A Critical Role for IFN Regulatory Factor 1 in NKT Cell-Mediated Liver Injury Induced by α-Galactosylceramide

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A Critical Role for IFN Regulatory Factor 1 in NKT Cell-Mediated Liver Injury Induced by \( \alpha \)-Galactosylceramide

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NKT cells are remarkably abundant in mouse liver. Compelling experimental evidence has suggested that NKT cells are involved in the pathogenesis of many liver diseases. Activation of NKT cells with \( \alpha \)-galactosylceramide (\( \alpha \)-GalCer) causes liver injury through mechanisms that are not well understood. We undertook studies to characterize the key pathways involved in \( \alpha \)-GalCer–induced liver injury. We found that expression of the transcription factor IFN regulatory factor 1 (IRF-1) in mouse liver was dramatically upregulated by \( \alpha \)-GalCer treatment. Neutralization of either TNF-\( \alpha \) or IFN-\( \gamma \) inhibited \( \alpha \)-GalCer–mediated IRF-1 upregulation. \( \alpha \)-GalCer–induced liver injury was significantly suppressed in IRF-1 knockout mice or in wild-type C56BL/6 mice that received a microRNA specifically targeting IRF-1. In contrast, overexpression of IRF-1 greatly potentiated \( \alpha \)-GalCer–induced liver injury. \( \alpha \)-GalCer injection also induced a marked increase in hepatic inducible NO synthase expression in C56BL/6 mice, but not in IRF-1 knockout mice. Inducible NO synthase knockout mice exhibited significantly reduced liver injury following \( \alpha \)-GalCer treatment. Finally, we demonstrated that both NKT cells and hepatocytes expressed IRF-1 in response to \( \alpha \)-GalCer. However, it appeared that the hepatocyte-derived IRF-1 was mainly responsible for \( \alpha \)-GalCer–induced liver injury, based on the observation that inhibition of IRF-1 by RNA interference did not affect \( \alpha \)-GalCer–induced NKT cell activation. Our findings revealed a novel mechanism of NKT cell-mediated liver injury in mice, which has implications in the development of human liver diseases.

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Immune-mediated liver injury particularly caused by T lymphocytes appears to play a pivotal role in the pathogenesis of various liver diseases. Attempts have been made to replicate the immune process in several animal models, including the hepatitis B surface Ag–based autoimmune hepatitis model and the liver injury model induced by LPS or the plant lectin Con A (14, 17). However, it has proven difficult to establish a reliable and reproducible animal model with an Ag-specific T cell response (14, 17). Administration of \( \alpha \)-GalCer causes a specific activation of NKT cells with subsequent liver damage, which has emerged as a robust model to investigate the fundamental pathophysiology of immune-mediated liver injury involved in many human liver diseases (14, 17–19). Studies on the basic mechanisms of \( \alpha \)-GalCer–induced liver injury could provide valuable insights into the development of human liver disorders. Recent studies have shed some light on the mechanisms of action of \( \alpha \)-GalCer–mediated hepatic damage (17, 19–21), but the detailed molecular and cellular mechanisms involved in the process remain to be fully elucidated.

IFN regulatory factor 1 (IRF-1) was originally identified as a transcription factor for type I IFN expression that mediates immune and inflammatory responses to viral and other pathogens (22–24). IRF-1 has been shown to be expressed in cultures of primary hepatocytes in response to inflammatory cytokine IFN-\( \alpha / \beta \), IFN-\( \gamma \), TNF-\( \alpha \), and IL-1\( \beta \) (25). Recent work in our laboratory suggested that IRF-1 contributes to liver damage induced by warm hepatic I/R injury (26, 27). Furthermore, we have also shown that IRF-1 plays a critical role in liver transplant preservation injury (27, 28). IRF-1 expression itself can be regulated by many factors, among which IFN-\( \gamma \) is the strongest IRF-1 inducer known so far (22). Upon activation by \( \alpha \)-GalCer, NKT cells are able to produce a broad range of cytokines, particularly IFN-\( \gamma \) and IL-4 (7–9). Therefore, we hypothesized that IRF-1 expression could be induced by \( \alpha \)-GalCer treatment. Indeed, in the present study we found that
IRF-1 expression was dramatically upregulated in mouse liver following treatment with even a low dose of α-GalCer. By using gene knockout mice and in vivo RNA interference, we were able to demonstrate that IRF-1 was an important mediator of α-GalCer-induced liver injury. Additionally, we provided evidence showing that α-GalCer–induced hepatic damage was mediated at least in part by inducible NO synthase (iNOS), one of the IRF-1 target genes. We also showed that both NKT cells and hepatocytes were able to produce IRF-1 in response to α-GalCer treatment, but it appeared that the hepatocyte-derived IRF-1 was mainly responsible for α-GalCer–induced hepatic damage.

