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IRF-1 promotes liver transplant ischemia/reperfusion injury via hepatocyte IL-15/IL-15R α production

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Abstract

Ischemia and reperfusion (I/R) injury following liver transplantation (LTx) is an important problem that significantly impacts clinical outcomes. Interferon regulatory factor-1 (IRF-1) is a nuclear transcription factor that plays a critical role in liver injury. Our objective was to determine the immunomodulatory role of IRF-1 during I/R injury following allogeneic LTx. IRF-1 was induced in liver grafts immediately after reperfusion in both human and mouse LTx. IRF-1 contributed significantly to I/R injury as IRF-1 KO grafts displayed much less damage assessed by serum alanine aminotransferase (ALT) and histology. In *vitro*, IRF-1 regulated both constitutive and induced expression of IL-15, as well as IL-15R α mRNA expression in murine hepatocytes and liver dendritic cells (DC). Specific knockdown of IRF-1 in human primary hepatocytes gave similar results. In addition, we identified hepatocytes as the major producer of soluble IL-15/IL-15R α complexes in the liver. IRF-1 KO livers had significantly reduced NK, NKT and CD8⁺T cell numbers, while rIL-15/IL-15R α restored these immune cells, augmented cytotoxic effector molecules, promoted systemic inflammatory responses, and exacerbated liver injury in IRF-1 KO graft recipients. These results indicate that IRF-1 promotes LTx I/R injury via hepatocyte IL-15/IL-15R α production and suggest that targeting IRF-1 and IL-15/IL-15R α may be effective in reducing I/R injury associated with LTx.

Introduction

Liver transplantation (LTx) is the established treatment for patients with end-stage liver disease and primary liver cancer. One of the major challenges limiting LTx in the United

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States is the shortage of donor organs (1). While >15,000 patients await LTx each year, <7,000 undergo the procedure according to data collected by Organ Procurement and Transplant Network. This critical organ shortage has led to use of extended criteria organs, such as those from elderly or non-heart beating donors, steatotic livers, or organs with extended cold storage time (2, 3).

Ischemia and reperfusion (I/R) injury remains one of the most understudied areas in organ transplantation, despite its clinical significance (3, 4). I/R injury following prolonged cold storage has a significant impact on early as well as late human liver transplant outcomes. Thus, prolonged cold ischemia time is not only associated with an increased incidence of re-transplantation and primary graft nonfunction during the first 2 weeks post-LTx (5), but also with increased mortality 3 and 12 months post-transplant (6). Whereas extended criteria donor organs are particularly susceptible to I/R injury (1–3), severe preservation injury leads to a higher incidence of rejection and lower survival rates among liver transplant recipients (7). Thus, there is a pressing need to elucidate molecular mechanisms of I/R injury following LTx and to develop effective therapeutic strategies. Prevention of I/R injury is likely to improve the outcome of LTx and potentially expand the limited donor pool by allowing wider use of marginal organs.

Interferon regulatory factor-1 (IRF-1) is a ubiquitous nuclear transcription factor identified originally as a regulator of the human IFN- β gene (8). It is expressed constitutively at low basal levels in various organs, as well as in a variety of immune cell types, including NK cells, T cells (9), macrophages (10) and dendritic cells (DC) (11–13). IRF-1 also regulates the development of certain lymphocyte subsets. Thus, previous reports have shown that IRF-1-deficient mice display severe NK and NKT cell deficiency in the liver, spleen and thymus (14, 15). There is also evidence that CD8⁺T cells are reduced in the thymus, peripheral blood, spleen and lymph nodes of these mice (16, 17). Some of these studies suggest that lack of inducible IL-15, especially in bone marrow (BM) stromal cells, is responsible for the deficiency of NK cells in IRF-1-deficient mice (14, 18, 19).

IL-15 is a pleiotropic cytokine in the common cytokine receptor γ_c -chain (γ_c) family that functions in the homeostasis and activation of innate and adaptive immune cells (19–21). The IL-15 gene is transcriptionally regulated by IRF-1 (8) and IRF-1 regulates the induction of IL-15 mRNA in BM cells in response to stimulation with LPS and IFN- γ *in vitro* (14, 15). IL-15R α is a receptor subunit with which IL-15 forms a heterodimer complex (22). This IL-15/IL-15R α complex binds to cells that express another heterodimeric receptor complex comprised of IL-2/IL-15R β and γ_c through which it signals (20). A recent study (22) indicates that this soluble IL-15/IL-15R α complex is the only form of circulating soluble IL-15 in mouse and human serum. IL-15R α also has the IRF responsive element sequence in its promoter region and overexpression of IRF-1 protein in COS-7 cells (monkey kidney fibroblast-like cells) can activate the IL-15R α promoter (23). However, it remains unclear whether IRF-1 regulates IL-15 and IL-15R α expression in hepatic immune and non-immune cells in the steady-state or under inflammatory conditions (24).

We have shown previously (25–29) that IRF-1 plays a critical role in various liver injury models. This includes hepatic warm I/R injury (25, 26), cold I/R injury during isograft LTx

(27, 28), and immune-mediated liver injury (29). However, the precise mechanism by which IRF-1 regulates hepatic lymphocyte populations, and the role of these hepatic lymphocytes in mediating liver injury is poorly understood (30). Moreover, the role of IRF-1 in allogeneic LTx has not been defined. Thus, we examined the role of IRF-1 in hepatic lymphocyte homeostasis in the steady-state and in the pathogenesis of cold I/R injury using a clinically-relevant, mouse orthotopic allogeneic LTx model. Our novel findings suggest that IRF-1 regulates hepatic NK cell, NKT cell and CD8⁺T cell homeostasis via hepatocyte and DC production of IL-15/IL-15R α complexes to promote proinflammatory cytokine production and the up-regulation of cytotoxic lymphocyte granules. These events contribute significantly to allograft liver I/R injury with prolonged cold ischemia time following allogeneic LTx.

Materials and Methods

IRF-1 staining of human liver allografts

Analysis of human liver allograft tissue (PRO10110393) and isolation of human primary hepatocytes (PRO012100076 and PRO08010372) were conducted under University of Pittsburgh Institutional Review Board protocols. Written informed consent was received from participants prior to inclusion in the study. Formalin-fixed, paraffin-embedded human liver allograft biopsy sections were obtained from 4 patients at two different time points (backtable and post-reperfusion (1–4 h)). 4 μ m sections were deparaffinized, hydrated, and treated with citrated buffer for antigen retrieval. Sections were then blocked with avidin and biotin block kit (Vector Laboratories, Inc., Burlingame, CA). Staining was performed by sequential incubation cycles of rabbit anti-IRF-1 primary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX), goat anti-rabbit biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), ABC kit (Vector Laboratories, Inc.), and AEC Substrate kit (ScyTek Laboratories, Inc., Logan, UT). Sections were then counterstained with aqueous hematoxylin. Digital images of whole staining slides were obtained with MIRAX MIDI digital whole slide scanning system (Carl Zeiss Microimaging, Jena, Germany) and analyzed with Panoramic Viewer (3D Histech, Ltd, Ramsey, NJ).

Animals

Male 8- to 12-wk old wild-type C57BL/6 mice (IRF-1^{+/+}; WT B6, H-2^b), IRF-1 deficient mice (IRF-1^{-/-}; IRF-1 KO, B6 background, H-2^b), IL-15R α deficient mice (IL-15R α ^{-/-}; IL-15R α KO, B6 background, H-2^b) and C3H/HeJ (H-2^k) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). They were maintained in the specific pathogen-free animal facility at the University of Pittsburgh School of Medicine. All animal studies were approved by University of Pittsburgh IACUC protocol # 14023395; PHS assurance #A3187-01 and in accordance with criteria outlined in Guide for the Care and Use of Laboratory Animals, a publication of the National Institutes of Health.

Orthotopic liver transplantation (LTx)

Liver harvesting and orthotopic liver transplantation without hepatic artery reconstruction were performed as described (31, 32), with minor modifications. WT B6 or IRF-1 KO mice were used as liver donors. The grafts were perfused with 5ml University of Wisconsin (UW)

solution via the inferior vena cava, stored for 24 h at 4°C, and then implanted orthotopically into C3H/HeJ recipients. The transplant recipients were euthanized 3 and 6 h after reperfusion to obtain blood and liver tissue.

Isolation of hepatocytes and non-parenchymal cells (NPC)

Hepatocytes and NPC were isolated from normal liver and liver grafts using the collagenase digestion method, as described (28, 33). The initial cell suspension was filtered using a cell strainer (70 µm, BD Bioscience, San Jose, CA) and hepatocytes and NPC separated by low speed centrifugation (x5; 45 g for 5 min).

Real-time RT-PCR

Total RNA was isolated using RNeasy Mini Kits (Qiagen Inc., Valencia, CA) and reverse transcribed into cDNA using RNA to cDNA EcoDry™ Premix (Double Primed) (Clontech Laboratories, Inc., Mountain View, CA). mRNA expression was quantified, as described (33), by SYBR Green RT-PCR with StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Expression of each gene was normalized to β-actin mRNA or GAPDH mRNA for mouse and human studies respectively and calculated with respect to normal liver tissue. Primer sequences are provided in Supplemental Table 1.

Confocal immunofluorescence imaging

Immunofluorescence staining and imaging of IRF-1 in mouse liver tissue was performed as described (26). Primary antibody was rabbit anti-IRF-1 (Santa Cruz Biotechnology, Inc.). A secondary antibody (goat anti-rabbit Cy3; Jackson ImmunoResearch Laboratories, Inc.) was applied with F-actin counterstain (Alexa Fluor 488 Phalloidin; Life Technologies, Grand Island, NY).

Circulating IL-15/IL-15Rα levels

IL-15/IL-15Rα levels in serum or culture supernatants were measured by mouse IL-15/IL-15R ELISA (eBioscience, Inc., San Diego, CA).

In vitro culture assays

Different liver cell populations (hepatocytes, bulk liver NPC [NPC], liver NPC depleted of CD11c⁺ DC [NPC-CD11c] and CD11c⁺ DC alone [CD11c]) were isolated from WT or IRF-1 KO mice and equal numbers of cells (5×10⁶ cells/ml) incubated at 37 °C for 6 or 24 h in media alone or with LPS (30µg/ml) and IFN-γ (100U/ml). CD11c⁺ DC were purified as described (32, 34).

Soluble IL-15/IL-15Rα complex preparation and in vivo administration

Human rIL-15 and r mouse IL-15Rα Fc were purchased from R&D Systems (Minneapolis, Minnesota, USA) and IL-15/IL-15Rα complexes prepared as described (35, 36). To assess the function of soluble IL-15/IL-15Rα in vivo, IRF-1 KO mice were given 2.5 µg IL-15 and 15 µg IL-15Rα Fc in 200µl PBS i.p. 4 days before cell isolation for flow cytometry or donor liver harvest for LTx.

