Activation of innate immunity (NK/IFN-γ) in rat allogeneic liver transplantation: contribution to liver injury and suppression of hepatocyte proliferation

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1Department of Hepatobiliary Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, China; 2Section on Liver Biology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland; 3Department of Surgery, Johns Hopkins University School of Medicine, Baltimore, Maryland; 4Department of Oncology, The Affiliated Provincial Hospital of Anhui Medical University, Anhui, China

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Shen K, Zheng S-S, Park O, Wang H, Sun Z, Gao B. Activation of innate immunity (NK/IFN-γ) in rat allogeneic liver transplantation: contribution to liver injury and suppression of hepatocyte proliferation. Am J Physiol Gastrointest Liver Physiol 294: G1070–G1077, 2008. First published February 21, 2008; doi:10.1152/ajpgi.00554.2007.—Liver transplantation is presently the only curative treatment for patients with end-stage liver disease. However, the mechanisms underlying liver injury and hepatocyte proliferation posttransplantation remain obscure. In this investigation, liver injury and hepatocyte proliferation in syngeneic and allogeneic animal models were compared. Male Lewis and Dark Agouti (DA) rats were subjected to orthotopic liver transplantation (OLT). Rat OLT was performed in syngeneic (Lewis-Lewis) and allogeneic (Lewis-DA or DA-Lewis) animal models. Allogeneic liver grafts exhibited greater injury and cellular apoptosis than syngeneic grafts but less hepatocyte proliferation after OLT. Expression of IFN-γ mRNA and activation of the downstream signal transducer and activator of transcription 1 (STAT1) and genes (interferon regulatory factor-1 and cyclin-dependent kinase inhibitor p21 (CDKN1A)) were also greater in the allogeneic grafts compared with the syngeneic grafts. In contrast, STAT3 activation was lower in the allogeneic grafts. Furthermore, in the allogeneic grafts, depletion of natural killer (NK) cells decreased IFN-γ/STAT1 activation but enhanced hepatocyte proliferation. These findings suggest that, compared with syngeneic transplantation, innate immunity (NK/IFN-γ) is activated after allogeneic transplantation, which likely contributes to liver injury and inhibits hepatocyte proliferation.

liver regeneration; NK cells; STAT1; IRF-1; p21

CHRONIC ALCOHOL DRINKING, nonalcoholic steatohepatitis, and viral hepatitis are the three major causes of chronic liver injury worldwide, causing end-stage liver diseases such as cirrhosis and hepatocellular carcinoma (46). Presently, the only effective treatment for these end-stage diseases is liver transplantation. Of note, due to the unique immunologic properties of the liver, the rejection rate for liver transplantation is lower compared with transplantation with other organs (1, 39, 40). The immune system of the liver is predominantly innate, enriched with innate immune cells such as Kupffer cells, natural killer (NK) cells, NKT cells, and γδ T cells (9, 15, 36). In contrast, the adaptive immune response is less active in the liver, the major site of induction of T cell apoptosis (4, 5). Reportedly, within an acute allograft rejection model, NK cells are activated posttransplantation, subsequently producing interferon-γ (IFN-γ) to trigger adaptive immune responses and ultimately acute rejection (32). However, the effects of immune cells on liver injury and hepatocyte proliferation posttransplantation remain obscure.

Liver lymphocytes are enriched in NK cells, ~5–10% in mice and 30–50% in rats and humans (9, 15, 36). Not only do NK cells play a pivotal role in host defenses against pathogen invasion and tumor transformation, they are also involved in liver injury and repair (11). In several different experimental models, NK cells have been shown to contribute to liver injury and inhibit liver fibrosis (3, 10, 29, 33, 37). Studies have also shown that activated NK cells can directly kill hepatocytes or activated hepatic stellate cells via tumor necrosis factor-related apoptosis-inducing ligand and NK cell-activating receptor NKG2D-dependent mechanisms (3, 10, 29, 33, 37). In addition, NK cell activation inhibits liver regeneration by blocking hepatocyte and oval cell proliferation in an IFN-γ-dependent manner (18, 41). NK cell activation produces IFN-γ, which targets hepatocytes by binding to IFN-γ receptors and activates the signal transducer and activator of transcription 1 (STAT1) signaling pathway. Activation of STAT1 induces cell cycle arrest and apoptosis in hepatocytes, thereby suppressing liver regeneration (6, 42). In contrast to STAT1, activation of STAT3 appears to promote liver regeneration (17, 27, 31, 47).

