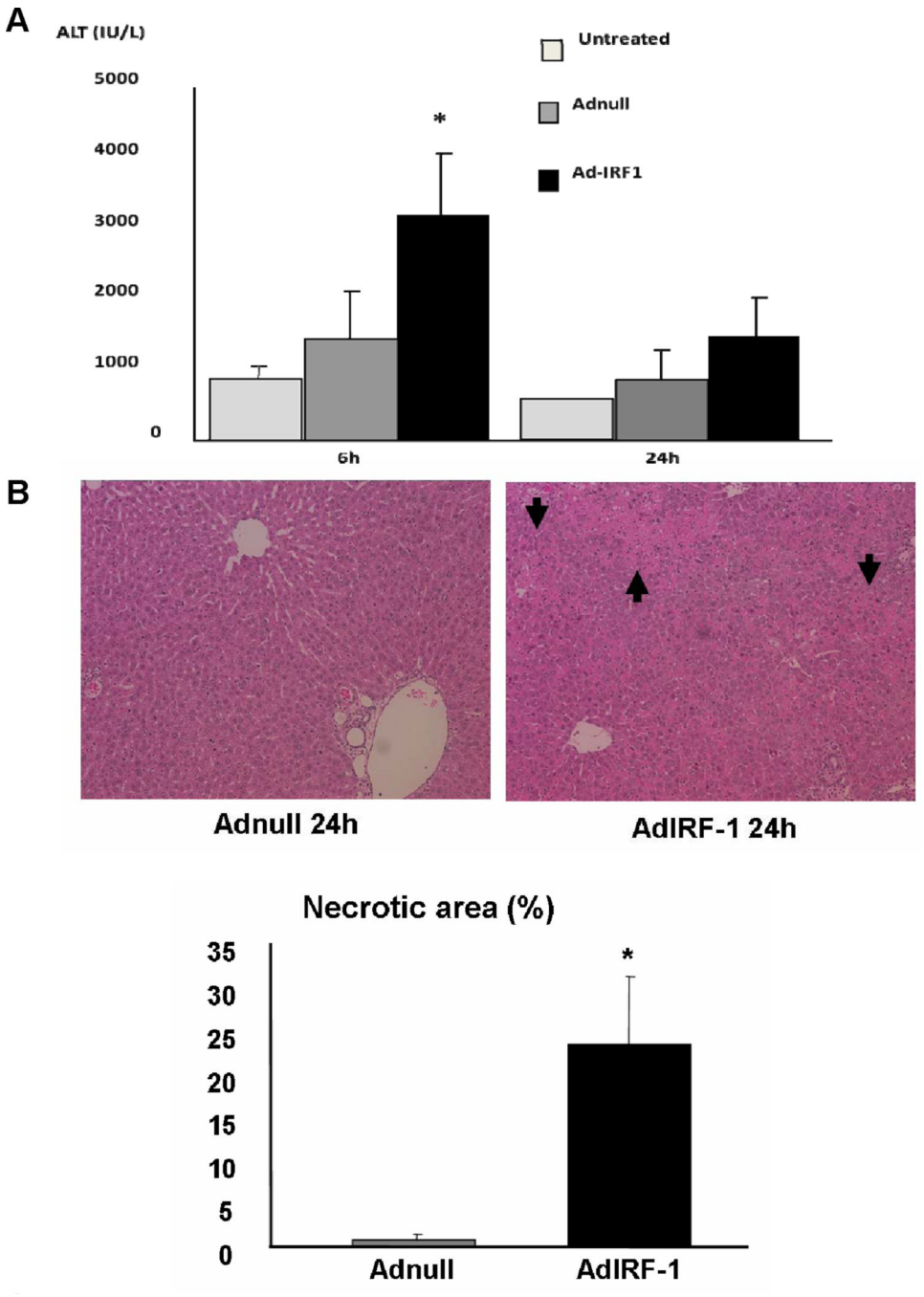

Figure 2. Donor livers pre-treated with AdIRF-1 have worse IR injury

(A) Serum ALT levels from rats transplanted with donor livers 4 days after pre-treatment with AdIRF-1 (3×10^9 pfu) show higher ALT levels than those pre-treated with Adnull ($p<0.05$) 6 hours after transplantation. Error bars show ±SD (n=4). (B) H&E staining 24 hours after liver transplantation demonstrate more necrosis (arrows) in donor livers pre-treated with AdIRF-1. Percentage of necrotic area was calculated in five high power fields for each animals using Adobe Photoshop, Version 7.0. Error bars show ±SD (n=4) (* $p<0.05$).