Flow cytometry

Mouse cell surface molecule and intracellular IFN- γ and FoxP3 staining was performed as described (32). Liver NPC were treated with Fc γ R-blocking rat anti-mouse CD16/32 mAb (2.4G2) to prevent non-specific Ab binding. For cell surface staining, the NPC were incubated for 30 min with FITC-, phycoerythrin (PE)-, allophycocyanin (APC) -, Pacific Blue-, or PE-cyanin (Cy)7-conjugated mAbs to detect expression of CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11c (HL3), CD45 (30-F11), H2-K^b (AF6-88.5), NK1.1 (PK136) (BD Biosciences, San Diego, CA), CD19 (eBio 1D3, eBioscience, San Diego, CA) and F4/80 (BM8, BioLegend, San Diego, CA). For intracellular cytokine staining, cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% saponin, then stained with anti-mouse IFN- γ Ab (XMG1.2) (BioLegend). For Foxp3 staining, cells were fixed and permeabilized using Foxp3 Fix Permkits (eBioscience) and stained with anti-Foxp3 mAb (FJK-16s) (eBioscience). Appropriate Ig isotype controls were obtained from BD Pharmingen (San Diego, CA). Flow cytometry was performed using a LSR Fortessa flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (version 7.6; TreeStar, Inc., Ashland, OR).

Assessment of liver injury

The extent of hepatic injury after I/R was determined by measuring serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels 3 and 6 h after reperfusion, as described (33). Liver tissue was obtained from graft recipients immediately after euthanasia, fixed in 10% formalin, embedded in paraffin, then sectioned and stained with H&E and TUNEL as described (28). Liver injury was assessed by Suzuki scores (37) in a “blinded” fashion.

Cytokine measurements

Serum cytokine levels (IFN- γ , IL-6, and TNF α) were measured by cytometric bead array (BD Bioscience).

MTT assay

Hepatocytes (WT B6 or IRF-1 KO) were cultured with or without IFN- γ (1000 U/ml) for 24 h when their viability was assessed by MTT assay (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s instructions.

Human primary hepatocyte culture

Human hepatocytes were isolated from histologically normal, surgically-resected liver tissue, as described (32) using a three-step collagenase perfusion technique and density-gradient centrifugation. They were cultured in media containing LPS (30 μ g/ml) and IFN- γ (250U/ml) and harvested 0, 1, 3, 6, or 24 h after stimulation for RNA isolation.

Hepatocyte transfection using adenovirus vector

A replication-deficient adenoviral vector (human adenovirus type 5, dE1/E3) containing the IRF-1 microRNA hairpin structure (Ad-IRF-1-shRNA) (Vector Biolabs, Philadelphia, PA) was prepared as described (29). The IRF-1 microRNA hairpin sequence is:

TGCTGTTGACAGTGAGCGACCTGGCTAGAGATGCAGATTATAGTGAAGCCACAG AT GTATAATCTGCATCTCTAGCCAGGGTGCCTACTGCCTCGGA. The target sequences are: *Homo sapiens* IRF-1 mRNA (NM_002198.1) 3'-untranslated region (UTR) 227–245; *Mus musculus* IRF-1 mRNA (NM_008390.1) 3'-UTR 235–253; *Rattus norvegicus* IRF-1 mRNA (NM_012591.1) 3'-UTR 227–245. Human primary hepatocytes were transfected with Ad-IRF-1-shRNA or control vector (Ad-SCR-ShRNA) for 48 h. The cells were then stimulated with LPS (30µg/ml) and IFN-γ (250U/ml) for 6 h before harvesting for RNA isolation and functional analysis.

Statistical analysis

Data are presented as means ± 1 SEM. Comparisons between groups were performed by using Student's 't' test. Non-parametric data (Suzuki score) were analyzed with the Mann-Whitney *U*-test. A log rank test was performed to assess differences in graft survival. A probability value of $p < 0.05$ was considered significant.

Results

IRF-1 is up-regulated in human LTx tissue after reperfusion

To determine if IRF-1 expression is up-regulated in human liver allografts after reperfusion, we performed IRF-1 immunohistochemistry on liver tissue specimens obtained from 4 human LTx patients (cold ischemia time range 8 to 16 h) at two time points, before perfusion (backtable) and immediately after reperfusion (post-perfusion). The liver pathologist grading the IRF-1 expression was blinded to the groups. As shown in Figure 1A, IRF-1 was strongly induced and mainly localized to nuclei of hepatocytes in samples obtained post-perfusion, whereas the expression was minimal to negligible in backtable samples. The number of IRF-1 positive hepatocytes increased significantly post-perfusion compared to backtable (Figure 1B).

IRF-1 is up-regulated in both hepatocytes and non-parenchymal cells (NPC) after allogeneic LTx with extended cold storage time

To determine the level of IRF-1 expression in liver grafts after mouse allogeneic (WT B6→C3H) LTx with extended cold storage (24 h preservation), we first performed RT-PCR of whole liver tissue collected 6 h after reperfusion. As we have shown previously with mouse syngeneic LTx (28), IRF-1 mRNA was markedly and significantly up-regulated (8-fold) compared with normal liver tissue (Figure 2A). Both hepatocytes and NPC isolated from the grafts 6 h after reperfusion exhibited marked elevations in IRF-1 expression. Hepatocytes showed significantly greater induction of IRF-1 mRNA (18-fold) than NPC (7-fold) (Figure 2B). Immunofluorescence staining of liver grafts 6 h after reperfusion confirmed that IRF-1 protein expression was significantly induced following LTx compared to normal liver and localized mainly to hepatocyte nuclei (Figure 2C). These data show that both IRF-1 mRNA and protein are up-regulated significantly after allogeneic LTx and that IRF-1 is expressed by both hepatocytes and NPC, with greater induction in hepatocytes.

IL-15 and IL-15R α expression is up-regulated in an IRF-1-dependent manner in both hepatocytes and NPC during LTx cold I/R injury

Although IRF-1 is known to induce the expression of both IL-15 and IL-15R α by BM cells or COS-7 cells *in vitro* (14, 15, (23)), it is unclear whether it induces IL-15 and/or IL-15R α in the liver during cold I/R injury associated with allogeneic LTx. We observed that both IL-15 and IL-15R α mRNA expression were induced significantly in hepatocytes 6 h after reperfusion in WT B6 \rightarrow C3H LTx compared to normal hepatocytes; IL-15 mRNA was up-regulated >15-fold and IL-15R α mRNA increased >50-fold compared to normal WT hepatocytes (Figure 3A). Hepatic NPC obtained from WT B6 \rightarrow C3H allografts also displayed significantly up-regulated IL-15 and IL-15R α mRNA expression after reperfusion compared to normal WT NPC (Figure 3A). However, the degree of IL-15 and IL-15R α mRNA induction was significantly greater in hepatocytes compared to NPC. We then used IRF-1 KO donor liver allografts to determine if IRF-1 was involved in promotion of IL-15 and IL-15R α expression in the liver after LTx. Expression of both IL-15 and IL-15R α mRNA was reduced significantly in hepatocytes and NPC from IRF-1 KO \rightarrow C3H compared to WT \rightarrow C3H grafts (Figure 3A). These data suggest that IRF-1 indeed regulates the induction of both IL-15 and IL-15R α mRNA in hepatocytes, as well as in liver NPC after LTx. We next measured circulating IL-15/IL-15R α protein complexes in sera collected from graft recipients 6 h after reperfusion. Levels were elevated significantly in WT \rightarrow C3H recipients compared to normal C3H mice (Figure 3B), while IRF-1 KO \rightarrow C3H recipients showed significantly reduced levels of circulating IL-15/IL-15R α complexes compared to WT \rightarrow C3H recipients. These data indicate that donor-expressed IRF-1 regulates not only IL-15 and IL-15R α mRNA expression in the liver, but also circulating IL-15/IL-15R α complexes in host serum 6 h after LTx.

Hepatocytes are the major source of soluble IL-15/IL-15R α complexes among liver cell populations

There is evidence that CD11c⁺ DC are important producers of circulating IL-15 in endotoxin shock (38) and CpG-induced immune activation (39), based on studies in CD11c-diphtheria toxin receptor transgenic (DTR tg) mice. Other reports indicate that liver parenchymal cells can express IL-15 and IL-15R α (40) and that they are equally responsible as hematopoietic cells in promoting hepatic NK and NKT cell development in the steady-state (41). Although previous studies have shown IL-15 production by human whole liver tissue (42) or human hepatocellular carcinoma cells (43), it remains unclear which cell population in the liver is the principal producer of soluble IL-15/IL-15R α complexes. Based on our findings and earlier published observations (38–43), we hypothesized that both liver DC and hepatocytes might be the main producers of secreted IL-15/IL-15R α complexes in the liver. To verify our hypothesis, we isolated different liver cell subsets and cultured these cells (5×10^6 cells/ml) with or without LPS and IFN- γ stimulation for 24 h. As shown in Figure 4, hepatocytes secreted significantly higher amounts of soluble IL-15/IL-15R α complexes compared to bulk liver NPC, liver NPC depleted of DC (NPC-CD11c⁺), or DC (CD11c⁺) alone. Among liver NPC, DC constituted one of main producers of IL-15/IL-15R α complexes, since secreted IL-15/IL-15R α complexes decreased significantly when DC were removed from NPC ($38 \pm 6\%$ decrease in media alone group and $57 \pm 6\%$ decrease with

LPS and IFN- γ group). Interestingly, the degree of soluble IL-15/IL-15R α production induced varied among different liver cell populations. Thus, in response to LPS and IFN- γ , induction was most pronounced in DC (3.7-fold), compared to hepatocytes (1.2-fold), bulk NPC (1.5-fold), or NPC-CD11c (1.0-fold) (Figure 4).

