INTERFERON REGULATORY FACTOR-2 (IRF2) IS PROTECTIVE AGAINST HEPATIC ISCHEMIA/REPERFUSION INJURY

John R. Klune#, Rajeev Dhupar#, Shoko Kimura, Shinya Ueki, Jon Cardinal, Atsunori Nakao, Gary Nace, John Evankovich, Noriko Murase, Allan Tsung, David A. Geller

#= These authors contributed equally to this work

Author Contributions:
RD/JRK/JC/DAG – Study Design
RD/JRK/SK/SU/JC/AN/GN/JE – Collection and assembly of data
RD/JRK/SK/JC/AN/GN/NM/AT/DAG – Analysis and interpretation of data
JRK/RD/JC – Drafting of manuscript
JRK/RD/NM/AT/DAG – Critical revision of manuscript

Department of Surgery, University of Pittsburgh, Starzl Transplantation Institute, Pittsburgh, PA

Running Title: IRF2 in Hepatic I/R

Corresponding Author: David A. Geller, MD, Starzl Transplantation Institute, 3459 Fifth Avenue, MUH 7 South, University of Pittsburgh, Pittsburgh, PA 15213

Phone: 412-692-2001    FAX: 412-692-2002          E-Mail: gellerda@upmc.edu
Abstract

Interferon regulatory factor-1 (IRF1) is a nuclear transcription factor that induces inflammatory cytokine mediators and contributes to hepatic ischemia-reperfusion injury (I/R). Currently, no strategies to mitigate IRF1-mediated liver damage exist. Interferon regulatory factor 2 (IRF2) is a structurally similar endogenous protein that competes with IRF1 for DNA binding sites in IRF-responsive target genes and acts as a competitive inhibitor. However, the role of IRF2 in hepatic injury during hypoxic or inflammatory conditions is unknown. In this study, we hypothesize that IRF2 overexpression may mitigate IRF1-mediated I/R damage. Endogenous IRF2 is basally expressed in normal livers, and is mildly increased by ischemia alone. Overexpression of IRF2 protects against hepatic warm I/R injury. Further, we demonstrate that IRF2 overexpression limits production of IRF1-dependent pro-inflammatory genes such as IL-12, IFNβ, and iNOS, even in the presence of IRF1 induction. Additionally, isograft liver transplantation with IRF2 heterozygote knockout (IRF2+/−) donor grafts with reduced endogenous IRF2 levels experience worse injury following cold I/R during murine orthotopic liver transplant. These findings indicate that endogenous intrahepatic IRF2 protein is protective because the IRF2-deficient liver donor grafts exhibited increased liver damage compared to the wild-type donor grafts. In summary, IRF2 overexpression protects against I/R injury by decreasing IRF1-dependent injury and may represent a novel therapeutic strategy.

Key Words:

Interferon regulatory factor 1 (IRF1), liver, transplantation, I/R injury
Introduction

Liver ischemia-reperfusion (I/R) injury occurs in several clinical settings and is known to involve both direct cellular ischemic damage as well as activation of the innate immune system leading to a complex system of inflammatory pathways (16; 37-41). While much has been learned regarding the initial events during hepatic I/R that lead to activation of the immune system and release of inflammatory mediators, the process is still not fully elucidated and possible strategies to reduce I/R injury are important to explore.

The interferon regulatory factors (IRFs) are a family of interferon (IFN)-inducible transcription factors that are known to be involved in the transcription of genes involved in antiviral defense, immune regulation, and malignancy (reviewed in (23)). Interferon regulatory factor 1 (IRF1) is upregulated in response to a variety of cytokines and in turn activates the transcription of multiple genes and inflammatory mediators, including type I interferons, iNOS, and others (5; 12; 19; 21). The involvement of IRF1 in liver I/R injury has been demonstrated in both cold (13; 35) and warm (34) I/R injury models. Additionally, the downstream products of IRF1 activation such as the type I interferons (39) and iNOS (31; 32) are known to contribute to liver I/R injury.