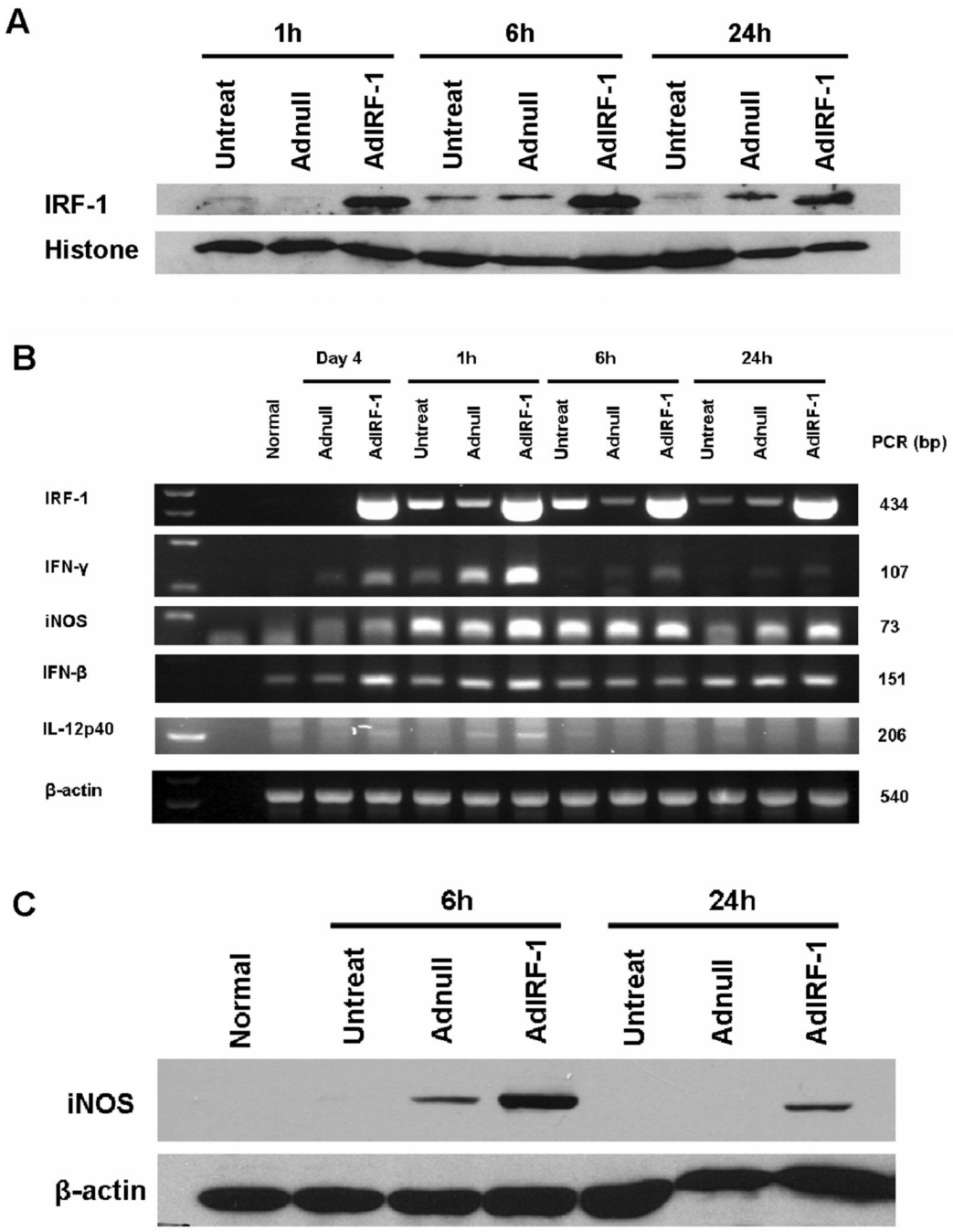

Figure 3. Donor livers pre-treated with AdIRF-1 have exaggerated expression of inflammatory mediators after transplantation

(A) Western blot of nuclear protein 1, 6, and 24 hours after transplantation shows low-level endogenous IRF-1 expression in untreated donor livers at early time-points as expected. However, donor livers pre-treated with AdIRF-1(3×10^9 pfu) have high levels of IRF-1 protein expression at all time-points. (B) After transplantation mRNA levels of inflammatory mediators are greater in donor livers pre-treated with AdIRF-1. IFN-γ, IL-12, and IFN-β expression are highest 1 hour after transplant with AdIRF-1 pre-treatment. iNOS expression is also highest in AdIRF-1 pre-treated donor livers, and is sustained for 24 hours. (C) Western

blot of iNOS protein from donor livers after transplantation shows sustained protein expression after 24 hours.

