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IRF-1 Promotes Liver Transplant Ischemia/Reperfusion Injury via Hepatocyte IL-15/IL-15Rα Production

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Ischemia and reperfusion (I/R) injury following liver transplantation (LTx) is an important problem that significantly impacts clinical outcomes. IFN 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 because IRF-1–knockout (KO) grafts displayed much less damage as assessed by serum alanine aminotransferase and histology. In vitro, IRF-1 regulated both constitutive and induced expression of IL-15, as well as IL-15Rα mRNA expression in murine hepatocytes and liver dendritic cells. 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-15Rα complexes in the liver. IRF-1–KO livers had significantly reduced NK, NKT, and CD8+ T cell numbers, whereas rIL-15/IL-15Rα 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-15Rα production and suggest that targeting IRF-1 and IL-15/IL-15Rα may be effective in reducing I/R injury associated with LTx. The Journal of Immunology, 2015, 194: 000–000.

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 States is the shortage of donor organs (1). Although >15,000 patients await LTx each year, <7,000 undergo the procedure according to data collected by the Organ Procurement and Transplant Network. This critical organ shortage has led to the 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 and late human LTx outcomes. Thus, prolonged cold ischemia time is associated with an increased incidence of retransplantation and primary graft nonfunction during the first 2 wk post-LTx (5), as well as with increased mortality at 3 and 12 mo posttransplant (6). Although 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 the 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 the wider use of marginal organs.

IFN 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 showed 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-chain (γc) 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-15Rα is a receptor subunit with which IL-15 forms a heterodimeric complex (22). This IL-15/IL-15Rα complex binds to cells that express another heterodimeric receptor complex consisting of IL-2/IL-15Rβ and γc, through which it signals (20). A recent study (22) indicates that this soluble IL-15/IL-15Rα complex is the only form of circu-
lating soluble IL-15 in mouse and human serum. IL-15Ra 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-15Ra promoter (23). However, it remains unclear whether IRF-1 regulates IL-15 and IL-15Ra expression in hepatic immune and nonimmune cells in the steady-state or under inflammatory conditions (24).

We showed 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 are 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 complexes to promote proinflammatory cytokine production and the upregulation 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 four patients at two time points: backtable and postreperfusion (1–4 h). Four-micron sections were deparaffinized, hydrated, and treated with citrated buffer for Ag retrieval. Sections were then blocked with an Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA). Staining was performed by sequential incubation cycles of rabbit anti–IRF-1 primary Ab (Santa Cruz Biotechnology, Dallas, TX), goat anti-rabbit biotinylated secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA), an ABC Kit (Vector Laboratories), and an AEC Substrate Kit (ScyTek Laboratories, Logan, UT). Sections were then counterstained with aqueous hematoxylin. Digital images of whole staining slides were obtained with the MIRAX MIDI (ImmunoResearch Laboratories) was applied with F-actin counterstain (Alexa Fluor 488 Phalloidin, Life Technologies, Grand Island, NY).

Circulating IL-15/IL-15Ra levels

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

In vitro culture assays

Different liver cell populations (hepatocytes, bulk liver 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 × 10^6 cells/ml) were incubated at 37°C for 6 or 24 h in media alone or with LPS (30 μg/ml) and IFN-γ (100 U/ml). CD11c+ DC were purified as described (32, 34).

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

Human rIL-15 and recombinant mouse IL-15RaFc were purchased from R&D Systems (Minneapolis, MN), and IL-15/IL-15Ra complexes were prepared as described (35, 36). To assess the function of soluble IL-15/IL-15Ra in vivo, IRF-1–KO mice were given 2.5 μg IL-15 and 15 μg IL-15Ra Fc in 200 μl PBS i.p. 4 d 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 FcγR-blocking rat anti-mouse CD16/32 mAb (2.4G2) to prevent nonspecific Ab binding. For cell surface staining, the NPC were incubated for 30 min with FITC-, PE-, allophycocyanin-, Pacific Blue–, or PE-Cy7–conjugated mAbs to detect the 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), and F4/80 (BM8; BioLegend, San Diego, CA). For intracellular cytokine staining, cells were fixed with 4% paraformaldehyde, permeabilized using 0.1% saponin, and stained with anti-mouse IFN-γ Ab (XMG1.2; BioLegend). For Foxp3 staining, cells were fixed and permeabilized using a Foxp3 Fix/Perm kit (eBioscience) and stained with anti-Foxp3 mAb (FK-16; eBioscience). Appropriate Ig isotype controls were obtained from BD Pharmingen (San Diego, CA). Flow cytometry was performed using an LSR Fortessa flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (version 7.6; TreeStar, 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, sectioned, and stained with H&E and TUNEL as described (33). Liver tissue was obtained from graft recipients immediately after euthanasia, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E and TUNEL as described (33). 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 cytokine bead array (BD Biosciences).