Interferon regulatory factor 2 (IRF2) was first described in 1989 as a structurally similar but functionally distinct nuclear factor from IRF1 (8). This transcription factor was found to bind the same regulatory sequences as IRF1, but suppressed, rather than activated, transcription of these IFN-inducible genes. However, more recent data has found several transcriptional activating roles for IRF2, in that it can function as an oncogene in activation of certain cell-cycle genes (36) as well as contributing to activation of cytokine production in some infectious and cytokine stimulation models (17; 24; 27). While IRF2 can function as either a transcription
repressor or activator depending on the cell type and inflammatory state, its role during hypoxic
or acute hepatic inflammation such as liver I/R injury is currently unknown.

With the understanding that IRF1 activation contributes to liver I/R injury and that IRF2 can act
as a competitive inhibitor of IRF1 binding to regulatory sequences, we hypothesized that
blocking the IRF1-mediated pro-inflammatory signaling pathways with enhanced IRF2
expression may serve as a novel therapeutic strategy to ameliorate liver I/R injury. In this study,
we demonstrate that endogenous intrahepatic IRF2 is protective, and that overexpression of IRF2
diminishes induction of IRF1-dependent genes and decreases subsequent liver injury.
Materials and Methods

Liver Function Tests. Hepatic function after warm and cold liver IR was assessed by serum alanine aminotransferase (ALT) levels using the HESKA 4000 Dri-chem veterinary chemistry analyzer (HESKA Corporation, Loveland, Colorado, USA).

Hepatocyte isolation. Hepatocytes were isolated from wild type mice by an in situ collagenase (type IV, Sigma) perfusion technique, modified as described previously (35). Hepatocytes were separated from the non-parenchymal cells by two cycles of differential centrifugation (50 g for 2 min) and further purified over a 30% Percoll gradient. Hepatocyte purity exceeded 98% as assessed by light microscopy, and viability was typically greater than 95% as determined by trypan blue exclusion assay.

Cell culture. Hepatocytes (3 x 10⁶) were plated on 60-mm gelatin-coated petri dishes in 3 ml of culture media. Media consisted of Williams media E (Gibco Life Technologies, Gaithersburg, MD) with L-arginine (0.5 mmol/l), insulin (10⁻⁶ mol/l), HEPES (15 mmol/l), L-glutamine, penicillin, streptomycin, and 10% low endotoxin calf serum (Hyclone Laboratories, Logan, UT). After a 24-h incubation (37°C, 95% air-5% CO2) the media were changed, and cells were treated as described.

Preparation of Adenoviral Vectors. An E1- and E3-deleted adenoviral vector carrying the human AdIRF1, AdIRF2, or AdLacZ cDNA were constructed as previously described (14). Concentrations of AdIRF1, AdIRF2, and the control adenovirus AdLacZ, were determined by plaque-forming assay, and expressed as plaque-forming units (pfu). All vectors were diluted with saline to the concentrations described for intravenous injection to the mouse. For cell culture treatment, cells were washed with warmed PBS, then the virus was diluted to the described
concentration in serum-free media (Opti-mem) and placed on the cell culture for 3 hours. After 3 hours, the virus was removed, and the cells were allowed to recover for 21 hours prior to further treatments/harvest.

Isolation of cytoplasmic and nuclear proteins. Frozen liver tissues or cell cultures were suspended in buffer containing 10mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, and 0.5% NonidetP-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 MgCl₂, 10 mM KCl, and 0.2 mM EDTA followed by 30 min 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 phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol, 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 nuclear or whole cell lysate from liver specimens (20-50 µg) as previously described (35). For IRF1 and IRF2, 20 µg of nuclear protein was used. Membranes were incubated with primary polyclonal antibody for IRF1 or histone3 (Santa Cruz Biotechnology, Santa Cruz, CA), IRF2 (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.

**Realtime RT-PCR.** The mRNA for interleukin (IL)-12, interferon-beta (IFN-β) and iNOS were quantified in duplicate using SYBR Green two-step, real-time RT-PCR, as previously described (22). Gene expression was normalized with GAPDH mRNA content.