Materials and Methods

Animals and reagents

Male C57Bl/6 mice and IRF-1 knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were maintained in a laminar-flow, specific pathogen-free environment. The research protocol was approved by the University of Pittsburgh Animal Care and Use Committee and was in compliance with National Institutes of Health (Bethesda, MD) guidelines. The reagent α-GalCer was purchased from Axxora (San Diego, CA). DMSO was initially used to dissolve α-GalCer to a concentration of 1 mg/ml, and the solution was then diluted to 0.2 mg/ml in 1× PBS containing 0.5% Tween-20. Neutralizing Abs for mouse TNF-α and IFN-γ were obtained from R&D Systems (Minneapolis, MN).

Animal treatment protocols

All animal treatment protocols were in accordance with our institutional animal care guidelines. To study the effect of α-GalCer on IRF-1 and iNOS expression, mice were treated by i.p. injection with 40 μg/kg α-GalCer over a time course from 30 min to 24 h. To induce liver damage, mice were treated by i.p. injection with 100 μg/kg α-GalCer for 24 h, while the control animals received a vehicle that contained an equal amount of DMSO. Neutralizing Abs against TNF-α or IFN-γ were administered i.p. at a dose of 150 μg/mouse 1 h before α-GalCer treatment. Recombinant mouse IFN-α or IFN-β (PBL InterferonSource, Piscataway, NJ) was injected i.p. with 4 × 10^5 U/mouse, and the mice were euthanized 24 h after the injection to get the serum samples for assaying alanine aminotransferase (ALT) levels. All of the reagents for i.p. injection were diluted in sterile normal saline in a final volume of 500 μl. For the use of adenoviral vectors in vivo, the recombinant adenovirus was diluted in 200 μl normal saline and administered by tail vein injection.

Protein sample preparation and Western blotting

Frozen liver tissues were homogenized and total cellular protein samples were prepared. Subcellular fractions were collected by differential centrifugation. The viruses were purified by centrifugation using two sequential cesium chloride gradients. The recombinant adenoviral DNA was linearized and transfected into 293 cells to generate viruses. The viruses were purified by centrifugation using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

RT-PCR

Total cellular RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. An aliquot of 1 μg total RNA was used as a template to synthesize the first-strand cDNA using the Titanium One-Step RT-PCR kit (Clontech, Mountain View, CA). The primers used for semiquantitative RT-PCR analysis of mouse IRF-1 are as following: sense, 5′-TTAGCCGGAGCACATTCTTCTGATTG-3′; antisense, 5′-GTTCC-CCTCGAOGGCCTGCAACTCTTCT-3′. PCR was performed for 35 cycles, with each cycle at 94˚C for 45 s, 60˚C for 45 s, and 68˚C for 1 min.

Isolation of hepatocyte mononuclear cells and flow cytometric analysis

Mice were anesthetized by isoflurane inhalation, their abdomens and thoraces were opened, and blood was drained by cardiac puncture before removal of the liver. The liver was cut into small pieces and gently pressed through a 200-gauge stainless steel mesh. The liver cell suspension was collected and centrifuged at 50 × g for 5 min to remove hepatocytes and tissue debris. The supernatant was then centrifuged at 300 × g for 10 min, and an RBC lysis buffer (eBioscience, San Diego, CA) was added to the cell pellet and resuspended for 5 min at room temperature. After washing twice in PBS, the cells were resuspended in 37% Percoll (GE Healthcare Bio-Sciences, Uppsala, Sweden) in RPMI 1640 medium. The cell suspension was gently overlayed onto 70% Percoll and centrifuged for 25 min at 800 × g. Purified hepatic mononuclear cells (MNCs) were collected from the interface, washed twice in PBS, and resuspended in a FACS staining buffer (PBS supplemented with 2% FBS and 0.09% sodium azide).