MTT assay

Hepatocytes (WT B6 or IRF-1 KO) were cultured or not with 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 μg/ml) and IFN-γ (250 U/ml) and harvested 0, 1, 3, 6, or 24 h after stimulation for RNA isolation.

Hepatocyte transfection using adenovirus vector

A replication-deficient adenovirus (Ad) vector (human Ad type 5, δE1/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 5′-TGCTTTGTG-ACAGTGACGGCACCTGCTAGAGATTGAAGTTAGACACACTACCTACCTGACGCTTCGGA-3′. The target sequences were 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, and 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 containing a scrambled (SCR) shRNA (Ad–SCR–shRNA) for 48 h. The cells were then stimulated with LPS (30 μg/ml) and IFN-γ (250 U/ml) for 6 h before harvesting for RNA isolation and functional analysis.

Statistical analysis

Data are presented as mean ± 1 SEM. Comparisons between groups were performed using the Student t test. Nonparametric data (Suzuki score) were analyzed with the Mann–Whitney U test. A log-rank test was performed to assess differences in graft survival. A p value < 0.05 was considered significant.

Results

IRF-1 is upregulated in human LTx tissue after reperfusion

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

IRF-1 is upregulated in both hepatocytes and NPC after allogenic LTx with extended cold storage time

To determine the level of IRF-1 expression in liver grafts after mouse allogenic (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 showed previously with mouse syngeneic LTx (28), IRF-1 mRNA was markedly and significantly upregulated (8-fold) compared with normal liver tissue (Fig. 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 did NPC (7-fold) (Fig. 2B). Immunofluorescence staining of liver grafts 6 h after reperfusion confirmed that IRF-1 protein expression was induced significantly following LTx compared with normal liver, and it localized mainly to hepatocyte nuclei (Fig. 2C). These data show that both IRF-1 mRNA and protein are upregulated significantly after allogeneic LTx and that IRF-1 is expressed by both hepatocytes and NPC, with greater induction in hepatocytes.

IL-15 and IL-15Ra expression are upregulated 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-15Ra by BM cells or COS-7 cells in vitro (14, 15, 23), it is unclear whether it induces IL-15 and/or IL-15Ra in the liver during cold I/R injury associated with allogeneic LTx. We observed that both IL-15 and IL-15Ra mRNA expression were induced significantly in hepatocytes 6 h after reperfusion in WT B6→C3H LTx hepatocytes compared with normal hepatocytes; IL-15 mRNA was upregulated >15-fold and IL-15Ra mRNA increased >50-fold compared with normal WT hepatocytes (Fig. 3A). Hepatic NPC obtained from WT B6→C3H allografts also displayed significantly upregulated IL-15 and IL-15Ra mRNA expression after reperfusion compared with normal WT NPC (Fig. 3A). However, the degree of IL-15 and IL-15Ra mRNA induction was significantly greater in hepatocytes compared with NPC. We then used IRF-1→KO donor liver allografts to determine whether IRF-1 was involved in the promotion of IL-15 and IL-15Ra expression in the liver after LTx. Expression of both IL-15 and IL-15Ra mRNA was reduced significantly in hepatocytes and NPC from IRF-1→KO→C3H grafts compared with WT→C3H grafts (Fig. 3A). These data suggest that IRF-1 regulates the induction of both IL-15 and IL-15Ra mRNA in hepatocytes, as well as in liver NPC, after LTx. We next measured circulating IL-15/IL-15Ra protein complexes in sera collected from graft recipients 6 h after reperfusion. Levels were elevated significantly in WT→C3H recipients compared with normal C3H mice (Fig. 3B), whereas IRF-1 KO→C3H recipients showed significantly reduced levels of circulating IL-15/IL-15Ra complexes compared with WT→C3H recipients. These data indicate that donor-expressed IRF-1 regulates IL-15 and IL-15Ra mRNA expression in the liver, as well as circulating IL-15/IL-15Ra complexes in host serum, 6 h after LTx.