**Animals.** Male WT (C57BL/6) mice (8 – 12 weeks old) were purchased from the Jackson Laboratory. Animal protocols were approved by the University of Pittsburgh IACUC and were performed according to the NIH guidelines for the use of laboratory animals. IRF2−/− mice were generated by targeted disruption as previously described (20). IRF2+/− mice, backcrossed to C57BL/6 mice for three to five generations at the time we received them, were the kind gift of Dr. T. Mak (Amgen Institute, Toronto, Ontario, Canada). All IRF2 mice bred in our colony were genotyped.

**In vivo gene delivery and warm I/R.** For *in vivo* transfection, mice were administered intravenous injection (tail vein) of either AdIRF2 or AdLacZ at doses of $1.5 \times 10^8$ or $7.5 \times 10^8$ pfu for 24 to 48 hours prior to assessment of predetermined endpoints. For warm liver I/R, mice were subjected to 1 hour of ischemia and sacrificed at predetermined reperfusion time-points for tissue sample collection. A nonlethal model of segmental (70%) hepatic warm ischemia was used as previously described (33). Sham animals underwent anesthesia, laparotomy, and exposure of the portal triad without hepatic ischemia. Baseline or untreated animals were given anesthesia and sacrificed without exposure of the portal triad.

**Orthotopic Liver Transplantation (OLTx).** The techniques of liver harvesting and murine OLTx without hepatic artery reconstruction were based on the method described by Qian et al (25). Briefly, after cholecystectomy and insertion of a polyethylene stent (PE10, Becton
Dickinson, Franklin Lakes, NJ) into the bile duct, donor animals were heparinized, and liver
grafts were perfused with 1.0 ml of University of Wisconsin (UW) solution via the portal vein.

Excised liver grafts were prepared with cuffs on the infrahepatic vena cava and portal vein and
then stored in UW solution for 24 hours at 4 °C. After the removal of native livers in recipient
animals, grafts were orthotopically implanted by anastamosing the suprahepatic vena cava with a
running 10–0 nylon suture and connecting the cuffs to the recipient portal vein and inferior vena
cava. Anhepatic time averaged 19.8±1.7 min. The bile duct was connected via the ligation over
the stent. The abdominal wall was then closed in two layers. Our group has recently shown this
murine OLTx technique results in 98% survival (35).

**Statistical analysis.** Results are expressed as means +/- SE. Group comparisons were performed
using Student’s t-test or ANOVA. Differences were considered significant at P <0.05.
Results

IRF2 can be safely overexpressed using adenoviral vector

Initial experiments were performed to demonstrate the safety of nuclear overexpression of IRF2 using recombinant adenovirus encoding murine IRF2 (AdIRF2). It is known that transfection with adenoviral vectors can elicit non-specific inflammation depending on viral titer levels, particularly in mice. Mice were injected with AdLacZ (control) or AdIRF2 at doses of $1.5 \times 10^8$ or $7.5 \times 10^8$ pfu as previously described (34). Mice injected with $1.5 \times 10^8$ pfu did not experience any hepatic damage at 36 hours as measured by serum ALT levels (Fig 1A). Also, there was no difference in liver injury between the AdLacZ and AdIRF2 groups. In contrast, mice injected with the higher dose of $7.5 \times 10^8$ pfu experienced a rise in ALT levels in both AdIRF2 and control AdLacZ injected mice, suggesting non-specific liver damage from the adenovirus. Therefore, the lower dose of $1.5 \times 10^8$ pfu was used for further experiments. To document in vivo gene transfection and transgene expression, mice were injected with a single dose of $1.5 \times 10^8$ pfu AdIRF2 or AdLacZ and were sacrificed at 24, 36, and 48 hours. Nuclear protein extracts from liver tissue were examined for expression of IRF2 protein. Nuclear levels of IRF2 protein were increased in AdIRF2 transfected mice compared to AdLacZ mice, confirming successful in vivo gene transfer (Fig 1B). The low level of hepatic IRF2 nuclear protein detected in the normal (baseline) and AdLacZ injected mice reflects constitutive expression of endogenous IRF2. These results suggest that IRF2 overexpression can be achieved in mouse livers without causing non-specific hepatic damage by using a low adenoviral titer.
IRF2 overexpression is protective in hepatic warm I/R injury