In this investigation, we examined the role of NK/IFN-γ on liver injury and hepatocyte proliferation in allogeneic and syngeneic orthotopic liver transplantation (OLT) models, where NK/IFN-γ is strongly activated in the allogeneic grafts but not in the syngeneic grafts. There are two well-established models of allogeneic OLT: the spontaneous acceptance model of Lewis-to-Dark Agouti (DA) (L-DA) rat liver transplants and the rejection model of DA-to-Lewis (DA-L) rat liver transplants. Livers transplanted from DA to Lewis rats are rejected within 9 to 12 days, followed by resulting death, but, conversely, L-DA liver transplants result in tolerance and survival (12, 32). The mechanisms underlying tolerance of L-DA liver transplants remain poorly understood. Previously, it was reported that, in the L-DA model, there is marked lymphocyte proliferation and infiltration, but it is unclear why this immune response does not lead to graft rejection (2, 39). Our findings from this investigation reveal that, although more liver injury...
was observed with the allogeneic (L-DA) grafts compared with the syngeneic grafts, hepatocyte proliferation was lower in the allogeneic grafts. Compared with the syngeneic grafts, higher levels of IFN-γ/STAT1 activation were detected in the allogeneic grafts. Depletion of NK cells decreased IFN-γ/STAT1 activation but enhanced hepatocyte proliferation, suggesting that NK/IFN-γ is likely involved in decreased hepatocyte proliferation in the allogeneic grafts.

MATERIALS AND METHODS

Animals. Inbred male Lewis and DA rats weighing 220–260 g were purchased from Harlan (Indianapolis, IN). All animals were kept in a temperature-controlled environment with a 12-h light-dark cycle, were allowed free access to food and water at all times, and were cared for in accordance with National Institutes of Health guidelines. All animal studies were approved by the ACUC committee of the National Institute on Alcohol Abuse and Alcoholism.

Surgical procedures. Rat OLT was performed under anesthesia with isoflurane inhalation (in O2) as described previously (43). Donor livers were flushed with 15 ml of cold saline solution (40 units/ml) and stored in 4°C until transplanted. The cold ischemia period lasted ~1 h. Three types of transplantation groups were designed: group I syngeneic (Lewis-to-Lewis, L-L), group II allogeneic (L-DA), and group III allogeneic (DA-L). Transplanted rats were killed posttransplantation on days 1, 2, 3, 4, 5, and 7. In our laboratory, transplantation of L-DA results in long-term survival, whereas transplantation of DA-L results in acute organ rejection within 9 to 12 days, consistent with well-established reports (12, 44).

Reverse transcription-polymerase chain reaction (RT-PCR). Experiments were performed as described previously (43). Rat primer sequences used in this study are listed in Table 1.

Immunohistochemistry. Paraffin-embedded sections were cut, deparaffinized, and hydrated by soaking in 100% xylene and descending ethanol, followed by heating treatment and incubation in 0.3% H2O2 in phosphate-buffered saline (PBS) to block endogenous peroxidase activity. Slides were then stained with anti-PCNA or anti-Ki67 antibodies and Avidin/Biotin complex reagent were then applied. Color development was induced by incubation with the DAB kit (Vector Laboratories, Burlingame, CA) for 3 to 5 min, and specific antibody sequences used in this study are listed in Table 1.