Hepatocytes are the major source of soluble IL-15/IL-15Ra 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 mice. Other reports indicate that liver parenchymal cells...
can express IL-15 and IL-15Rα (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 showed 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-15Rα 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-15Rα complexes in the liver. To verify our hypothesis, we isolated different liver cell subsets and cultured these cells (5 × 10^6 cells/ml) or not with LPS and IFN-γ stimulation for 24 h. As shown in Fig. 4, hepatocytes secreted significantly higher amounts of soluble IL-15/IL-15Rα complexes compared with bulk liver NPC, liver NPC depleted of DC (NPC-CD11c+), or DC (CD11c+) alone. Among liver NPC, DC constituted one of the main producers of IL-15/IL-15Rα complexes, because secreted IL-15/IL-15Rα complexes decreased significantly when DC were removed from NPC (38 ± 6% decrease in media-alone group and 57 ± 6% decrease with LPS and IFN-γ group). Interestingly, the degree of soluble IL-15/IL-15Rα 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 with hepatocytes (1.2-fold), bulk NPC (1.5-fold), or NPC-CD11c (1.0-fold) (Fig. 4).

**FIGURE 2.** IRF-1 is induced in hepatocytes and 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). (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 with normal WT B6 hepatocytes or liver NPC, respectively (n = 3). (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 (original magnification ×400) of three similar experiments are shown. Dotted rectangles show IRF-1+ cells. *p < 0.05, **p < 0.01.

**FIGURE 3.** IL-15 and IL-15Rα 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-15Rα mRNA 6 h after liver reperfusion determined by real-time RT-PCR. Data shown are fold increases compared with normal WT B6 hepatocytes or liver NPC, respectively (n = 3). (B) Concentration of IL-15/IL-15Rα complexes in serum 6 h after reperfusion determined by ELISA (n = 4). *p < 0.05, **p < 0.01.
IRF-1 regulates basal and induced expression of IL-15 and IL-15Ra mRNA and IL-15/IL-15Ra secretion by mouse primary hepatocytes and liver DC

Although previous studies (14, 23) indicated that IL-15 and IL-15Ra gene sequences both contain IRF-1–binding elements, it remains unclear whether IRF-1 regulates IL-15 and/or IL-15Ra 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 transcripationally regulate IL-15 and IL-15Ra expression, as well as IL-15/IL-15Ra complex secretion, by these cell populations. First, liver DC isolated from WT B6 or IRF-1–KO mice were cultured or not with IFN-γ and LPS for 6 h to determine their constitutive and induced IL-15 and IL-15Ra mRNA expression. As shown in Fig. 5A, WT liver CD11c+ DC upregulated both IL-15 and IL-15Ra mRNA markedly (~7-fold) and in response to stimulation with IFN-γ and LPS. The magnitude of basal and stimulated IL-15/IL-15Ra mRNA expression was diminished in liver DC lacking IRF-1. WT or IRF-1–KO liver DC were cultured or not with IFN-γ and LPS stimulation for 24 h, and secreted IL-15/IL-15Ra complexes in the culture supernatants were measured. WT liver DC secreted significantly higher levels of IL-15/IL-15Ra complexes compared with IRF-1–KO liver DC, with or without IFN-γ and LPS stimulation (Fig. 5B). As shown in Fig. 5C and 5D, similar findings were seen using mouse primary hepatocytes. These results indicate that IRF-1 transcriptionally regulates constitutive expression and induced expression of IL-15 and IL-15Ra 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, whereas systemic administration of soluble IL-15/IL-15Ra complexes restores these hepatic lymphocyte populations