We have previously shown that IRF1 activation occurs early following both warm (34) and cold (13; 35) liver I/R injury and contributes to liver damage. However, the expression pattern of IRF2 following I/R injury has not been described. The time course of nuclear expression of IRF1 and IRF2 proteins was determined using Western blot analysis of nuclear extracts from hepatic parenchyma following warm I/R injury. Nuclear IRF1 expression increased at 1 hr following I/R with maximum expression occurring 3 hrs after reperfusion (Fig 2A). IRF2 expression, in contrast, is induced slightly above sham levels by ischemia alone but does not increase further with reperfusion (Fig 2A). To determine the effect of IRF2 overexpression on hepatic I/R, mice were pre-treated with AdIRF2 vector or AdLacZ and subjected to hepatic warm I/R. Overexpression of IRF2 significantly reduced serum ALT levels 3.7-fold, with a 73% decrease from 3,700 (IU/L) in the AdLacZ treated mice to 1000 (IU/L) in the AdIRF2 treated mice (Fig 2B). Hence, overexpression of IRF2 is protective during I/R injury.

IRF2 overexpression results in decreased cytokine and IRF1 target gene production

IRF1 results in the transcriptional activation of a variety of target genes, including many that have been implicated in hepatic I/R injury such as iNOS, type I interferons, and IL-12. IRF2 is known to bind to the same cis-acting DNA sequences and competitively inhibit IRF1 protein-DNA binding. We examined the expression of hepatic IRF1 target genes using quantitative RT-PCR in mice injected with AdIRF2 or AdLacZ and subjected to warm I/R. Sham-treated mice had low basal levels of hepatic IL-12, which was not significantly affected by IRF2 overexpression (Fig 3A). Hepatic IL-12 mRNA was markedly increased by I/R, and IRF2 overexpression significantly decreased IL-12 mRNA levels compared to AdLacZ treated
animals. Additional specific IRF1 target genes associated with I/R injury include IFN-β and iNOS. Both IFNβ and iNOS mRNA were increased after hepatic I/R compared to baseline levels, and AdIRF2 treated mice also demonstrated significantly decreased transcriptional activity of these genes (Fig 3B,C). These data suggest that IRF2 overexpression is protective during hepatic I/R by inhibiting activation of IRF1 driven pro-inflammatory genes.

IRF2 overexpression does not alter nuclear IRF1 levels in vivo or in vitro

IRF1 and IRF2 compete for binding to DNA regulatory elements, and the ultimate expression of IFN-responsive genes depends on the balance between these two regulatory transcription factors (9). While IRF1 and IFNβ work in a positive feedback loop where each induce transcriptional activation of the other, it is unknown if IRF1 and IRF2 can alter the expression of the other. Additionally, while increased IRF1 expression in response to stimulation with IFNγ is well known, IRF2 expression following the same stimulation is not established (9; 15; 28). To determine whether IRF1 expression is affected by IRF2 overexpression, nuclear protein extracts from hepatic parenchyma from mice injected with AdLacZ or AdIRF2 were analyzed by Western blot analysis. As expected, AdIRF2 treated mice had higher levels of hepatic nuclear IRF2 protein, but IRF1 nuclear protein was not affected (Fig 4A). Similar experiments were done in vitro where hepatocytes were transfected with AdIRF2 and nuclear samples analyzed by Western blot. Overexpression of IRF2 at MOI of 50 or 100 showed strong nuclear IRF2 protein levels, but there was no detectable IRF1 protein (Fig 4B). In order to further characterize IRF1 and IRF2 expression following IFNγ stimulation in hepatocytes, and to confirm that IRF2 did not affect nuclear IRF1 levels, hepatocytes were stimulated with IFNγ and/or AdIRF2. Western blot analysis of nuclear protein extracts confirmed increased IRF1 in IFNγ stimulated hepatocytes,
but IFN\(_{\gamma}\) did not have any effect on IRF2 levels (Fig 4C, 2\(^{nd}\) lane). AdIRF2 treatment resulted in increased IRF2 expression in a dose-dependent fashion, but this did not alter IFN\(_{\gamma}\)-induced nuclear IRF1 expression at any dose (Fig 4C). These results confirm that IFN\(_{\gamma}\) induces hepatocyte IRF1 expression as expected, and that overexpression of IRF2 does not alter basal or IFN\(_{\gamma}\)-induced IRF1 expression.