IRF-1 regulates basal and induced expression of IL-15 and IL-15R α mRNA and IL-15/IL-15R α secretion by mouse primary hepatocytes and liver DC

Although previous studies (14, 23) have indicated that IL-15 and IL-15R α gene sequences both contain IRF-1 binding elements, it remains unclear whether IRF-1 regulates IL-15 and/or IL-15R α expression equally in liver hematopoietic and parenchymal cells under steady-state and inflammatory conditions (24). Thus, we isolated primary hepatocytes and DC from mouse liver to determine whether IRF-1 could transcriptionally regulate IL-15 and IL-15R α expression, as well as IL-15/IL-15R α complex secretion by these cell populations. First, liver DC isolated from WT B6 or IRF-1 KO mice were cultured with or without IFN- γ and LPS for 6 h to determine their constitutive and induced IL-15 and IL-15R α mRNA expression. As shown in Figure 5A, WT liver CD11c⁺ DC up-regulated both IL-15 and IL-15R α mRNA markedly and (~7-fold) in response to stimulation with IFN- γ and LPS. The magnitude of basal and stimulated IL-15/IL-15R α mRNA expression was diminished in liver DC lacking IRF-1. We then cultured WT or IRF-1 KO liver DC, with or without IFN- γ and LPS stimulation for 24 h, and measured secreted IL-15/IL-15R α complexes in the culture supernatants. WT liver DC secreted significantly higher levels of IL-15/IL-15R α complexes compared with IRF-1 KO liver DC, with or without IFN- γ and LPS stimulation (Figure 5B). As shown in Figure 5C and 5D, similar findings were seen using mouse primary hepatocytes. These results indicate that IRF-1 transcriptionally regulates not only constitutive expression but also induced expression of IL-15 and IL-15R α in both DC and primary hepatocytes isolated from mouse liver.

IRF-1-deficient mice lack hepatic NK, NKT and CD8⁺T cells in the steady-state, while systemic administration of soluble IL-15/IL-15R α complexes restores these hepatic lymphocyte populations

Although previous studies (14, 15) have shown that IRF-1 KO mice are deficient in hepatic NK and NKT cells, it is not clear whether other hepatic lymphocyte populations, such as CD8⁺T cells or CD4⁺CD25⁺ regulatory T cells (Treg), that are known to be regulated by IRF-1 in other organs (17, 44), are also affected in the liver. It is also unknown whether deficiency of certain hepatic lymphocytes in IRF-1 KO mice can be restored by systemic administration of IL-15/IL-15R α complexes. To answer these questions, we determined the absolute numbers of hepatic lymphocyte subsets isolated from normal WT B6, untreated IRF-1 KO, and IRF-1 KO mice given IL-15/IL-15R α complexes systemically. When compared to WT livers, IRF-1 KO livers showed reduced absolute numbers of total liver NPC (Figure 6A). There were no significant differences in the absolute numbers of macrophages, DC, B cells or Treg between livers of WT and IRF-1 KO mice (data not shown) whereas absolute numbers of NK, NKT, and CD8⁺T cells were all reduced significantly in IRF-1 KO compared to WT livers (Figure 6B, 6C). There were no differences in the absolute numbers of CD4⁺ T cells between WT and IRF-1 KO livers (Figure 6C). IL-15/IL-15R α complex administration increased the total number of liver

NPC significantly in IRF-1 KO mice. This number was similar to the total liver NPC number in WT mice (Figure 6A). As shown in Figure 6C, soluble IL-15/IL-15R α complex administration increased the absolute numbers of NK, NKT and CD8⁺T cells, but not CD4⁺T cells in IRF-1 KO mice. These data suggest that the deficiency of NK, NKT, and CD8⁺T cells in the livers of IRF-1 KO mice is due to reduced IL-15/IL-15R α expression and that systemic administration of IL-15/IL-15R α complexes can restore these hepatic lymphocytes in IRF-1 KO mice *in vivo*.

IRF-1 KO liver allografts are protected against cold I/R injury, whereas pretreatment with soluble IL-15/IL-15R α complexes partially reverses the protective effect

Previous studies have shown that liver-resident lymphocytes, including NK (45), NKT (46), and CD4⁺T cells (47) play important roles in the pathogenesis of hepatic partial warm I/R injury (3). Although partial warm I/R injury and cold I/R injury following LTx may share common mechanism(s) (3), these do not always correlate between the two conditions (48, 49). Few studies have investigated the role of donor-derived lymphocytes during cold I/R injury after LTx (33, 50), and it remains uncertain how liver-resident NK, NKT and CD8⁺T cells regulated by IRF-1 via IL-15/IL-15R α complexes might impact the outcome of LTx-induced cold I/R injury. Thus, we performed allogeneic LTx following 24 h extended cold storage using donor livers from WT B6, IRF-1 KO or IRF-1 KO mice pretreated with soluble IL-15/IL-15R α complexes and C3H recipients. As shown in Figure 7A, IRF-1 KO grafts were markedly protected compared to WT grafts after 6 h reperfusion, as evidenced by significantly reduced levels of serum ALT and AST. Histological analysis confirmed significantly lower Suzuki scores for congestion and necrosis in IRF-1 KO compared to WT grafts (Fig 7B, 7C). However, when IRF-1 KO liver grafts pretreated with soluble IL-15/IL-15R α complexes were transplanted into C3H recipients, protection was reversed, as demonstrated by significantly elevated serum ALT and AST levels and worse liver injury and Suzuki scores compared to IRF-1 KO grafts (Figure 7A, 7B, 7C).

Survival of IRF-1 KO donor liver allografts is prolonged but this effect is reversed by soluble IL-15/IL-15R α complexes

We further tested liver graft survival to determine whether IRF-1 deficiency in the allograft could prolong host survival in our extended cold storage model. Transplantation of WT grafts without extended cold storage resulted in 100% survival, while WT grafts with 24 h extended cold storage did not survive beyond 24 h (Fig 7D). In contrast, recipients of IRF-1 KO grafts showed significantly improved survival compared to WT grafts. However, when IRF-1 KO grafts were pretreated with soluble IL-15/IL-15R α complexes, the recipients had markedly shortened survival.

IL-15R α KO liver allografts are protected against cold I/R injury

We also compared liver injury between recipients of WT and IL-15R α KO allografts to test whether the effects observed in IRF-1 KO mice could be reproduced with allografts deficient in IL-15R α . Compared to recipients that received WT grafts, those that received IL-15R α KO liver grafts were significantly protected 6 h after reperfusion with significantly

lower ALT and AST levels (ALT $30,827 \pm 1,988$ vs. $18,967 \pm 1,810$ IU/L, AST $13,067 \pm 965$ vs. $8,353 \pm 392$ IU/L, respectively; $n = 3$ transplant/group, $p < 0.05$)

IRF-1 KO liver allografts exhibit diminished donor-derived NK1.1⁺ cells, cytotoxic effector molecules, and pro-inflammatory cytokines that are restored by pretreatment with soluble IL-15/IL-15R α complexes

Flow cytometric analysis of intra-graft mononuclear cells 6 h after reperfusion showed markedly decreased NK and NKT cell numbers in IRF-1 KO compared to WT grafts (Figure 8A). By contrast, no significant differences in absolute numbers of macrophages, DC, or CD4⁺T cells were observed. There was also no significant difference in the absolute numbers of CD8⁺T cells between WT and IRF-1 KO grafts, despite the significant difference observed in non-transplanted livers (Figure 6C). We hypothesized that this discrepancy might be due to differences in population dynamics between hepatic NK1.1⁺ and CD8⁺ cells. Thus, we next determined the proportions of these donor- and recipient-derived lymphocyte populations. As shown in Figure 8B, 6 h after reperfusion, the vast majority (approximately 95%) of graft NK 1.1⁺ cells were donor-derived (H2^{b+}), whereas CD8⁺T cells were predominantly recipient-derived graft-infiltrating cells (H2^{b-}).

We then determined IFN- γ ⁺ populations among NK cells and NKT cells and found that IRF-1 KO grafts had significantly reduced incidences of these cells (Figure 8C). Notably, donor pretreatment with soluble IL-15/IL-15R α complexes restored both the absolute number of NK 1.1⁺ cells and their IFN- γ positivity. Consistent with the reduction in absolute numbers of NK and NKT cells in IRF-1 KO livers 6 h after reperfusion, gene transcripts for cytotoxic lymphocyte-activating receptor NKG2D, IFN- γ and cytotoxic mediators (granzyme B, perforin) were all reduced significantly in IRF-1 KO grafts compared with WT grafts (Figure 9A). However, donor pretreatment with soluble IL-15/IL-15R α complexes significantly reversed expression of each of these cytotoxic effector molecules in IRF-1 KO grafts.

As shown in Figure 9B, circulating levels of proinflammatory cytokines (IFN- γ , IL-6, and TNF α) in serum were also reduced substantially in recipients of IRF-1 KO grafts compared with those given WT livers. However, donor pretreatment with IL-15/IL-15R α complexes fully (IFN- γ , TNF α) or partially restored (IL-6) serum cytokine levels in recipients of IRF-1 KO grafts.

Pretreatment of IRF-1 KO grafts with IL-15/IL-15R α complexes partially reverses hepatocyte cell death

We next examined whether the increase in cytotoxic effector molecules and proinflammatory cytokines observed with soluble IL-15/IL-15R α complexes correlated with the extent of hepatocyte cell death assessed by TUNEL staining. As shown in Figure 10A and 10B, IRF-1 KO grafts exhibited significantly reduced incidences of TUNEL⁺ hepatocytes compared with WT grafts, while treatment of IRF-1KO donors with IL-15/IL-15R α complexes partially reversed the effect. This partial reversal of TUNEL⁺ hepatocytes in grafts from IRF-1 KO donors given IL-15/IL-15R α complexes was consistent with the differences in liver injury between the groups, as determined by serum

transaminase levels (Figure 7A). We speculated that this partial rather than full restoration of hepatocyte cell death and liver injury might reflect IRF-1 KO hepatocyte resistance to cell death. Thus, IRF-1 is a crucial mediator of apoptosis (28) and IRF-1 KO primary hepatocytes are resistant to apoptosis induced by IFN- α (51) or IFN- γ (52). To confirm whether IRF-1 KO primary hepatocytes were resistant to cell death induced by IFN- γ , we examined the viability of primary hepatocytes from WT or IRF-1 KO mice exposed to IFN- γ *in vitro*. After incubation with IFN- γ for 24 h, the viability of WT hepatocytes decreased substantially (~80%), whereas IRF-1 KO hepatocytes remained viable and demonstrated resistance to IFN- γ -induced cell death (Figure 10C).