Although previous studies (14, 15) showed 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, which are known to be regulated by IRF-1 in other organs (17, 44), are also affected in the liver. It is also unknown whether a deficiency of certain hepatic lymphocytes in IRF-1–KO mice can be restored by systemic administration of IL-15/IL-15Ra 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-15Ra complexes systemically. Compared with WT livers, IRF-1–KO livers showed reduced absolute numbers of total liver NPC (Fig. 6A). There were no significant differences in the absolute numbers of macrophages, DC, B cells, or regulatory T cells between livers from WT and IRF-1–KO mice (data not shown), whereas the absolute numbers of NK, NKT, and CD8+ T cells were reduced significantly in IRF-1–KO livers compared with WT livers (Fig. 6B, 6C). There were no differences in the absolute numbers of CD4+ T cells between WT and IRF-1–KO livers (Fig. 6C). IL-15/IL-15Ra 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 (Fig. 6A). As shown in Fig. 6C, soluble IL-15/IL-15Ra 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-15Ra expression and that systemic administration of IL-15/IL-15Ra 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-15Ra complexes partially reverses the protective effect

Previous studies showed 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 mechanisms (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-15Ra complexes might impact the outcome of LTx-induced cold I/R injury. Thus, we performed allogeneic LTxs following 24 h of extended cold storage using donor livers from WT B6, IRF-1–KO, or IRF-1–KO mice pretreated with soluble IL-15/IL-15Ra complexes and C3H recipients. As shown in Fig. 7A, IRF-1–KO grafts were markedly protected compared with WT grafts after 6 h of 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 grafts compared with WT grafts (Fig. 7B, 7C). However, when IRF-1–KO liver grafts pretreated with soluble IL-15/IL-15Ra 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 with IRF-1–KO grafts (Fig. 7A–C).
**FIGURE 5.** IRF-1 regulates IL-15 and IL-15Rα mRNA expression and the secretion of IL-15/IL-15Rα complexes by liver DC and hepatocytes. (A) Liver DC expression of IL-15 and IL-15Rα mRNA 6 h after the start of culture in either media alone or media containing LPS (30 µg/ml) and IFN-γ (100 U/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 with WT liver DC with media alone. (B) Concentrations of IL-15/IL-15Rα complexes in supernatants of cultures containing equal numbers (5 × 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 µg/ml) and IFN-γ (100 U/ml). Data are representative of three independent experiments. (C) Hepatocyte expression of IL-15 and IL-15Rα mRNA 6 h after the start of culture in either media alone or with media containing LPS (30 µg/ml) and IFN-γ (100 U/ml) determined by real-time RT-PCR. Hepatocytes were isolated from WT or IRF-1–KO mice. Data are fold increases compared with WT hepatocytes with media alone. (D) Concentrations of IL-15/IL-15Rα complexes in supernatants of cultures containing equal numbers (5 × 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 µg/ml) and IFN-γ (100 U/ml). Representative of three independent experiments. *p < 0.05, **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-15Rα complex production. (A) Absolute numbers of total liver NPC isolated from normal WT B6 mice, IRF-1–KO mice, or IRF-1–KO mice given soluble IL-15/IL-15Rα complex protein 4 d before cell isolation (mean ± SEM; n = 5 mice/group). (B) Representative data (n = 5 independent experiments) of liver-resident lymphocyte frequencies represented as a percentage of hepatic CD45+ cells. (C) Bar graphs showing absolute numbers of each lymphocyte subset (mean ± SEM; n = 5 mice/group). *p < 0.05, **p < 0.01.

1–KO mice could be reproduced with allografts deficient in IL-15Rα. Compared with recipients that received WT grafts, those that received IL-15Rα–KO liver grafts were significantly protected 6 h after reperfusion, with significantly lower ALT and AST levels (ALT: 30,827 ± 1,988 versus 18,967 ± 1,810 IU/l; AST: 13,067 ± 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 proinflammatory cytokines that are restored by pretreatment with soluble IL-15/IL-15Rα complexes

Flow cytometric analysis of intragraft mononuclear cells 6 h after reperfusion showed markedly decreased NK and NKT cell numbers in IRF-1–KO grafts compared with WT grafts (Fig. 8A). In contrast, no significant differences in the absolute numbers of macrophages, DC, or CD4+ T cells were observed. There also was no significant difference in the absolute numbers of CD8+ T cells between WT and IRF-1–KO grafts, despite the significant difference observed in nontransplanted livers (Fig. 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 Fig. 8B, 6 h after reperfusion, the vast majority (∼95%) of graft NK 1.1+ cells were donor derived (H2b+), whereas CD8+ T cells were predominately recipient-derived graft-infiltrating cells (H2b2).