**IRF2 overexpression decreases IRF1-dependent iNOS transcription in vitro**

Stimulation of hepatocytes by IFN\(_{\gamma}\) has previously been shown to activate transcription of IRF1 (6) and iNOS (7) by mRNA analysis. IFN\(_{\gamma}\) dependent iNOS expression is mediated by IRF1 binding in the iNOS promoter (12; 19; 29; 30), and IRF1\(^{-}\) mice show diminished iNOS induction (12; 26). As expected, *in vitro* stimulation of hepatocytes with IFN\(_{\gamma}\) resulted in iNOS protein induction after 24 hours of stimulation (Fig 5A). This induction was inhibited in a dose-dependent fashion in response to IRF2 overexpression using AdIRF2 transfection. In contrast, AdLacZ control vector did not alter expression of iNOS in response to IFN\(_{\gamma}\) stimulation. These findings confirm a suppressive role for IRF2 in down-regulating IFN\(_{\gamma}\)-induced IRF1-dependent iNOS transcriptional activation in hepatocytes. Next, we sought to show that IRF2 would also inhibit iNOS gene expression stimulated by exogenous IRF1 overexpression. Hepatocytes transfected with AdIRF1 demonstrated increased iNOS expression in a dose-dependent fashion (Fig 5B). In contrast, AdIRF2 transfection alone did not induce iNOS expression in hepatocytes. Co-transfection with AdIRF1 and AdIRF2 with MOI 50 resulted in barely detectable iNOS expression, confirming a role for IRF2 in repressing IRF1-mediated transcriptional activation of iNOS. These findings show that IRF2 functions to suppress IRF1-mediated transcriptional activity in hepatocytes. Additionally, we wanted to confirm that this effect was not limited to
hepatocytes but also applies in other cell types such as macrophages or Kupffer cells, which are also known to contribute to I/R injury. RAW 264.7 cells did over express IRF2 with AdIRF2 transfection compared with control (data not shown), and this IRF2 overexpression inhibited expression of iNOS following stimulation with LPS, IFNγ, or a combination of both. Therefore, we found that IRF2 expression is able to block IRF1 activity in other important cell types as well.

IRF2 heterozygote liver grafts result in increased injury after hepatic transplantation

The previous experiments demonstrated that IRF2 overexpression could block IRF1-mediated inflammatory signaling and decrease liver I/R injury; however, they do not address the functional role of endogenous IRF2. Therefore, IRF2+/− mice were used to determine the effect of decreased IRF2 expression on liver I/R injury. Heterozygote mice were used because IRF2−/− homozygous mice bred poorly and were phenotypically abnormal. To determine the baseline expression of IRF2 in these mice, hepatic nuclear protein extracts from IRF2+/− mice were examined and showed that baseline expression of IRF2 was significantly decreased in IRF2+/− mice compared to wild-type control mice (Fig 6A). Additionally, nuclear protein extracts from an IRF2−/− mouse demonstrated no IRF2 protein. In contrast, baseline levels of nuclear IRF1 were not affected in any of the nuclear protein extracts including the IRF2+/− mice. Therefore, the IRF2 heterozygote mice were used for liver graft donation for OLTx into wild-type mice. Murine OLTx using IRF2+/− donor grafts showed a two-fold increase in liver injury measured by serum ALT compared to wild-type donor grafts (Fig 6B). These results confirm a protective role for endogenous hepatic IRF2 because IRF2 relative deficiency in the IRF2+/− mice resulted in increased liver damage.
Liver I/R injury occurs in the clinical settings of liver transplantation, hepatic resection surgery, and hemorrhagic trauma. Previously, we have shown that the nuclear transcription factor IRF1 contributes to liver damage in both warm and cold I/R injury through multiple mechanisms, including expression of pro-inflammatory cytokine mediators of inflammation, activation of intracellular signaling cascades, and promoting apoptosis through expression of death receptors and ligands (13; 14; 34; 35; 39). Currently, no strategies aimed at decreasing IRF1-mediated liver injury have been defined. IRF2 is a ubiquitous endogenous protein antagonist of IRF1 (8; 9). Therefore, we examined the role of IRF2 in liver I/R injury. The major and novel findings of this study are: 1) endogenous intrahepatic IRF2 is protective against liver I/R injury as IRF2+/− deficient mice exhibited worse liver damage after OLTx; 2) IRF2 overexpression reduces hepatic I/R injury; 3) IRF2 overexpression significantly decreases induction of IRF1 responsive hepatic inflammatory mediators IL-12, IFNβ, and iNOS.