Human primary hepatocytes express IRF-1, IL-15 and IL-15R α and IRF-1 gene silencing reduces the expression of both IL-15 and IL-15R α

We next assessed the expression of IRF-1, IL-15, and IL-15R α in human primary hepatocytes isolated from histologically normal, surgically-resected tissue. In response to LPS and IFN- γ stimulation, IRF-1 mRNA expression was up-regulated within 1 h and peaked at 3 h (Figure 11A). These data are consistent with our earlier observations (25, 28) using mouse or rat primary hepatocytes. Induction of IL-15 and IL-15R α occurred within 3 h of stimulation and peaked 6 h after stimulation (Figure 11B, 11C). To determine whether IRF-1 directly regulated IL-15 and IL-15R α expression in human primary hepatocytes, we used an adenoviral (Ad) vector containing a microRNA designed to specifically knock down IRF-1 expression (Ad-IRF1-shRNA) (29). Gene delivery efficiency was determined by GFP expression of infected hepatocytes and was >90% (Figure 11D). The Ad-IRF1-shRNA vector effectively knocked down both basal (data not shown) and LPS- and IFN- γ -induced IRF-1 expression (Figure 11E) in human primary hepatocytes compared to an Ad vector containing a scrambled (SCR) shRNA (Ad-SCR-ShRNA). Gene-silencing of IRF-1 also significantly diminished both basal (data not shown) and induced expression of IL-15 and IL-15R α (Figure 11F, 11G), thus confirming an important role for IRF-1 in regulating human hepatocyte IL-15 and IL-15R α expression.

Discussion

The IRF family of transcription factors consists of nine members in mammals and plays a central role in the regulation of hematopoietic cell development, innate and adaptive immune responses, and oncogenesis (53). IRF-1, the first member of this family to be identified, is expressed constitutively at low levels in a variety of cell types, including both parenchymal and non-parenchymal cells. It is strongly induced by type I and II IFN, LPS, viral infection, double-stranded RNA and by various cytokines, such as TNF α , IL-1 β and IL-6 (12, 54). IRF-1 exhibits remarkably diverse functions as it targets a variety of genes including iNOS, IL-12p40, and caspase 1 that are involved in anti-bacterial responses, Th1-type immunity and the induction of apoptosis (8). We have shown previously (27, 28) that IRF-1 plays a central role in promoting hepatocyte apoptosis following cold I/R injury in syngeneic mouse or rat orthotopic liver transplantation. However, the immunomodulatory role of hepatic IRF-1 in the steady-state or during cold I/R injury following allogeneic LTx is not well understood. Here, we demonstrate that IRF-1 KO donor allografts are markedly protected (approximately 90%) from cold I/R injury compared to WT allografts. Our novel

findings indicate that this protective effect is due in large part to reduced expression of IL-15/IL-15R α complexes in the IRF-1 KO grafts and consequent, diminished innate immune responses and hepatic lymphocyte insufficiency during cold I/R injury.

The liver is a unique organ that receives blood supply containing food and microbial products from the intestine via the portal vein (55–57). Its immune cell constituency is enriched in NK cells, NKT cells and CD8⁺T cells compared to other parenchymal organs (58–60) and previous studies have suggested that the hepatic microenvironment can support the maintenance of a unique lymphocyte repertoire (42, 57, 58). However, the exact mechanism of hepatic lymphocyte homeostasis is not fully understood. To our knowledge, no previous studies have examined the relative contribution of different liver cell populations to the production of IL-15/IL-15R α complexes (24). Thus, we examined both parenchymal and non-parenchymal cells as potential sources of soluble IL-15/IL-15R α complexes within the liver. Our study provides evidence, for the first time, that hepatocytes are the major producers of soluble IL-15/IL-15R α complexes in both the steady-state (without stimulation) and under inflammatory conditions *in vitro* (IFN- γ and LPS stimulation). We also found that IRF-1 transcriptionally regulates not only the induced expression, but also the constitutive expression of both IL-15 and IL-15R α mRNA in hepatocytes, as well as in liver DC. Additionally, we have confirmed that IRF-1 is important for basal and induced production of IL-15/IL-15R α complex protein by both hepatocytes and liver DC. Importantly, using human primary hepatocyte cultures we have confirmed that these findings in mice also apply to human cells. Specific knockdown of IRF-1 allowed us to demonstrate that IRF-1 controls both basal and induced IL-15 and IL-15R α mRNA expression in human primary hepatocytes.

These findings provide new insights into our understanding of IL-15 biology. Based on BM chimera experiments, a traditional view has been that IRF-1 expression by radiation-resistant BM stromal cells is important for the induction of IL-15 expression and subsequent maturation of NK cell precursors in the BM (8, 12, 14, 18, 19). However, these studies did not exclude the possibility that other radio-resistant parenchymal cells, such as hepatocytes, might also play an important role in development of lymphocytes within an organ such as the liver. Evidence has accumulated that parenchymal cells, including hepatocytes, can express both IL-15 and IL-15R α protein (40) and thus provide a microenvironment favorable to T cell survival and CD8⁺T differentiation (61). Indeed, we found that hepatocytes could secrete significantly higher amounts of IL-15/IL-15R α complex protein than various immune cells, including DC, a commonly-studied source of IL-15 (38, 39). Interestingly, our finding that hepatocytes are the major source of soluble IL-15/IL-15R α complexes correlates well with a report (62) that identified hepatocytes as a new source of IL-7, another member of the common cytokine-receptor γ_c family, that prolongs the survival of naïve and memory CD4⁺ and CD8⁺ T cells. Our findings are also consistent with those of a recent study (63) showing that IRF-1 regulates the induced expression of IL-15R α by Huh7 (human hepatocellular carcinoma cell line) cells and that IRF-2, another member of the IRF family, controls basal expression of both IL-7 and IL-15R α . The latter study shows that IRF-2 is significantly down-regulated in the liver in hepatitis C virus (HCV) infection and subsequently impaired expression of IL-7 and IL-15R α in hepatocytes may explain poor

HCV-specific CD8⁺T cell responses, inability to eliminate HCV and perpetuation of infection (63).

Considering that hepatocytes constitute the majority (60–80%) of the total cell population in the liver (57), the present findings and these earlier reports together suggest that IRF-1 expression by hepatocytes may contribute significantly to the development and homeostasis of NK, NKT, and CD8⁺T cells in the liver through soluble IL-15/IL-15R α complex production. Notably, lymphocytes isolated from livers of normal IRF-1 KO mice showed significantly reduced numbers of NK, NKT, and CD8⁺ T cells compared to those from livers of WT B6 mice. When IRF-1 KO mice were treated with soluble IL-15/IL-15R α complexes *in vivo*, absolute numbers of hepatic NK, NKT, and CD8⁺ T cells became similar to those in WT B6 mice. These results suggest that the reduced cell numbers in IRF-1 KO livers are due to diminished expression of IL-15/IL-15R α complexes and that IRF-1 KO hematopoietic cells retain the ability to develop and proliferate in response to exogenous soluble IL-15/IL-15R α complexes. Our results are consistent with similar *in vitro* observations made using IRF-1 KO BM cells and rIL-15 (14). They do not, however, delineate the relative contributions of BM stromal cells and liver parenchymal cells to the development of NK, NKT, and CD8⁺T cells in the liver. Generation of a mouse strain with hepatocyte-specific deletion of IRF-1 may be useful in addressing this issue.

Our *in vivo* results also show that IL-15 and IL-15R α mRNA is up-regulated in liver allograft hepatocytes and NPC in an IRF-1-dependent manner during I/R injury following extended cold storage. IL-15 and IL-15R α expression was significantly higher in hepatocytes than NPC, suggesting that hepatocytes may also be the major producers of IL-15 and IL-15R α *in vivo*. Circulating IL-15/IL-15R α complex levels were increased significantly in WT but not IRF-1 KO allograft recipients 6 h post reperfusion. These results indicate that the predominant source of circulating IL-15/IL-15R α complexes is the allograft, rather than host-derived cells. Normal IL-15/IL-15R α complex levels in IRF-1 KO liver recipients were associated with significantly improved survival, reduced liver injury, less TUNEL⁺ hepatocytes, decreased numbers of graft NK and NKT cells, and reduced expression of cytotoxic effector molecules (NKG2D, granzyme B, perforin), and pro-inflammatory cytokines (IFN- γ , IL-6, TNF α). When IRF-1 KO livers were exposed to soluble IL-15/IL-15R α complexes, cytotoxic molecules and proinflammatory cytokines were increased significantly and liver injury and hepatocyte cell death partially restored and recipient survival shortened. These findings indicate that exogenous IL-15/IL-15R α complexes can promote the development and proliferation of immune cells in the liver and restore innate immune responses in IRF-1 KO liver recipients, which in turn, affects the viability of hepatocytes during liver transplant I/R injury. These findings from *in vivo* experiments are in accord with a previous report that showed proinflammatory effects of exogenous IL-15 in the liver; daily administration of rIL-15 for 7 days to healthy, WT mice significantly increased hepatic expression of proinflammatory cytokines (IFN- γ and TNF α), the number of inflammatory foci, and caspase-3 positive cells in the liver (40). We further tested whether the proinflammatory effects of soluble IL-15/IL-15R α complexes in IRF-1 KO could be reproduced in IL-15R α KO allografts. Recipients of IL-15R α KO grafts were significantly protected compared to those that received WT transplants, as indicated by

significantly lower ALT and AST levels. These results suggest that deficiency of IL-15R α in donor liver grafts is indeed protective and supports the effects observed in IRF-1 KO mice. These findings from our study are also consistent with previous reports that IL-15 promotes the survival (64, 65) and activation of NK1.1⁺ cells (66, 67) and CD8⁺T cells (68) and up-regulates NKG2D(69), granzyme B, perforin (70) and IFN- γ (36, 71, 72) in various models of inflammation and infection. We hypothesized that the partial restoration of liver injury and hepatocyte cell death observed in recipients of IRF-1 KO livers treated with IL-15/IL-15R α complexes was due to the resistance of IRF-1 KO hepatocytes to apoptosis, as described (28, 51, 52). Indeed, we found that IRF-1 KO hepatocytes were more resistance to cell death in response to IFN- γ compared to WT hepatocytes. These results indicate that IRF-1 regulates innate immune responses via soluble IL-15/IL-15R α complexes and plays a significant role in the pathogenesis of LTx cold I/R injury.