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 (Fig. 8C). Notably, donor pretreatment with soluble IL-15/IL-15Rα 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 reduced significantly in IRF-1–KO grafts compared with WT grafts (Fig. 9A). However, donor pretreatment with soluble IL-15/IL-15Rα complexes significantly reversed the expression of each of these cytotoxic effector molecules in IRF-1–KO grafts.

As shown in Fig. 9B, circulating levels of proinflammatory cytokines (IFN-γ, IL-6, and TNF-α) in serum also were reduced substantially in recipients of IRF-1–KO grafts compared with those given WT livers. However, donor pretreatment with IL-15/IL-15Rα complexes fully restored (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-15Rα 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-15Rα complexes correlated with the extent of hepatocyte cell death assessed by TUNEL staining. As shown in Fig. 10A and 10B, IRF-1–KO liver recipients and partial restoration of injury by IRF-1–KO donor exposure to IL-15/IL-15Rα complexes was consistent with the differences in liver injury between the groups, as determined by serum transaminase levels (Fig. 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 (Fig. 10C).

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

We next assessed the expression of IRF-1, IL-15, and IL-15Rα 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 (Fig. 11A). These data are consistent with our earlier observations (25, 28) using mouse or rat primary hepatocytes. Induction of IL-15 and IL-15Rα occurred within 3 h of stimulation and peaked 6 h after stimulation (Fig. 11B, 11C). To determine whether IRF-1 directly regulated IL-15 and IL-15Rα expression in human primary hepatocytes, we used an Ad vector containing a microRNA designed to specifically knock down IRF-1 expression (Ad–IRF-1–shRNA) (29). Gene delivery efficiency was determined by GFP expression of infected hepatocytes and was 90% (Fig. 11D). The Ad–IRF-1–shRNA vector effectively knocked down both basal (data not shown) and LPS- and IFN-γ–induced IRF-1 expression (Fig. 11E) in human primary hepatocytes compared with an Ad vector containing 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-15Rα (Fig. 11F, 11G), thus confirming an important role for IRF-1 in regulating human hepatocyte IL-15 and IL-15Rα expression.

**FIGURE 8.** IRF-1 regulates donor-derived NK and NKT cells and their IFN-γ production via IL-15/IL-15Rα complexes. (A) Absolute numbers of various leukocyte populations isolated from liver allografts 6 h posttransplant. Total liver NPC were isolated from WT, IRF-1–KO, or IL-15/IL-15Rα–treated IRF-1–KO liver recipients (mean ± SEM; n = 3 mice/group). (B) Representative data (n = 3 independent experiments) showing donor-derived (H2b+) 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 three transplant groups (mean ± SEM; n = 3 mice/group). *p < 0.05, **p < 0.01.

**FIGURE 9.** IL-15/IL-15Rα 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 (mean ± SEM; n = 3 mice/group). (B) Corresponding systemic (serum) IFN-γ, IL-6, and TNF-α levels determined by cytokine bead array (mean ± SEM; n = 5 transplants/group). *p < 0.05, **p < 0.01.
Findings from the current study and their interpretation are summarized in Fig. 12.

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 cells and NPC. It is strongly induced by types I and II IFN, LPS, viral infection, and dsRNA, as well as by various cytokines, such as TNF-α, IL-1β, and IL-6 (12, 54). IRF-1 exhibits remarkably diverse functions, because it targets a variety of genes, including iNOS, IL-12p40, and caspase 1, that are involved in antibacterial responses, Th1-type immunity, and the induction of apoptosis (8). We showed 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 LTx. 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. In this study, we demonstrate that IRF-1–KO donor allografts are markedly protected (~90%) from cold I/R injury compared with WT allografts. Our novel findings indicate that this protective effect is due, in large part, to reduced expression of IL-15/IL-15Rα 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.
IRF-1 PROMOTES LIVER I/R INJURY VIA IL-15/IL-15Rα PRODUCTION

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 complex 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 (NK2GD, granzyme B, perforin, IFN-γ) and systemic proinflammatory markers in the recipient’s serum (IL-6, TNF-α). Serum ALT and AST levels increase significantly, reflecting worsened liver injury as a result of these inflammatory responses.