A major mechanism of action of IRF2 is to competitively inhibit induction of IRF1 inflammatory target genes. While IRF2 was originally described as an IRF1 antagonist (8; 9), it has also been shown to function as an IRF1 agonist or transcriptional activator in response to infection or certain cytokine stimulation. In macrophages, IRF2 contributes to IL-12 and IFNγ expression in response to LPS with or without co-stimulation with IFNγ (27), and contributes to IRF1 activation and IFNα release in lipoteichoic acid stimulation in a model of Staphylococcus aureus infection (17). IRF2 functions to upregulate IL-7 expression in human intestinal epithelial cells in response to IFNγ stimulation (24). While IRF2 has the potential to function as either a transcriptional inhibitor or activator in different inflammatory states, its role during the immune response to liver I/R injury has not been addressed.
Since IRF1 contributed to hepatic injury during warm and cold I/R (13; 34; 35), we initially sought to block the actions of IRF1 by overexpressing IRF2. In vivo expression of IRF2 was achieved with AdIRF2 delivery at a dose of 1.5x10^8 pfu which did not induce any non-specific inflammation (Fig. 1A). We recognize the limitations of not being able to use adenoviral delivery in patients; however, it provides excellent gene delivery for in vivo hepatic transfection and therefore was used to establish proof-of-principle in this study. Strong AdIRF2 expression was observed 24-48 hrs after injection. Mice that were overexpressing IRF2 were then subjected to warm I/R injury and showed over 70% decrease in serum ALT levels compared to AdLacZ-treated animals. This protection was mediated in part by IRF2 down-regulating induction of IRF1-mediated inflammatory cytokines IL-12, IFNβ, and iNOS in vivo. Moreover, IRF2 inhibited the expression of the IRF1-dependent iNOS gene in vitro in a dose-dependent manner.

Since overexpressed IRF2 was successful in blocking IRF1-mediated inflammatory gene activation and decreasing subsequent liver injury, we next sought to determine the role of endogenous IRF2 on hepatic I/R injury. We utilized the IRF2+/- heterozygous KO mice which have lower IRF2 protein levels compared to wild-type B6 mice. Donor liver grafts deficient in IRF2 (+/-) showed a doubling of liver damage after OLTx compared to the wild-type B6 mice, which indicates that the endogenous IRF2 is protective because a relative deficiency results in worse liver damage. To our knowledge, this is the first study showing a beneficial physiologic role for endogenous IRF2 during hepatic I/R injury, and we also show that IRF2 can be used to protect against I/R injury.

In addition to the roles described above, it is also clear that both IRF1 and IRF2 contribute to immune system development. Both IRF1−/− and IRF2−/− mice demonstrate defective differentiation of T helper type 1 cells (18). Interestingly, while IRF2−/− have reduced numbers of natural killer
(NK) cells, IRF1-/- mice do not have reduced numbers of these cells but the NK cells present have decreased function in response to a variety of stimuli (4; 18). While IRF1 and IRF2 clearly have related functions in immune system development, there are differences in the mechanisms which lead to their immune deficient state. One limitation of this study is that we focus our in vitro investigations on the role of IRF2 in hepatocytes and only briefly considered the role in immune cells, specifically macrophages or Kupffer cells, which also contribute to I/R injury. In these cell types, IRF2 signaling is complex and varies according to stimulus. In response to TLR2 activation with lipoteichoic acid, IRF2 appears to be upregulated and lead to activation of IRF1 and secretion of type I IFNs (17). In contrast, in response to LPS, IRF2-/- KO mice had significantly increased TNFα levels and greater numbers of apoptotic Kupffer cells (3). These anti-apoptotic effects of IRF2 appear to signal through STAT1/3 and Caspase-1 (2). We have previously demonstrated the importance of IRF1 in both hepatocytes and non-parenchymal cells in contributing to liver damage during I/R (35). Future investigations may be warranted to more fully elucidate the role of IRF2 in these other cell types, but was outside the limits of this investigation.