In conclusion, our study provides new evidence that IRF-1 regulates the homeostasis of NK, NKT and CD8⁺T cells in the liver through IL-15/IL-15R α production by liver parenchymal and non-parenchymal cells and also contributes to liver injury during liver allograft cold I/R through innate immune cell activation. Findings from the present study and their interpretation are summarized in Fig 12. Our novel observations suggest that targeting of IRF-1 and/or IL-15/IL-15R α may be an effective approach to reducing I/R injury associated with LTx.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BM	bone marrow
DC	dendritic cell
IRF-1	interferon regulatory factor 1
I/R	ischemia and reperfusion
LTx	liver transplantation
NPC	non-parenchymal cells
WT	wild-type

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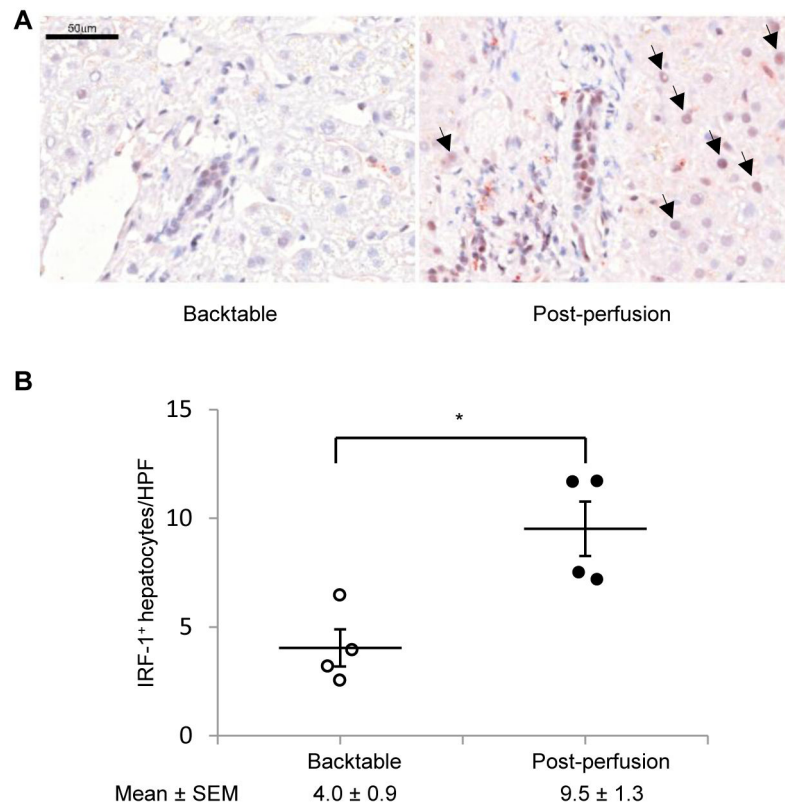


FIGURE 1. IRF-1 is induced in human liver allografts following reperfusion

(A) Immunohistochemical staining of IRF-1 (red color) in human liver allograft biopsy samples obtained at backtable and post-perfusion. Representative images of four similar experiments are shown. Scale bar: 50 µm. Arrows show IRF-1 positive cells. (B) Incidences of IRF-1 positive hepatocytes per high power field (x400) were quantified (means ± SEM; 15–20 fields per patient, n=4 patients each time point); *p<0.05.

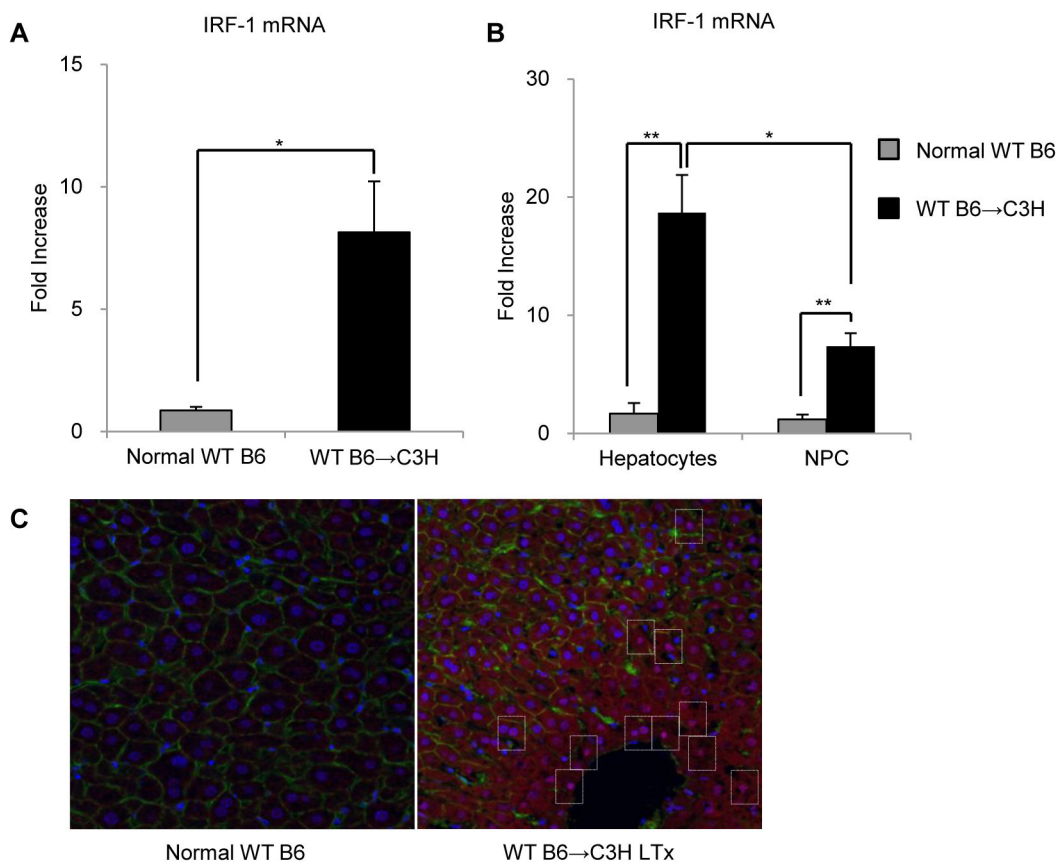


FIGURE 2. IRF-1 is induced in both hepatocytes and non-parenchymal cells (NPC) following allogeneic liver transplantation in mice

(A) Whole liver tissue IRF-1 mRNA expression 6 h after reperfusion determined by real-time RT-PCR (n=3); *p<0.05. (B) Hepatocyte and liver NPC IRF-1 mRNA expression 6 h after reperfusion determined by real-time RT-PCR. Data shown are fold increases compared to normal WT B6 hepatocytes or liver NPC, respectively (n=3); **p<0.01, *p<0.05. (C) Immunofluorescence staining of IRF-1 (red color, using anti-IRF-1 and Cy3 goat anti-rabbit), DAPI (blue color), and actin (green color, using Alexa Fluor 488 Phalloidin) in normal WT B6 livers and WT B6 liver allografts 6 h after reperfusion. Representative images (x400) of three similar experiments are shown. Dotted rectangles show IRF-1 positive cells.

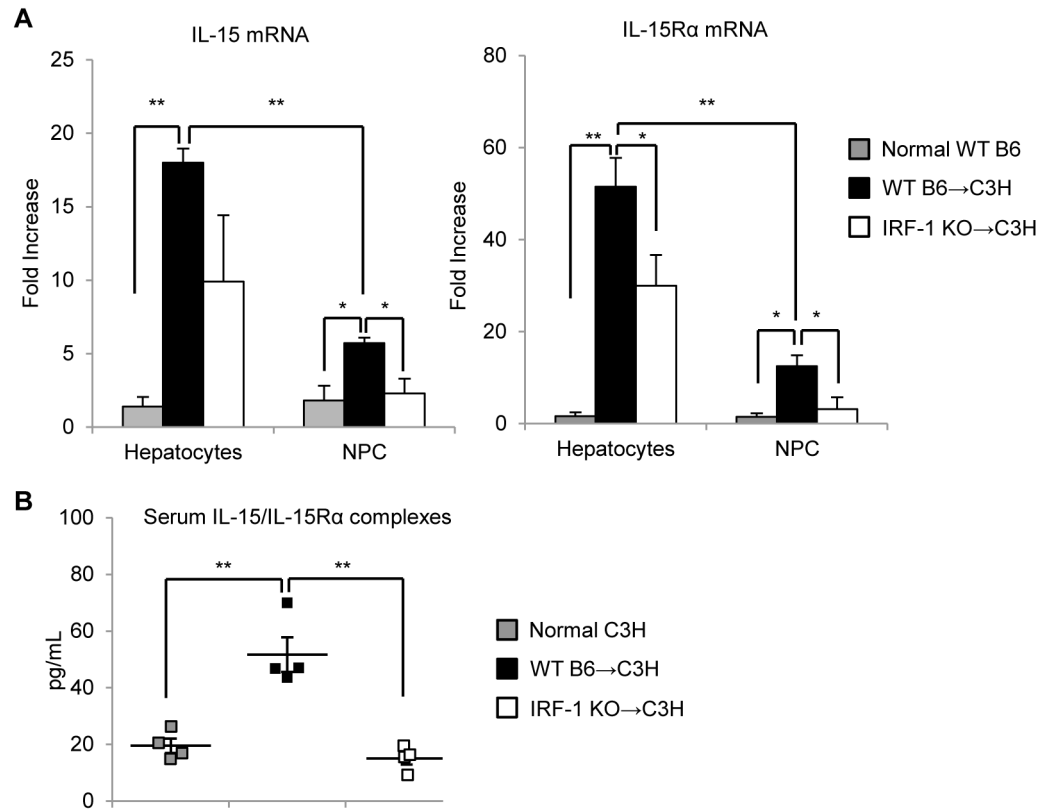


FIGURE 3. IL-15 and IL-15R α expression is induced in hepatocytes and NPC in an IRF-1-dependent manner during liver transplantation I/R injury

(A) Hepatocyte and liver NPC expression of IL-15 and IL-15R α mRNA 6 h after liver reperfusion determined by real-time RT-PCR. Data shown are fold increases compared to normal WT B6 hepatocytes or liver NPC, respectively (n=3); **p<0.01, *p<0.05. (B) Concentration of IL-15/IL-15R α complexes in serum 6 h after reperfusion determined by ELISA (n=4); **p<0.01.

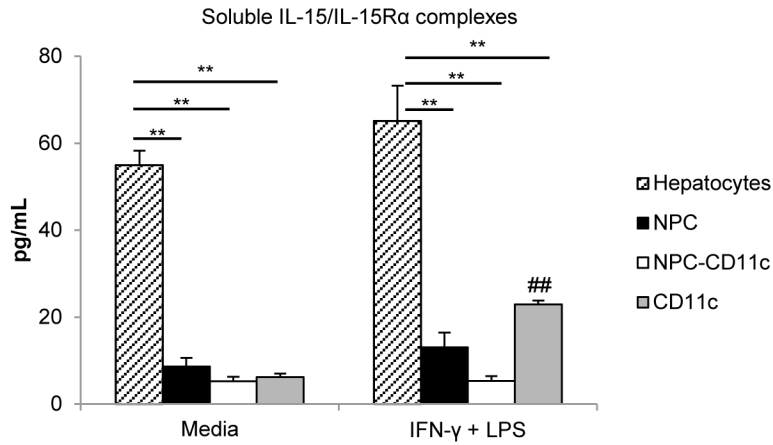


FIGURE 4. Hepatocytes are the principal producers of IL-15/IL-15R α complexes among different liver cell populations

Concentrations of IL-15/IL-15R α complexes in supernatants of cultures containing equal numbers (5×10^6 cells/ml) of different liver cell populations isolated from WT B6 mice (hepatocytes, bulk liver NPC [NPC], liver NPC depleted of DC [NPC-CD11c] and CD11c⁺ DC alone [CD11c]). Cells were incubated for 24 h with media alone or with culture media containing LPS (30 μ g/ml) and IFN- γ (100U/ml). Data are means obtained from 3 independent experiments; **p<0.01 vs hepatocytes; ## p<0.01 stimulated CD11c vs CD11c with media alone.