To analyze intracellular IFN-γ expression in NKT cells, the hepatic MNCs were first stained with a FITC-conjugated anti-CD3 mAb and anallophycocyanin-conjugated NK1.1 mAb and an allophycocyanin-conjugated NK1.1 mAb (eBioscience) at 4˚C for 30 min. The cells were washed twice in the staining buffer and were then fixed in a fixation/permeabilization solution (BD Biosciences) for 20 min at 4˚C. After fixation, the cells were washed with BD Perm/Wash solution (BD Biosciences) and resuspended in 50 μl of the solution containing either a PE-Cy7–conjugated anti-IFN-γ mAb or a PE-Cy7–conjugated isotype control IgG for 30 min at 4˚C. The stained cells were analyzed on an LSR II system (BD Biosciences) with FACSDiva software (BD Biosciences).

Construction of an adenovirus vector expressing a microRNA to block IRF-1 expression

The IRF-1 microRNA hairpin structure was obtained from Open Biosystems (Huntsville, AL). The IRF-1 microRNA hairpin sequence is: TCCTGTGTTGATATGCTGACCTGCTGTCGACATTCTAGATGTCAGATTTCTATAGGTGACGAGCACAAAGAGTGTATAATCTGCACTCAGCCGAGTTGCTACTGCTCGCGA. The target sequences are: Homo sapiens IRF-1 mRNA (NM_0012918.1) 3′-untranslated region (UTR) 227–245; Mus musculus IRF-1 mRNA (NM_0008590.1) 3′-UTR 235–245; Rattus norvegicus IRF-1 mRNA (NM_012591.1) 3′-UTR 227–245. The IRF-1 microRNA hairpin structure was subcloned into the adenoviral shuttle vector (Vector Biolabs, Philadelphia, PA). The expression cassette was then transfected into a replication-deficient adenovirus genome vector (human adenovirus type 5, dE1E3). The recombinant adenoviral DNA was linearized and transfected into 293 cells to generate viruses. The viruses were purified by centrifugation using two sequential cesium chloride gradients.

Statistical analysis

All data are expressed as mean ± SEM. Comparison of means between two groups was performed using a Student t test. Comparisons between multiple groups were analyzed using one-way ANOVA with a Tukey’s post hoc test. Statistical significance was inferred by p < 0.05. All of the statistical analyses were performed using the GraphPad Prism software (GraphPad Software, San Diego, CA).

Results

Activation of NKT cells in vivo with α-GalCer significantly induces IRF-1 expression in mouse liver

To examine whether IRF-1 expression can be modulated by NKT cell activation, we treated C57BL/6 mice with an i.p. injection of α-GalCer, a synthetic glycolipid that has been used as a potent agonist to selectively activate NKT cells both in vitro and in vivo.
(7–9, 16). The mice received a dose of 40 μg/kg α-GalCer over a time course that lasted 30 min to 24 h. We found that IRF-1 mRNA expression in the liver was dramatically increased 2 h after α-GalCer treatment and remained elevated for up to 24 h (Fig. 1A). Consistent with the mRNA levels, IRF-1 protein expression was also markedly increased in the liver 2–24 h after α-GalCer administration (Fig. 1B). The induction of IRF-1 expression in the liver was not observed in Jc281 knockout mice that are known to have deficient NKT cell development (Fig. 1C). These data demonstrate that α-GalCer treatment induces IRF-1 expression in mouse liver by specifically activating NKT cells.