Previous reports demonstrated that IRF2 expression can be induced by IFNβ, IFNγ, TNFα, or LPS in some cell types under specific conditions (1; 9; 10), while other reports indicate constitutive expression (11). The expression and induction patterns are not well described for IRF2 in hepatocytes. In this study, IRF2 is expressed at a low baseline level in untreated or sham liver tissue, and induced following liver ischemia, but the expression does not increase further following reperfusion; rather, it is stably induced over this period. It is unknown what leads to the induction of IRF2 during I/R, and this could be due to cytokines, general cellular stress, or oxidative stress among other possibilities. The factors leading to increased IRF2 will be the
subject of further investigation. Furthermore, other pharmacologic methods for induction of
IRF2 may lead to potential therapeutic options in liver I/R injury.
As already mentioned, a limitation of this study is the use of adenovirus for over-expression of
IRF2. While the data clearly indicate a beneficial effect, other viral or non-viral delivery methods
will have to be pursued in the clinical setting. Nonetheless, the current data provide confirmation
that IRF2 functions as an antagonist to IRF1-mediated signaling pathways in this setting.
Importantly, this offers a novel therapeutic strategy for combating hepatic I/R injury by
decreasing IRF1-mediated injury through IRF2 overexpression. Further studies are warranted to
optimize IRF1 blockade and IRF2 delivery.
Acknowledgements

Special thanks to Dr. Tak Mak of the Ontario Cancer Institute for providing IRF2 knock-out mice for the colony. Funded by NIH AI081678, DK62313, GM52021 (DAG), NIH DK071753 (NM), ACS Resident Research Scholarship (JRK), and SUS Ethicon Research Fellowship (RD).
Figure Legends

**Figure 1: IRF2 overexpression does not affect IRF1 levels.** C57B/6 mice (8-12 weeks old) underwent adoptive transfer of IRF2 or control vector LacZ by tail vein injection of varying doses of AdIRF2 or AdLacZ for predetermined time points. (A) Serum ALT levels were determined 36 hours after treatment with AdIRF2 or AdLacZ at doses 1.5 x 10^8 or 7.5 x 10^8 pfu. Levels are expressed as mean +/- SE (n=3 per treatment). (B) Liver nuclear extracts were taken 24, 36, or 48 hours after AdIRF2 or AdLacZ injection at 1.5x10^8 pfu. Western blot analysis on nuclear extracts was performed for IRF2 to determine expression in the liver (n=3 per treatment).

**Figure 2: IRF2 overexpression is protective in warm liverI/R injury.** C57B/6 mice (8-12 weeks old) underwent partial warm liver I/R. (A) Untreated mice were subjected to 1 hour of partial ischemia with predetermined sacrifice timepoints after 0, 1, 3, and 6 hours of reperfusion. Liver nuclear protein extracts were analyzed by Western blot analysis for IRF1 and IRF2 to determine a timecourse of activation. (B) Mice treated with either AdIRF2 or AdLacZ control subjected to 1 hour of partial warm ischemia followed by 6 hours of reperfusion. Serum ALT levels from these mice were determined. Levels are expressed as mean +/- SE (n=3 per treatment). *p<0.05