TUNEL assay. Staining was performed with an in situ apoptosis detection kit according to the manufacturer’s instructions (Chemicon International, Temecula, CA) and examined by a light microscopy. The terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive hepatocytes were counted randomly in 10 fields (×200) of each slide. The percentage was calculated as number of TUNEL-positive hepatocytes per total number of hepatocytes.

Western blotting. Liver tissues were homogenized in protein lysis buffer (30 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). Western blot analyses were performed with 60 μg of protein from liver homogenates using STAT1, STAT3, phosphorylated STAT (pSTAT1), and pSTAT3 antibodies (1:1,000 dilution; Cell Signaling Technology, Danvers, MA).

Alanine aminotransferase and aspartate aminotransferase. Serum levels of alanine aminotransferase (ALT) and aspartateaminotransferase (AST) were measured using a kit from Drew Scientific (Barrow-in-Furness, UK).

Enzyme-linked immunosorbent assay. Serum levels of IFN-γ were measured using an Enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA). All serum samples were analyzed in triplicate. This assay was determined to have a sensitivity of 10 pg/ml using recombinant rat IFN-γ as a standard (BioSource).

Depletion of NK cells and flow cytometric analysis. To deplete NK cells, donor and recipient rats were injected with anti-Rat NK receptor I (NKRPI) antibody (100 μg/rat) (Endogen, Rockford, IL). After 24 h, depletion of NK cells was confirmed by flow cytometric analysis by anti-rat CD3 and anti-rat NKRPI antibodies (BD Biosciences, San Jose, CA).

Statistical analysis. Data are expressed as means ± SE. To compare values obtained from two groups, the Student’s t-test was performed. To compare values obtained from three or more groups, one-way ANOVA was performed. A value of P < 0.05 was considered significant.

RESULTS

Liver injury and apoptosis are higher in the allogeneic grafts vs. the syngeneic grafts. Results show that serum ALT and AST levels in the allogeneic groups (L-DA, DA-L) on day 1 postsurgery were slightly higher than the syngeneic group (L-L), but this difference did not reach statistical significance (Fig. 1, A–B). On the following days, serum ALT and AST levels in the syngeneic transplant group rapidly decreased. In contrast, ALT and AST values declined on days 2–3 but then started to increase on days 4, 5, and 7 in the allogeneic group. Increased serum levels of ALT and AST were more evident in the DA-L group than the L-DA group.

In view of the increased liver injury found in the allogeneic grafts, we next examined whether the allografts had more apoptosis than the syngeneic grafts by TUNEL assay to detect the presence of apoptotic cells. As shown in Fig. 1C, peak hepatocyte apoptosis occurred four days posttransplant, with the most apoptosis observed in the DA-L group (6%), followed by the L-DA group (3%), and the lowest in the L-L group (1%), which are consistent with previous reports (25). Percentages of apoptotic TUNEL-positive hepatocytes were also higher in the DA-L and DA-L groups on days 3, 5, and 7 posttransplant compared with the L-L group. Similar to previous reports (22, 30), we also observed significantly increased