We and others have shown that mice injected with α-GalCer can produce a broad range of circulating cytokines, including IFN-γ and TNF-α (7–9, 17, 30). To examine whether IFN-γ and TNF-α play a role in α-GalCer–induced IRF-1 expression in vivo, the mice were treated with a neutralizing Ab to either TNF-α or IFN-γ prior to α-GalCer injection. As shown in Fig. 1D, neutralization of TNF-α resulted in a moderate (~30%) decrease in α-GalCer–induced IRF-1 expression, whereas neutralization of IFN-γ caused a more significant (~70%) inhibition of IRF-1 expression induced by α-GalCer. These data indicate that both TNF-α and IFN-γ are involved in α-GalCer–mediated IRF-1 expression in vivo, with IFN-γ being a much stronger mediator, which is in agreement with the well-documented role of IFN-γ as a key inducer of IRF-1 expression (22).

**IRF-1–deficient mice are resistant to α-GalCer–induced liver injury**

We next wanted to determine whether the α-GalCer–mediated IRF-1 expression plays a role in α-GalCer–induced hepatic injury. Serum ALT levels were initially measured to assess liver damage. In wild-type (WT) C57BL/6 mice, a significant increase in serum ALT (mean value, ~2500 IU/liter) was detected 24 h after injection with 100 μg/kg α-GalCer (Fig. 2A). In contrast, IRF-1–deficient mice were markedly protected from liver damage following α-GalCer treatment (Fig. 2A). The liver damage induced by α-GalCer was confirmed histologically by the presence of hepatic necrosis (Fig. 2B). In accordance with the reduced liver injury, IRF-1–deficient mice produced minimal levels of proinflammatory cytokine IFN-γ, TNF-α, and IL-6 both in the circulation and in the liver after α-GalCer treatment (Fig. 2C, 2D). As expected, α-GalCer caused no liver damage in NKT knockout mice, and α-GalCer injection failed to elicit cytokine production in the mice (Fig. 2). These data suggest that IRF-1 plays an essential role in α-GalCer–induced liver damage.

IRF-1 deficiency is associated with defects in multiple cell types involved in Th1 responses, including defective development of NK cells (31, 32). To examine whether there are any defects in NKT cells in the IRF-1–deficient mice, we performed flow cytometric analysis of the mononuclear cells isolated from the mouse liver. Consistent with previous reports, we observed that the basal number of NK (NK1.1+CD3−) cells in the liver was significantly reduced in IRF-1–deficient mice (0.96% vs 5.85% in WT mice, Fig. 2E). Remarkably, we also found a significant decrease in the basal number of NKT cells (NK1.1+CD3+) in the liver of IRF-1–deficient mice (Fig. 2E). Following α-GalCer treatment, the NKT cell fraction decreased in both WT and IRF-1 knockout mice (Fig. 2E). These data suggest that the suppressed α-GalCer–induced liver injury observed in IRF-1–deficient mice could be due in part to the decreased hepatic NKT cell abundance that resulted in inadequate immune responses to the glycolipid Ag α-GalCer.

**Modulation of IRF-1 expression in the liver dictates the severity of liver injury following NKT cell activation by α-GalCer**

Because the IRF-1–deficient mice lack normal numbers of NKT cells in the liver, we decided to block IRF-1 in WT mice to assess the role of IRF-1 in animals with a normal NKT cell complement. Therefore, we developed an in vivo small interfering RNA silencing strategy to specifically knockdown IRF-1 expression in WT mice. We constructed an adeno virus (Ad) vector that