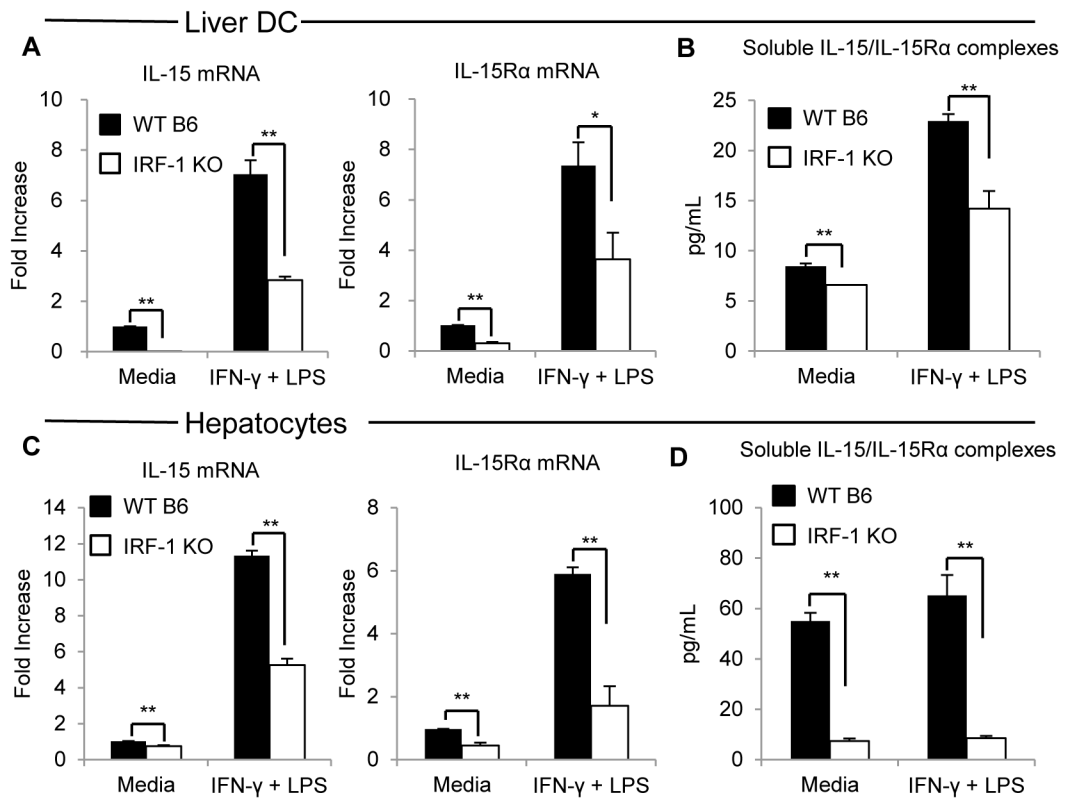


FIGURE 5. IRF-1 regulates IL-15 and IL-15R α mRNA expression and the secretion of IL-15/IL-15R α complexes by liver DC and hepatocytes

(A) Liver DC expression of IL-15 and IL-15R α mRNA 6 h after the start of culture in either media alone or media containing LPS (30 μ g/ml) and IFN- γ (100U/ml) determined by real-time RT-PCR. Liver DC were isolated from WT mice or IRF-1 KO mice. Data are shown as fold increases compared to WT liver DC with media alone; ** p <0.01, * p <0.05. (B) Concentrations of IL-15/IL-15R α complexes in supernatants of cultures containing equal numbers (5 \times 10⁶ cells/ml) of liver DC isolated from WT or IRF-1 KO mice. DC were incubated for 24 h with either media alone or media containing LPS (30 μ g/ml) and IFN- γ (100U/ml). Data are representative of 3 independent experiments; ** p <0.01. (C) Hepatocyte expression of IL-15 and IL-15R α mRNA 6 h after the start of culture in either media alone or with media containing LPS (30 μ g/ml) and IFN- γ (100U/ml) determined by real-time RT-PCR. Hepatocytes were isolated from WT or IRF-1 KO mice. Data are fold increases compared to WT hepatocytes with media alone; ** p <0.01. (D) Concentrations of IL-15/IL-15R α complexes in supernatants of cultures containing equal numbers (5 \times 10⁶/ml) of hepatocytes isolated from WT or IRF-1 KO mice. Hepatocytes were incubated for 24 h with either media alone or media containing LPS (30 μ g/ml) and IFN- γ (100U/ml). Representative of 3 independent experiments; ** p <0.01.

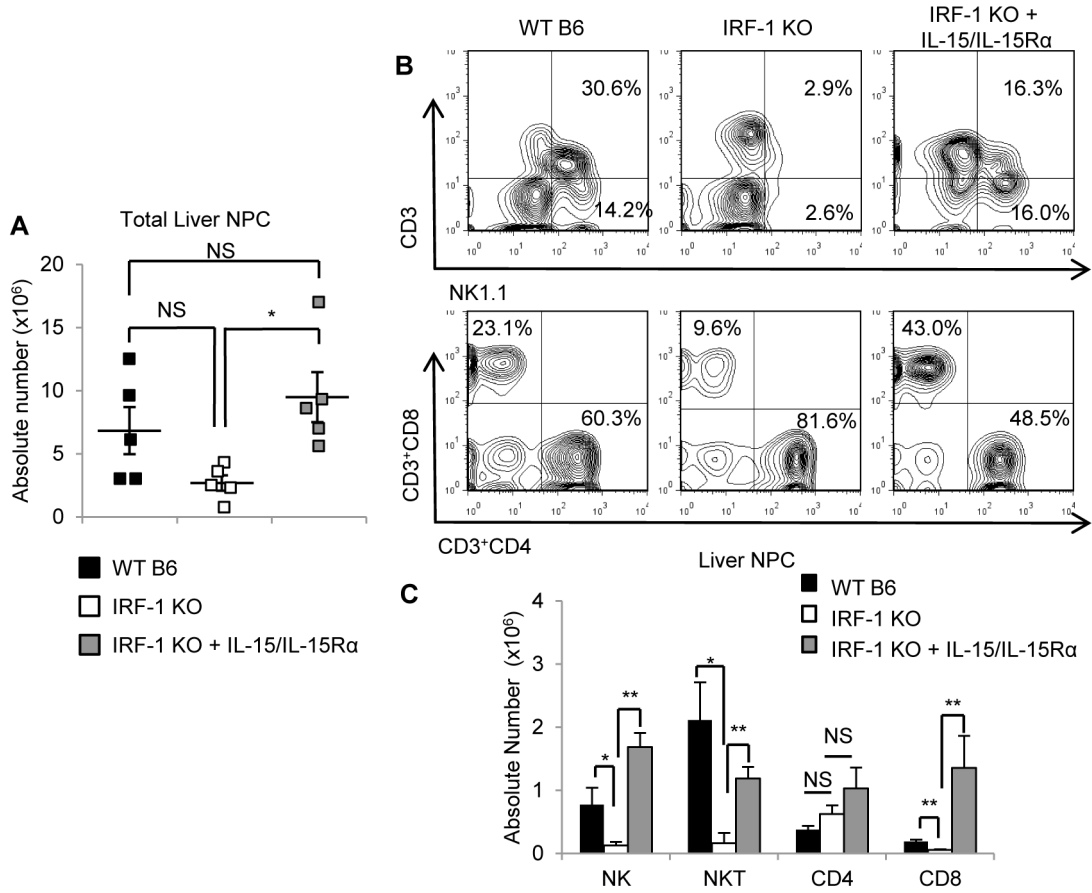


FIGURE 6. IRF-1 regulates NK, NKT, and CD8⁺T cell numbers in the liver in the normal steady-state via IL-15/IL-15R α complex production

(A) Absolute numbers of total liver non-parenchymal cells (NPC) isolated from normal WT B6 mice, IRF-1 KO mice, or IRF-1 KO mice given soluble IL-15/IL-15R α complexes protein 4 days before cell isolation (means \pm SEM; n=5 mice per group); *p<0.05. (B) Representative data (n=5 independent experiments) of liver-resident lymphocyte frequencies represented as a percentage of hepatic CD45⁺ cells. (C) The bar graph represents absolute numbers of each lymphocyte subset (means \pm SEM; n=5 mice per group); *p<0.05, **p<0.01.