**Figure 3: IRF2 overexpression results in decreased cytokine and IRF1 target gene production.** C57B/6 mice (8-12 weeks old) underwent partial warm ischemia/reperfusion. Animals were sacrificed at 6 hours of reperfusion and liver tissue sampled. The tissue was processed to recover mRNA and subjected to PCR for (A) IL-12; (B) IFNβ; and (C) iNOS. *p<0.05
**Figure 4: IRF2 overexpression does not alter nuclear IRF1 levels in vitro.** (A) Liver nuclear protein extracts were analyzed by Western blot analysis for expression of IRF1, IRF2, and histones to determine relative expression of these proteins after treatments with AdIRF2 vs. AdLacZ at doses of $1.5 \times 10^8$ pfu. (B) Primary cultured hepatocytes were subjected to gene transfer with $1.5 \times 10^8$ pfu AdIRF2. Cells were incubated for 24 hours prior to nuclear protein extraction. Western blot analysis for IRF1 or IRF2 was performed on nuclear extracts. (C) Primary cultured hepatocytes underwent gene transfer with AdIRF2. After incubation for gene transfer, cells were treated with IFN$\gamma$ (100 Units/mL). Nuclear protein extracts were analyzed for IRF1 and IRF2 by Western blot analysis.

**Figure 5: IRF2 overexpression decreases IRF1 dependent iNOS transcription in vitro.** (A) Primary cultured hepatocytes were pretreated with AdIRF2 or AdLacZ at indicated doses for 24 hours. After incubation period, cells were treated with IFN$\gamma$ (100 Units/mL) for 2 additional hours and whole cell protein was isolated. Western blot analysis of cellular protein was performed for iNOS. (B) Primary cultured hepatocytes were treated with AdIRF1 or AdIRF2 for 24 hours and whole cell protein was isolated. Western blot analysis of cellular protein was performed for iNOS. (C) RAW 264.7 cells were cultured and transfected with AdIRF2 or AdLacZ with MOI 100 for 24 hours. Cells were then cultured in normoxia, hypoxia (1% O$_2$), or normoxia with LPS (100 ng/mL), IFN$\gamma$ (100 Units/mL), or both for an additional 8 hours. Cells were then harvested and whole cell protein isolated. Western blot analysis of cellular protein was performed for iNOS.

**Figure 6: IRF2 heterozygote liver grafts result in increased injury after transplantation.** (A) Wild-type, IRF2 heterozygote, and IRF2 knock-out mice were sacrificed and liver tissue sampled. Nuclear protein extracts were obtained and baseline levels of IRF1 and IRF2 were
determined by Western blot analysis. (B) Serum ALT levels were determined following liver
transplants in wild-type (WT → WT) (n=5) and IRF2 heterozygote (IRF2+/− → WT) (n=3) liver
transplants.


Figure 1

A

![ALT (IU/L) vs Virus (pfu) Graph]

B

<table>
<thead>
<tr>
<th></th>
<th>AdLacZ 1.5 x 10^8</th>
<th>AdIRF2 1.5 x 10^8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 hr</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nuclear IRF-2

Histone
Figure 2

A

<table>
<thead>
<tr>
<th>(+) Control</th>
<th>60 Min Ischemia</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>3 hr</td>
<td>6 hr</td>
</tr>
<tr>
<td>Sham</td>
<td>Reperfusion</td>
<td>ALT (IU/L)</td>
</tr>
</tbody>
</table>

Nuclear IRF-2

Nuclear IRF-1

Histone

B

 ALT (IU/L)

- AdLacZ
- AdIRF2

*
Figure 3

A

![IL-12 mRNA](chart)

B

![IFN-β mRNA](chart)

C

![iNOS mRNA](chart)
Figure 5

A

<table>
<thead>
<tr>
<th>MOI 100</th>
<th>MOI 10</th>
<th>MOI 50</th>
<th>MOI 100</th>
</tr>
</thead>
</table>

- iNOS
- β-actin
- AdIRF2
- AdLacZ
- IFN γ (100U/CC)

B

<table>
<thead>
<tr>
<th>MOI 10</th>
<th>MOI 50</th>
<th>MOI 100</th>
</tr>
</thead>
</table>

- iNOS
- AdIRF1
- AdIRF2

C

<table>
<thead>
<tr>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>LPS</th>
<th>IFNγ</th>
<th>IFN γ + LPS</th>
</tr>
</thead>
</table>

- AdLacZ
- AdIRF2

- iNOS
- β-actin