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**FIGURE 1.** α-GalCer injection induces IRF-1 expression in mouse liver. A, C57BL/6 mice were treated by i.p. injections with either vehicle or 40 μg/kg α-GalCer. The mice were euthanized at the indicated time points after the treatment. Total cellular RNA was prepared from the liver tissues, and IRF-1 mRNA levels were assayed by RT-PCR. β-actin mRNA was assayed as an internal control. B, Aliquots of the liver tissues were homogenized to extract nuclear proteins for IRF-1 analysis by Western blot. The nuclear protein histone H3 levels were detected to show equal protein loading. C, C57BL/6 mice and NKT knockout mice (Jc281−/−) were treated by i.p. injection with either vehicle or α-GalCer for 6 h. Nuclear IRF-1 and histone H3 proteins in the liver tissues were assayed by Western blot. D, C57BL/6 mice were pretreated with a neutralizing Ab against mouse IFN-γ, TNF-α, or a control IgG Ab for 1 h, and were then treated with vehicle or α-GalCer for 6 h. The liver tissues were collected and nuclear IRF-1 and histone H3 proteins were assayed by Western blot. The values shown below the blot represent relative intensities of the Western blot signals quantified by using ImageJ software (National Institutes of Health).
expressed a microRNA selectively targeting the IRF-1 message (Ad-IRF1–short hairpin RNA [shRNA]). This recombinant adenovirus was able to effectively knockdown IRF-1 expression induced by IFN-γ in mouse hepatocytes in a dose-dependent manner (Fig. 3A). Infection with an adenovirus vector expressing a scrambled (SCR) shRNA (Ad-SCR–shRNA) had no effect on IRF-1 expression (data not shown). We then injected C57BL/6 mice with the Ad-IRF1–shRNA 3 d prior to α-GalCer treatment and found that prediministration of Ad-IRF1–shRNA significantly inhibited α-GalCer–induced liver injury (Fig. 3B). Importantly, NKT cell abundance in the liver was not altered by infection of the mice with Ad-IRF1–shRNA (Fig. 3C). Additionally, Ad-IRF1–shRNA prediministration did not affect NKT cell activation in response to α-GalCer treatment (Fig. 3C). These experiments demonstrate that the enhanced IRF-1 expression is required for α-GalCer–mediated liver injury.

To further explore the impact of IRF-1 on α-GalCer–induced hepatic damage, we used an adenoviral vector to overexpress WT IRF-1 protein in the liver followed by α-GalCer treatment (Fig. 4A). We found that overexpression of the exogenous IRF-1 significantly enhanced α-GalCer–mediated liver injury (Fig. 4B). Taken together, these data suggest that α-GalCer–induced IRF-1 expression in the liver exerts an important role in mediating hepatic injury following NKT cell activation by α-GalCer.

iNOS expression in mouse liver is upregulated by α-GalCer in an IRF-1–dependent manner

As a transcriptional factor, IRF-1 regulates the expression of many important genes, including iNOS (22, 32, 33). Increased iNOS activity in the liver has been implicated as a contributing factor in a variety of liver diseases, including alcoholic hepatitis and hepatic I/R injury (34, 35). Hence, we sought to determine whether NKT cell activation by α-GalCer had any effects on iNOS expression in the liver. We performed an immunoblotting analysis of iNOS protein levels in mouse livers following a similar time course study of α-GalCer–induced IRF-1 expression. The basal levels of hepatic iNOS protein were undetectable in the control animals; the iNOS protein expression could be detected 4 h after α-GalCer injection and increased thereafter in a time-dependent manner up to 24 h after treatment (Fig. 5A). The iNOS induction by α-GalCer was dependent on IRF-1, as evidenced by the absence of iNOS expression in IRF-1–deficient mice treated by α-GalCer (Fig. 5B). However, α-GalCer treatment was able to induce IRF-1 expression in iNOS knockout mice (Fig. 5C), further supporting that α-GalCer–induced iNOS expression is downstream of IRF-1. Neutralization of IFN-γ in vivo inhibited α-GalCer–induced iNOS expression, which is consistent with the inhibitory effect of IFN-γ on IRF-1 expression (Figs. 1D, 5D). Taken together, these experiments demonstrate that α-GalCer