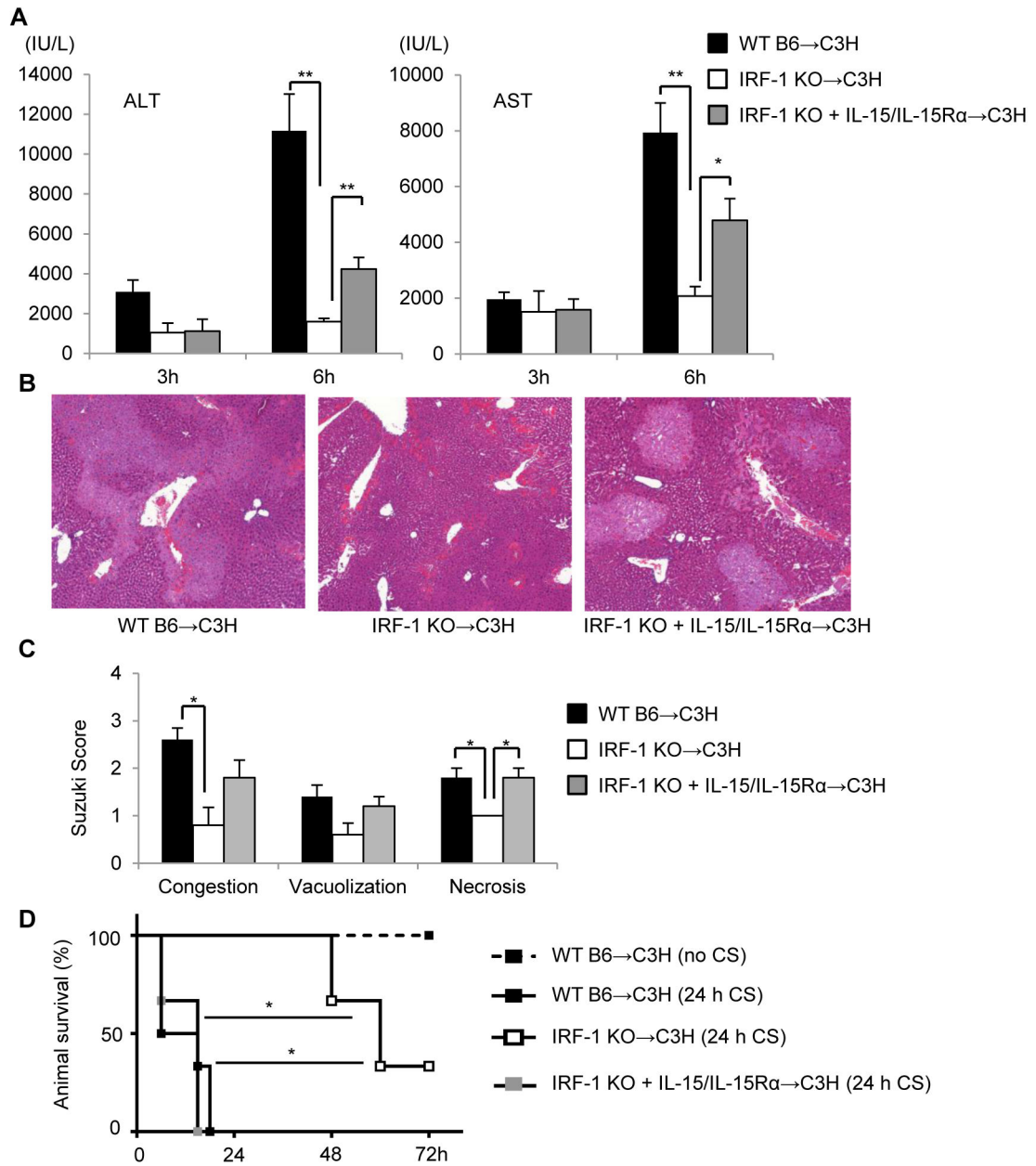


FIGURE 7. IRF-1 mediates liver cold I/R injury via IL-15/IL-15R α complex production
(A) Liver allograft recipients' serum ALT and AST levels 3 and 6 h after reperfusion (means \pm SEM; n=3–5 transplants per group); *p<0.05, **p<0.01. **(B)** Representative histology 6 h after reperfusion showing significantly reduced allograft damage in IRF-1 KO liver recipients and partial restoration of injury by IRF-1 KO donor exposure to IL-15/IL-15R complexes. **(C)** Liver injury assessed by Suzuki score (means \pm SEM; n=5 transplants per group); *p<0.05. **(D)** Survival rates of recipients receiving liver grafts from WT B6 without extended cold storage (CS) (no CS) (n=3), WT B6 with 24 h CS (24h CS) (n=6), IRF-1 KO with 24 h CS (n=3), or IRF-1 KO pretreated with IL-15/IL-15R α with 24 h CS (n=3); *p<0.05, log rank test.

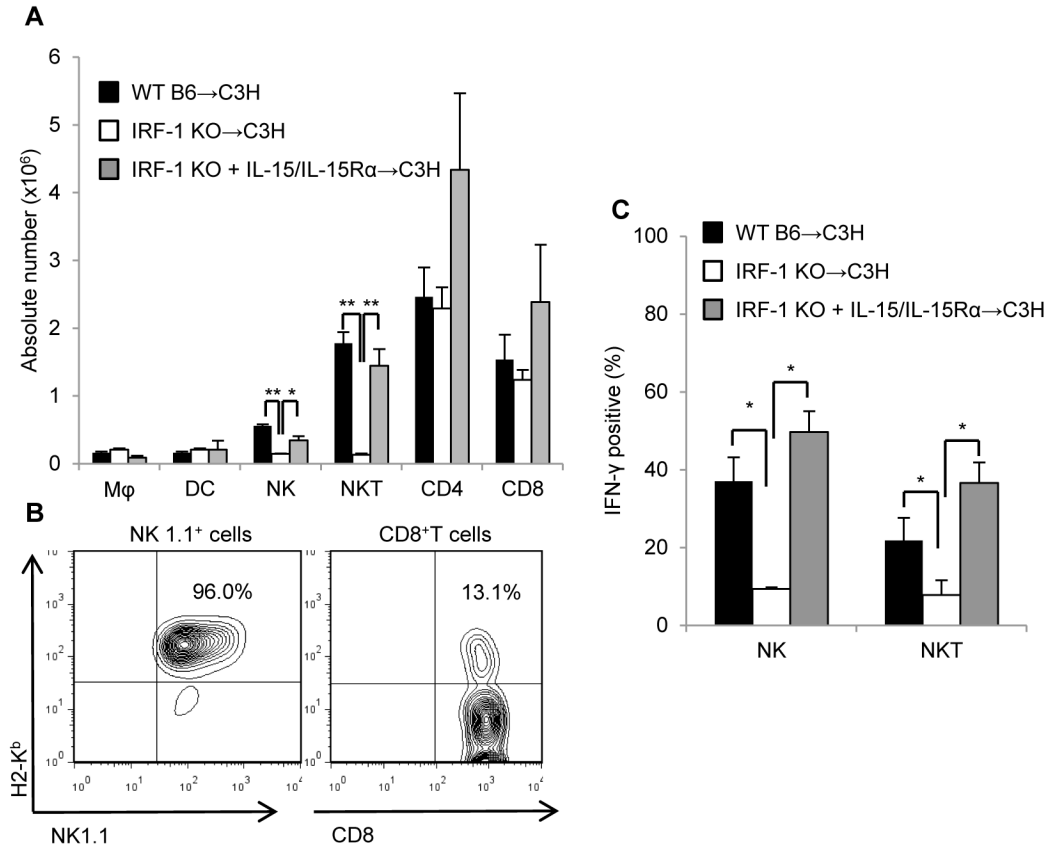


FIGURE 8. IRF-1 regulates donor-derived NK and NKT cells and their IFN- γ production via IL-15/IL-15R α complexes

(A) Absolute numbers of various leukocyte populations isolated from liver allografts 6 h post-transplant. Total liver NPC were isolated from WT, IRF-1KO or IL-15/IL-15R-treated IRF-1KO liver recipients (means \pm SEM; n=3 mice per group); *p<0.05, **p<0.01. (B) Representative data (n=3 independent experiments) showing donor-derived (H2^{b+}) NK1.1⁺ and CD8⁺T cell frequencies as percentages of hepatic CD45⁺ cells isolated from liver graft recipients. (C) Incidences of IFN- γ ⁺ NK and NKT cells in liver allografts of the 3 transplant groups (means \pm SEM; n=3 mice per group); *p<0.05.

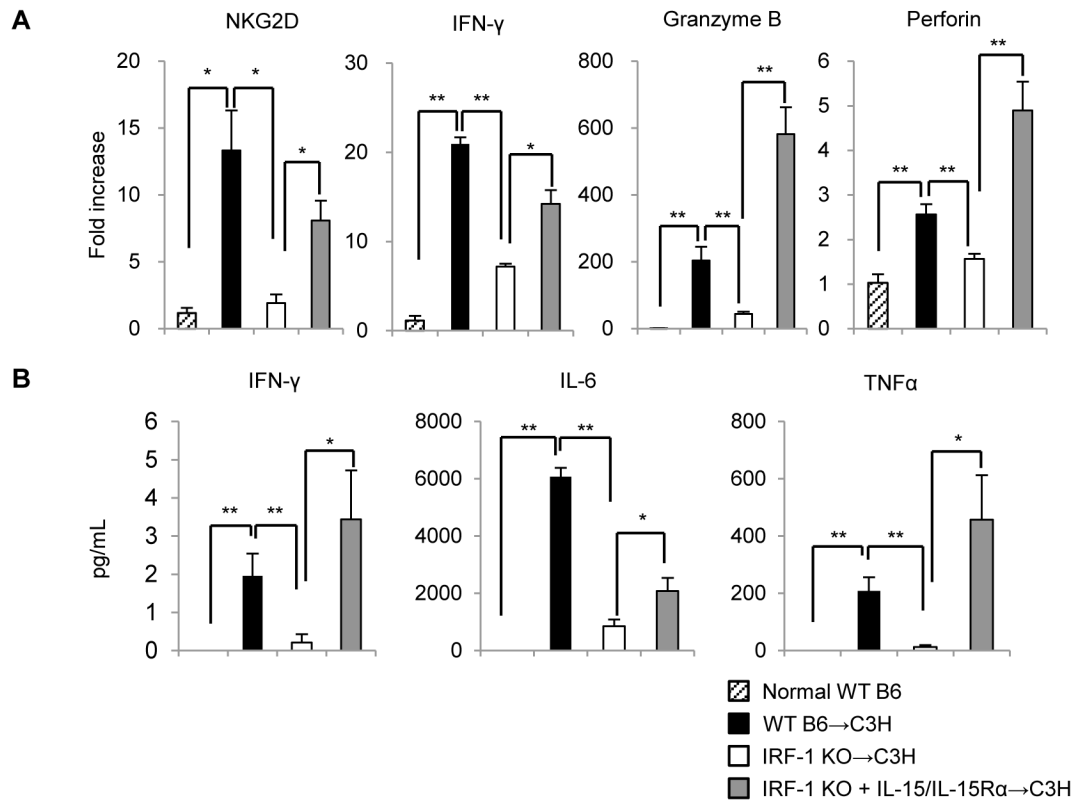


FIGURE 9. IL-15/IL-15R α complexes augment cytotoxic effector molecule expression in liver allografts and promote systemic inflammatory responses in allograft recipients

(A) Expression of cytotoxic molecules in liver grafts 6 h after LTx (means \pm SEM; n=3 mice per group); *p<0.05, **p<0.01. (B) Corresponding systemic (serum) IFN- γ , IL-6, and TNF α levels determined by cytokine bead array (means \pm SEM; n=5 transplants per group); *p<0.05, **p<0.01.