(55–57). Its immune cell constituency is enriched in NK cells, NKT cells, and CD8+ T cells compared with other parenchymal organs (58–60), and previous studies 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 study has examined the relative contribution of different liver cell populations to the production of IL-15/IL-15Rα complexes (24). Thus, we examined both parenchymal cells and NPC as potential sources of soluble IL-15/IL-15Rα complexes within the liver. Our study provides evidence for the first time, to our knowledge, that hepatocytes are the major producers of soluble IL-15/IL-15Rα 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 the induced expression, as well as the constitutive expression, of both IL-15 and IL-15Rα mRNA in hepatocytes and liver DC. Additionally, we confirmed that IRF-1 is important for basal and induced production of IL-15/IL-15Rα complex protein by both hepatocytes and liver DC. Importantly, using human primary hepatocyte cultures, we confirmed that these findings in mice also apply to human cells. Specific knock down of IRF-1 allowed us to demonstrate that IRF-1 controls both basal and induced IL-15 and IL-15Rα 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, also play an important role in the 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-15Rα 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-15Rα 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-15Rα complexes correlates well with a report (62) that identified hepatocytes as a new source of IL-7, another member of the common cytokine-receptor γc family, which prolongs the survival of naive 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-15/IL-15Rα 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-15Rα. The latter study shows that IRF-2 is significantly downregulated in the liver in hepatitis C virus (HCV) infection, and subsequently impaired expression of IL-7 and IL-15Rα in hepatocytes may explain the poor HCV-specific CD8+ T cell responses, the inability to eliminate HCV, and the 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-15Rα 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 with those from livers of WT B6 mice. When IRF-1–KO mice were treated with soluble IL-15/IL-15Rα 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 the diminished expression of IL-15/IL-15Rα complexes and that IRF-1–KO hematopoietic cells retain the ability to develop and proliferate in response to exogenous soluble IL-15/IL-15Rα complexes. Our results are consistent with similar in vitro observations made using IRF-1–KO BM cells and rIL-15 (14). However, they do not 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-15Rα mRNA are upregulated in liver allograft hepatocytes and NPC in an IRF-1–dependent manner during I/R injury following extended cold storage. IL-15 and IL-15Rα expression was significantly higher in hepatocytes than NPC, suggesting that hepatocytes may also be major producers of IL-15 and IL-15Rα in vivo. Circulating IL-15/IL-15Rα complex levels were increased significantly in WT but not in IRF-1–KO allograft recipients 6 h postreperfusion. These results indicate that the predominant source of circulating IL-15/IL-15Rα complexes is the allograft, rather than host-derived cells. Normal IL-15/IL-15Rα 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 (NK2GD, granzyme B, perforin) and proinflammatory cytokines (IFN-γ, IL-6, TNF-α). When IRF-1–KO livers were exposed to...
solute IL-15/IL-15Rα complexes, cytotoxic molecules and proinflammatory cytokines were increased significantly, liver injury and hepatocyte cell death were partially restored, and recipient survival was shortened. These findings indicate that exogenous IL-15/IL-15Rα 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, affect 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 d to healthy, WT mice significantly increased hepatic expression of proinflammatory cytokines (IFN-γ and TNF-α), the number of inflammatory foci, and caspase-3+ cells in the liver (40). We further tested whether the proinflammatory effects of soluble IL-15/IL-15Rα complexes in IRF-1–KO allografts could be reproduced in IL-15Rα–KO allografts. Recipients of IL-15Rα–KO grafts were significantly protected compared with those that received WT transplants, as indicated by significantly lower ALT.

Our novel observations and NPC and contributes to liver injury during liver allograft cold I/R through innate immune cell activation. Our novel observations suggest that targeting of IRF-1 and/or IL-15/IL-15Rα may be an effective approach to reduce liver I/R injury associated with LTx.

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Disclosures

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In conclusion, our study provides new evidence that IFN-1 regulates the homeostasis of NK, NKT, and CD8+ T cells in the liver through IL-15/IL-15Rα production by liver parenchymal cells and NPC and contributes to liver injury during liver allotagraft cold I/R through innate immune cell activation. Our novel observations suggest that targeting of IRF-1 and/or IL-15/IL-15Rα may be an effective approach to reduce liver I/R injury associated with LTx.

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