FIGURE 2. IRF-1–deficient mice are resistant to α-GalCer–induced liver injury. A, WT C57BL/6 mice, IRF-1 knockout mice (IRF1−/−), and NKT knockout mice (Jα281−/−) were treated by i.p. with 100 μg/kg α-GalCer for 24 h. Serum ALT levels were assayed and are presented as mean ± SE (n = 8 mice/group for C57BL/6 mice, n = 6 mice/group for IRF1−/− mice, and n = 4 mice/group for Jα281−/− mice). B, The mouse livers were sectioned and stained with H&E. Liver damage induced by α-GalCer injection was assessed by light microscopy (original magnification ×100). C, Aliquots of serum samples from mice treated as described above were also used to assay IFN-γ, TNF-α, and IL-6 levels by ELISA. D, Aliquots of the mouse liver tissues were homogenized to prepare total protein samples. An aliquot of 1 mg of each protein samples was used to assay IFN-γ, TNF-α, and IL-6 levels by ELISA. E, C57BL/6 mice and IRF-1 knockout mice were treated by i.p. with 100 μg/kg α-GalCer for 6 h. Liver mononuclear cells were isolated and stained for cell surface marker CD3 and NK1.1 for FACS analysis. The numbers shown in the upper right quadrants of each plot are the percentages of CD3+/NK1.1+ NKT cells in the hepatic mononuclear cell population. Shown are representative results of two independent experiments.
induces iNOS expression in mouse liver through IRF-1 in an IFN-γ-dependent manner.

iNOS is an important mediator for α-GalCer–induced liver injury

Next, we wanted to determine whether α-GalCer–induced iNOS expression plays a role in α-GalCer–mediated hepatic injury. We found that iNOS knockout mice had significantly lower transaminase responses to α-GalCer injection compared with the WT controls (Fig. 6A). The iNOS knockout mice had similar numbers of hepatic NKT cells compared with those of the WT mice, and the NKT cells were effectively activated by α-GalCer in iNOS knockout mice (Fig. 6B). These data suggest that iNOS is an important mediator of α-GalCer–induced liver injury.

The cellular origin of IRF-1 expression induced by α-GalCer in mouse liver

We also performed experiments to identify the cell populations within mouse liver that produce IRF-1 upon α-GalCer treatment. After treatment with α-GalCer, C57BL/6 mice were euthanized and the liver was perfused with a collagenase solution. Hepatoocytes were separated from the nonparenchymal mononuclear cells, and the latter fraction of cells was then used to isolate NKT cells by using the MACS system (Miltenyi Biotec, Auburn, CA). We found that both NKT cells and hepatocytes had increased IRF-1 expression upon α-GalCer treatment (Fig. 7A). However, the increased IRF-1 expression in NKT cells themselves did not seem to influence their activation by α-GalCer, because we observed that inhibition of IRF-1 by Ad-IRF1-shRNA did not affect NKT cell activation by α-GalCer (Fig. 3C), and the remaining NKT cells in IRF-1 knockout mice were still able to undergo activation-induced cell death upon α-GalCer treatment (Fig. 2E). Therefore, we propose the following sequential molecular and cellular events that contribute to α-GalCer–induced liver injury (summarized in Fig. 7B): the injected α-GalCer is taken up by the APCs within the liver and is presented to NKT cells in a CD1d-restricted manner; NKT cells are activated to produce cytokines, such as IFN-γ and TNF-α; the cytokines, particularly IFN-γ, in turn stimulate hepatocytes to express IRF-1, which then turns on the expression of many target genes, including iNOS, that ultimately lead to the hepatocyte damage.

Discussion

The liver has been well recognized not only as an important metabolic organ, but also as an important site for immune responses. It is widely accepted that immune-mediated liver injury plays a central role in the pathophysiology of many liver diseases.
The remarkable enrichment of NKT cells in mouse liver has attracted much research attention to explore the role of this unique cell population in the pathogenesis of hepatic diseases, and increasing evidence has suggested that NKT cells are indeed involved in the development of many liver diseases (14). Activation of NKT cells with α-GalCer causes moderate liver injury, which has been recognized as a useful model to investigate immune-mediated liver injury (14, 17–19). The major and novel findings of this study are: 1) α-GalCer injection causes a remarkable increase in IRF-1 expression in mouse liver; 2) α-GalCer-induced IRF-1 upregulation in vivo is TNF-α- and IFN-γ-dependent; 3) IRF-1 plays an important role in mediating α-GalCer-induced liver injury; 4) the IRF-1 target gene iNOS is involved in α-GalCer-induced hepatic damage; 5) IRF-1 is expressed in both hepatocytes and NKT cells following α-GalCer stimulation; and 6) upregulation of IRF-1 in hepatocytes accounts for a major component of the α-GalCer-induced hepatic damage.