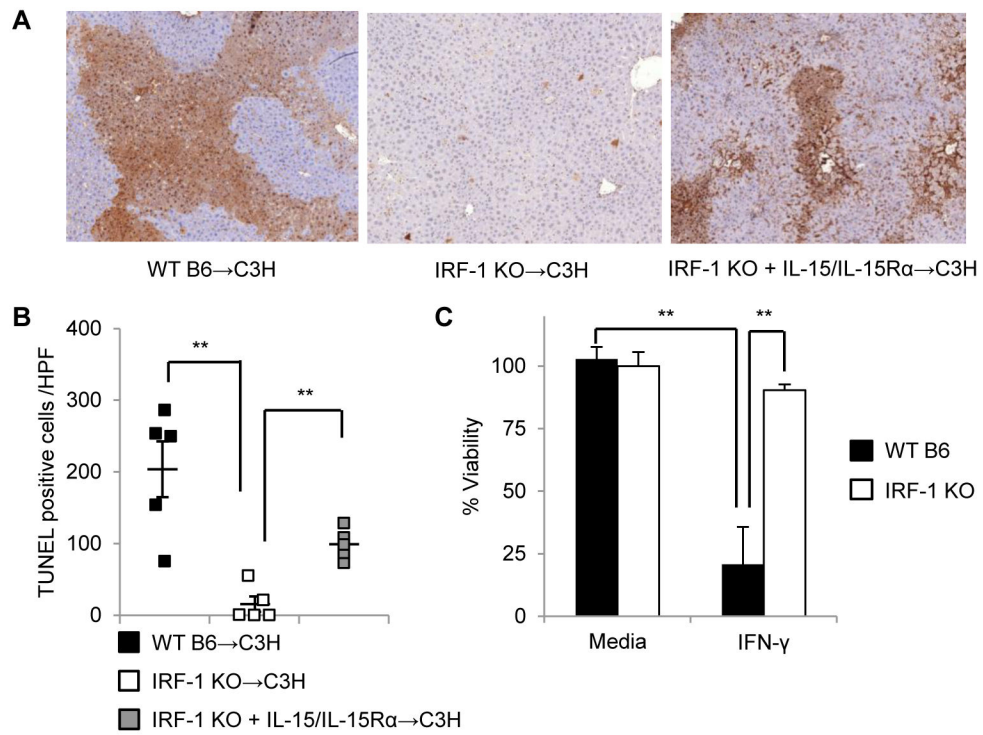


FIGURE 10. IL-15/IL-15R α complexes promote hepatocyte cell death in liver allografts
(A) Representative TUNEL-stained sections of liver grafts from WT, IRF-1KO or IL-15/IL-15R α -treated IRF-1KO donors **(B)** Incidences of hepatocytes undergoing cell death (TUNEL⁺ cells) per high power field (x200) in the 3 transplant groups were quantified (means \pm SEM; n=5 transplants per group); **p<0.01. **(C)** Hepatocyte viability determined by MTT assay. Hepatocytes isolated from WT or IRF-1 KO mice were cultured in media with or without IFN- γ (1000U/ml) for 24 h; **p<0.01.

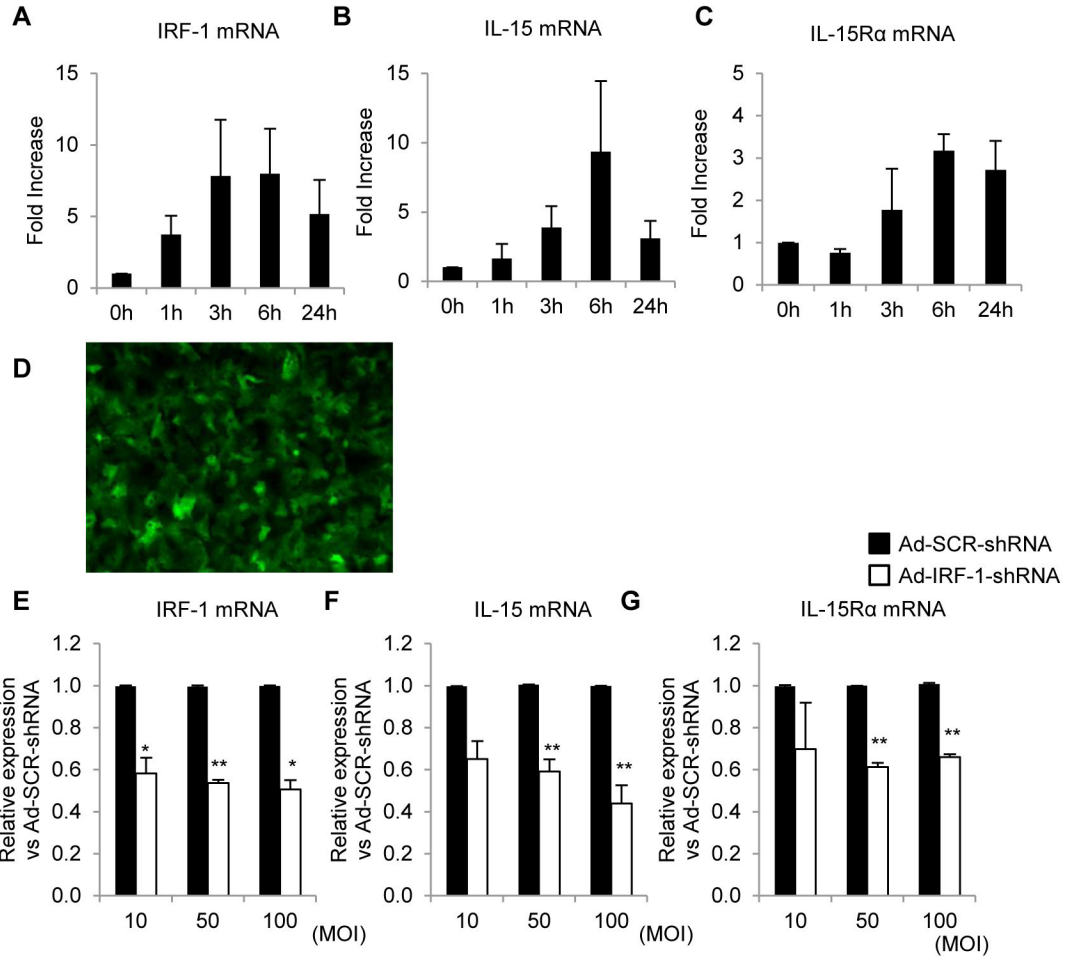


FIGURE 11. IRF-1 regulates IL-15 and IL-15Rα expression by human primary hepatocytes (A–C) Time courses of human primary hepatocyte IRF-1 (A), IL-15 (B) and IL-15Rα mRNA (C) expression by real-time RT-PCR. The hepatocytes were cultured in media containing LPS (30μg/ml) and IFN-γ (250U/ml). Data are fold increases compared to hepatocytes without treatment (means ± SEM; n=3 independent experiments). (D–G) Analysis of human primary hepatocyte IRF-1 (E), IL-15 (F), and IL-15Rα mRNA expression (G) by real-time RT-PCR following transfection with Ad-IRF-1-shRNA or control vector (Ad-SCR-ShRNA) for 48 h. Gene transfection efficiency was confirmed by GFP expression in hepatocytes (D). The cells were then stimulated with LPS (30μg/ml) and IFN-γ (250U/ml) for 6 h before harvesting for analysis. Data are shown as relative expression vs Ad-SCR-ShRNA (means ± SEM; n=3 independent experiments); *p<0.05, **p<0.01. MOI = multiplicity of infection.

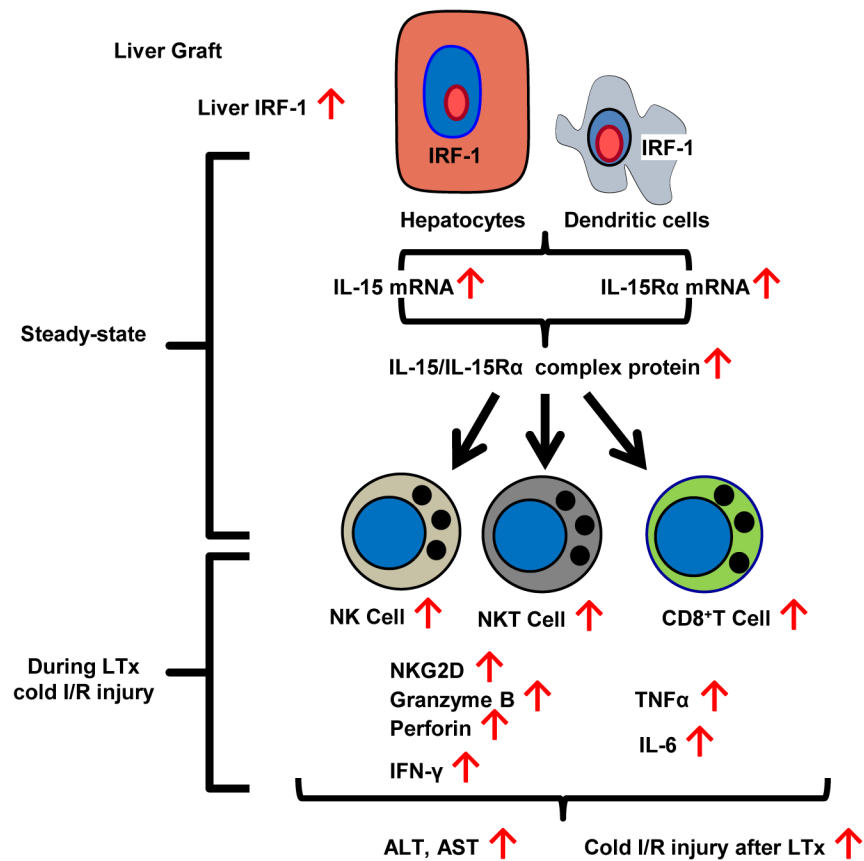


FIGURE 12. Model depicting the proposed role of IRF-1 in hepatic lymphocyte homeostasis and innate immune cell activation during liver transplant cold I/R injury

IRF-1 in hepatocytes and DC is crucial in regulating both IL-15 and IL-15Rα mRNA expression and subsequent production of soluble IL-15/IL-15Rα complexes by these cells. Hepatocytes are the principal producers of soluble IL-15/IL-15Rα complexes among different liver cell populations in both the steady-state and under inflammatory conditions (LTx cold I/R injury with 24 h of cold storage). Soluble IL-15/IL-15Rα complexes contribute to the homeostasis of hepatic NK, NKT, and CD8⁺T cells and the activation of these cells during LTx cold I/R injury determined by increased cytotoxic effector molecule expression in the liver graft (NKG2D, Granzyme B, Perforin, IFN-γ) and systemic proinflammatory markers in the recipient's serum (IFN-γ, TNFα, IL-6). Serum ALT and AST levels significantly increase, reflecting worsened liver injury as a result of these inflammatory responses.