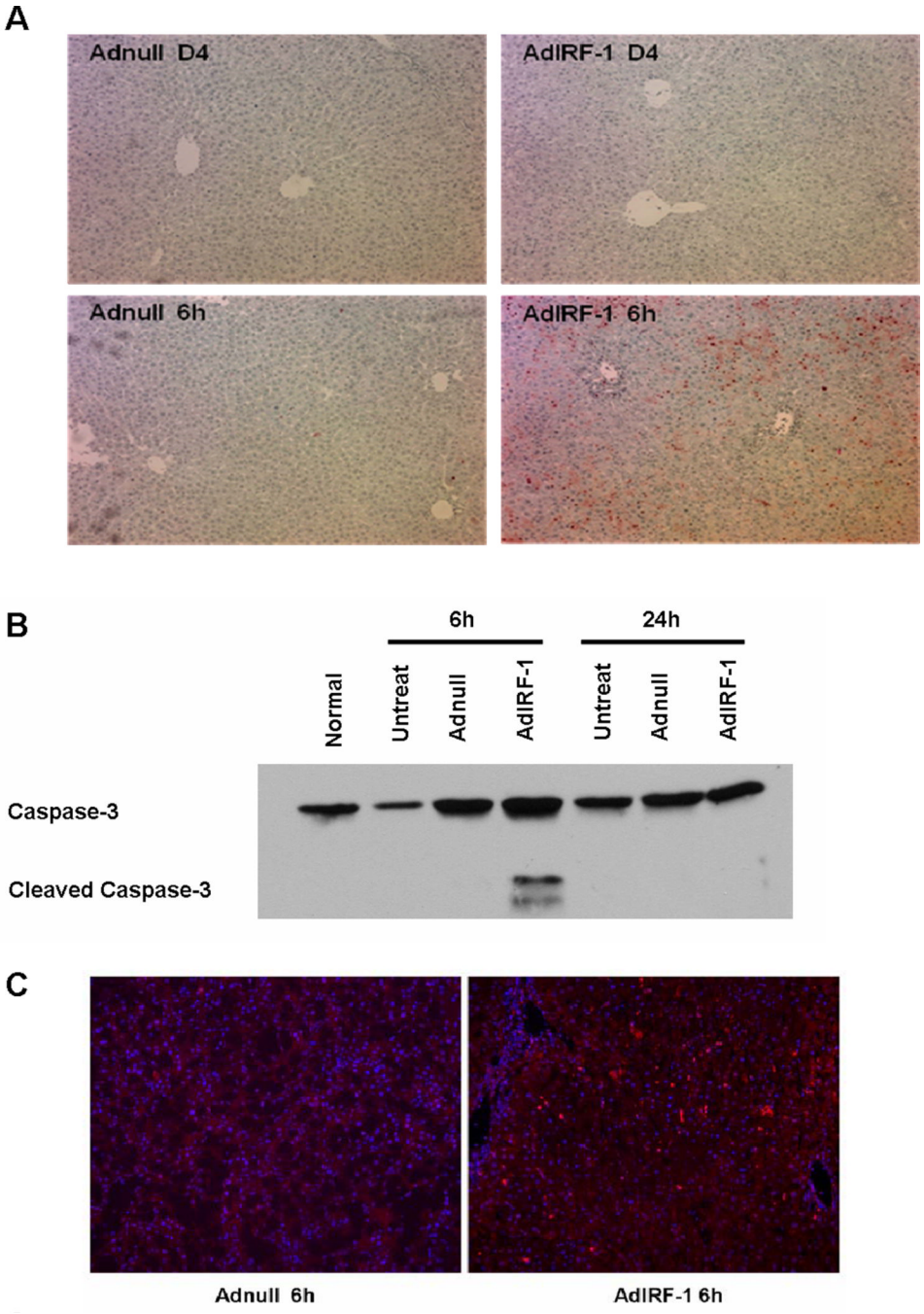

Figure 4. Donor livers pre-treated with AdIRF-1 have increased apoptosis 6 hours after transplantation

(A) TUNEL staining prior to transplantation shows no difference in livers pre-treated with AdIRF-1 vs. Adnull. (upper panels) However, 6 hours after transplantation there is increased TUNEL staining in donor livers pre-treated with AdIRF-1 (lower panels). (B) Western blot for cleaved caspase-3 and total caspase-3 using whole cell lysate isolated from the liver grafts 6 or 24 hours after reperfusion shows higher levels of cleaved caspase-3 in donor livers pre-treated with AdIRF-1. (C) Confocal immunofluorescence of liver tissue 6 hours after transplantation shows more intense staining of cleaved caspase-3 (red color) in donor livers pre-treated with AdIRF-1.