IRF-1 is a nuclear transcription factor that has been implicated in host defense against viral pathogens, immune modulation, as well as tumor suppression (22–24). IRF-1 is expressed at a low basal level in most resting cells, and its expression is increased in response to stimuli such as type I and type II IFNs, dsRNA, and certain hormones (22, 38). In this study, we identified that IRF-1 expression can also be strongly induced in vivo by α-GalCer–mediated NKT cell activation. We showed that α-GalCer treatment caused a rapid increase in IRF-1 mRNA and protein expression in mouse liver. This observation is in agreement with previous findings that IRF-1 protein has a very short half-life and that IRF-1 levels are regulated primarily at the transcriptional level (22, 38). IFN-γ is well documented to be a strong inducer for IRF-1 expression (22), and we have previously shown that IRF-1 mRNA is upregulated in cultured rat hepatocytes in response to IFN-γ, TNF-α, and IL-1β stimulation (25). In the present work, we found that TNF-α and IFN-γ are also involved in α-GalCer–mediated IRF-1 expression, with IFN-γ having a much stronger effect. Additionally, we demonstrated that α-GalCer–induced IRF-1 expression is specifically mediated by CD1d-restricted NKT cell activation, as evidenced by the lack of IRF-1 induction in Jα281 knockout mice.

IRF-1 knockout mice have been used as a valuable model to investigate the physiologic functions of IRF-1. To determine the functional significance of α-GalCer–induced IRF-1 expression, we compared the severity of liver injury in WT versus IRF-1 knockout mice following α-GalCer treatment. As compared with WT mice, α-GalCer treatment only resulted in minimal ALT elevation in IRF-1 knockout mice, which is comparable to that in Jα281 knockout mice. These data generated from IRF-1 knockout mice suggest an essential role of IRF-1 in α-GalCer–mediated liver damage.

IRF-1 deletion has been previously shown to result in dysfunction of the immune response. To determine whether a defect in
immune cell populations could account for the dramatic protection in IRF-1 knockout mice, we performed FACS analysis of hepatic lymphocytes. Strikingly, we found that the basal number of hepatic NKT cells was severely reduced in IRF-1 knockout mice. This NKT cell deficiency would be expected to lead to an inadequate immune response to α-GalCer, which likely explains the suppressed α-GalCer–induced liver injury observed in the IRF-1 knockout mice. In addition to the defect in NK and NKT cells, IRF-1 knockout mice have also been characterized to have functional defects in CD4^+ T cells, APCs, and macrophages (22, 23, 31, 39, 40). It is possible that defects in other aspects of the immune response may also account for the blunted response to α-GalCer in the IRF-1 knockout mice. To more precisely define the role of IRF-1 in α-GalCer–induced liver injury, we developed an adenovirus-mediated in vivo shRNA delivery strategy to inhibit IRF-1 in WT mice with intact NKT cell populations. An adenovirus vector expressing a scrambled shRNA was used as a control for nonspecific effects of adenovirus on immune function and liver physiology. We were able to show that transient knockdown of IRF-1 expression in WT mice significantly reduced liver damage induced by α-GalCer, while both NKT cell abundance and activation remained unaltered. Additionally, we observed further enhancement of α-GalCer–induced hepatic injury when exogenous IRF-1 protein was overexpressed in the liver. Taken together, these experiments have established an important role of IRF-1 in α-GalCer–induced liver injury.

As a transcriptional factor, IRF-1 regulates expression of many genes that are involved in immune and inflammatory responses, such as type I and type II IFNs (22, 38). Interestingly, we found that administration of exogenous type I IFN for 24 h did not result in significant liver damage in either WT or IRF-1 knockout mice (data not shown), suggesting that other IRF-1 target genes rather than type I IFN might be involved in the liver injury observed in mice treated with α-GalCer. In murine macrophages, IRF-1 has been shown to control the transcription of iNOS in response to IFN-γ and LPS stimulation (32, 33). We have previously shown that iNOS is upregulated by IRF-1 in cultured murine hepatocytes (26). Increased iNOS activity in the liver has been implicated as a contributing factor for a variety of liver diseases, such as hepatic I/R, hemorrhagic shock, and alcoholic hepatitis (34). In the present study, we found that the NKT cell agonist α-GalCer could stimulate a robust iNOS expression in mouse liver, which is also IRF-1–dependent. We further showed that α-GalCer–induced liver injury was significantly suppressed in iNOS knockout mice, suggesting that iNOS is an important mediator of α-GalCer–induced liver injury, presumably by acting as a downstream effector of IRF-1.

The cytokine TNF-α, but not IFN-γ, has been shown to be an important mediator of α-GalCer–induced hepatotoxicity (17, 20, 21). The effect of TNF-α was linked to an induction of Fas ligand on NKT cells, which may then induce hepatocyte damage by direct cytotoxicity (11, 17, 20). In this work, we showed for the first time that IRF-1 is also critically involved in α-GalCer–induced liver injury and that the upregulation of IRF-1 by α-GalCer is dependent on both TNF-α and IFN-γ signaling. Interestingly, neutralization of IFN-γ was more effective in inhibiting α-GalCer–induced IRF-1 expression compared with TNF-α neutralization. On the basis of these findings, we proposed that there might be multiple mechanisms by which IRF-1 mediates α-GalCer–induced liver injury. First, the effect of IRF-1 appears to be partly achieved by acting as a downstream effector of TNF-α. Second, as described above, the IRF-1 target gene iNOS is implicated in the contribution of IRF-1 to α-GalCer–induced liver injury. Third, IRF-1 might be involved in the regulation of NKT cell-mediated apoptotic damage to hepatocytes in response to α-GalCer treatment. IRF-1 is known to promote apoptosis in many cell types, including hepatocytes (22). Increased Fas ligand expression on activated NKT cells, which then could induce hepatocyte apoptosis by direct cytotoxicity, is considered a potential mechanism for α-GalCer–induced liver injury (17, 20). Our present work showed that increased IRF-1 expression in hepatocytes seems to be directly associated with α-GalCer–induced hepatic damage. The upregulation of IRF-1 in hepatocytes is most likely a result of cytokines released by activated NKT cells. Therefore, the hepatic damage appears to be due to NKT cell-mediated cytotoxicity in combination with the consequences of IRF-1 upregulation in the hepatocytes. Further work will be required to define all the specific mechanisms of IRF-1–mediated toxicity in the liver.

As mentioned before, NKT cell-mediated liver injury has been implicated in the pathogenesis of many liver diseases based on work in animal models. These models include Con A-induced hepatitis and hepatotoxin models using ethanol or acetaminophen (14). Recent evidence also suggests an important role of NKT cells in initiating injury following hepatic warm I/R, as well as limiting liver graft survival following transplantation (12, 13, 15, 41). We have previously shown that IRF-1 expression is upregulated in the liver following both warm and cold I/R injury, and increased IRF-1 expression contributes to the hepatic damage whereas the IRF-1 knockout mice were protected from the injury (26, 27). Importantly, it was the specific presence of IRF-1 in the donor liver graft, but not the recipient host, that accounted for the liver damage (27, 28). Moreover, overexpression of IRF-1 exacerbated routine preservation injury (27, 28). Based on our present and previous observations and known roles for NKT cells in liver I/R injury, it...
is reasonable to hypothesize that NKT cell-induced IRF-1 upregulation is also involved in liver injury in the setting of I/R.

In summary, the present work illustrates that IRF-1 expression in the liver is upregulated following NKT cell activation by o-GaICer. The liver injury induced by o-GaICer is significantly inhibited in IRF-1 knockout mice or in WT mice receiving a microRNA targeting IRF-1, whereas overexpression of IRF-1 worsens the hepatic injury. We also provided evidence showing that the IRF-1 target gene iNOS is involved in o-GaICer–induced liver injury. These findings reveal a novel mechanism for NKT cell–mediated immune liver injury and have implications in the pathophysiology of human liver diseases, such as viral and alcoholic hepatitides and liver I/R injury.

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Disclosures

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References