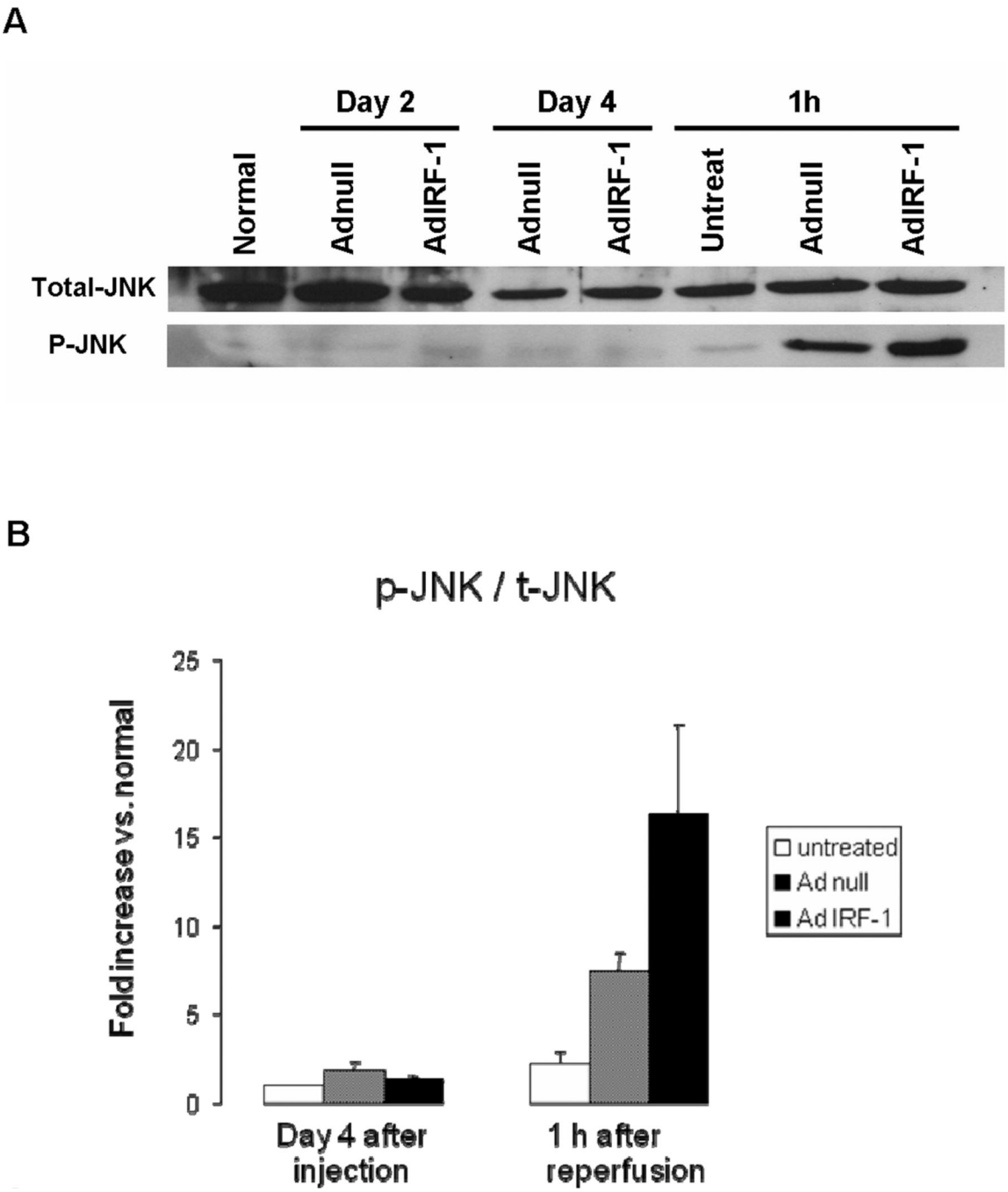

Figure 5. Increased IRF-1 expression after transplantation is associated with JNK activation

(A) Western blot of liver whole cell lysates for phosphorylated and total c-Jun NH_2-terminal kinase (JNK) prior to transplantation and 1 hour after transplantation shows that AdIRF-1 (3×10^9 pfu) pre-treatment increases the amount of phosphorylated JNK (p-JNK) after transplantation. (B) Quantification of phosphorylated JNK bands demonstrates a several fold increase in JNK activation in donor rat livers pre-treated with AdIRF-1 when compared with controls.