Table 1. Rat primers for RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
<th>PCR Size</th>
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<tr>
<td>IFN-γ</td>
<td>5′-GCC CTC TCT GCC TCT TAG TG-3′</td>
<td>5′-CTG ATG GCC TGG TGG TCT TT-3′</td>
<td>221 bp</td>
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<tr>
<td>IL-6</td>
<td>5′-GCC GAG AGG AGA CTT CAC AG-3′</td>
<td>5′-ACA GTG CAT CAT CCC TGG TCT-3′</td>
<td>161 bp</td>
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<tr>
<td>IL-2</td>
<td>5′-AAAC CTC CTC GTG ATT ATG CTC AC-3′</td>
<td>5′-GAA ATT CCC TCC AGC TGC CTC CA-3′</td>
<td>156 bp</td>
</tr>
<tr>
<td>IL-15</td>
<td>5′-ATG TGA GGA GCT GGA GGA GA-3′</td>
<td>5′-TCA ACC GTG TCC TGG TAG GC-3′</td>
<td>205 bp</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5′-GCT GCA ACA GAT GAT GAT GA-3′</td>
<td>5′-CTA TCT GGA TGG CCC CTC CA-3′</td>
<td>202 bp</td>
</tr>
<tr>
<td>p21</td>
<td>5′-TCA GTG GAC GAG AGG AGG AC-3′</td>
<td>5′-GCT GCA CAT CCC AGA TAA GT-3′</td>
<td>197 bp</td>
</tr>
<tr>
<td>SOCS1</td>
<td>5′-TGG TAG AGC CTA AGG AGG TG-3′</td>
<td>5′-GAA GGT GGG GAA GAT ATG GT-3′</td>
<td>176 bp</td>
</tr>
<tr>
<td>SOCS3</td>
<td>5′-TGT CAC TGT ACC AGC AGC AA-3′</td>
<td>5′-TGA CAG TCT TGG GAC AA-3′</td>
<td>165 bp</td>
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IFN-γ, IFN regulatory factor-1; p21, cyclin-dependent kinase inhibitor p21CDKN1A; SOCS, suppressor of cytokine signaling.
numbers of apoptotic lymphocytes in the allogeneic grafts. Because this paper is mainly focused on hepatocyte proliferation and injury, the number of apoptotic lymphocytes was not counted in this paper.

Hepatocyte proliferation is lower in the allogeneic grafts vs. the syngeneic grafts. Hepatocyte proliferation was determined by immunostaining with Ki-67 or PCNA antibodies. Results shown in Fig. 2 reveal that peak Ki-67 staining occurred on day 3 after

Fig. 1. Liver injury and apoptosis are higher in the allogeneic grafts vs. the syngeneic grafts. Three pairs of orthotopic liver transplantation (OLT) were performed (Lewis-to-Lewis (L-L), Lewis-to-Dark Agouti (DA) (L-DA), and DA-to-Lewis (DA-L)) for various time points. A and B: sera were harvested for alanine aminotransferase (ALT)/aspartate aminotransferase (AST) measurement. C: liver tissues were harvested and stained with terminal uridine deoxyribonucleotidyl transferase dUTP nick end labeling (TUNEL) assay to examine apoptosis. Top: representative photomicrographs (arrows indicate TUNEL+ hepatocytes). Bottom: number of TUNEL+ hepatocytes was counted. Values in A–C are shown as means ± SE (n = 3 pairs on day 1; n = 5 pairs on days 2 and 5; n = 6 pairs on days 3, 4, and 7). *P < 0.05, **P < 0.01, ***P < 0.001 compared with values from corresponding L-L syngeneic grafts.

Fig. 2. Hepatocyte proliferation is lower in the allogeneic grafts vs. the syngeneic grafts. The liver tissues from Fig. 1 were stained with anti-PCNA or anti-Ki67 antibodies. Left: representative photomicrographs. Right: numbers of Ki67+ and PCNA+ hepatocytes were counted. Values are shown as means ± SE (n = 3–6 as described in Fig. 1). **P < 0.01, ***P < 0.001 compared with values from corresponding L-L syngeneic grafts.
transplantation in all three groups, with the highest in the L-L group (~12%) but only 2–5% in the L-DA and DA-L groups. Similarly, peak PCNA staining was also observed on day 3 posttransplant in all three groups, with the most staining detected in the L-L group.

Upregulation of STAT1 activation and downregulation of STAT3 in the allogeneic grafts vs. the syngeneic grafts. The STAT3 protein has been implicated in promoting liver regeneration, whereas STAT1 has been implicated in inhibition of liver regeneration (6, 17, 27, 31, 42, 47). To define the molecular mechanism by which liver regeneration is suppressed in allogeneic grafts, we examined STAT1 and STAT3 activation in all three groups. As shown in Fig. 3, A–B, low levels of STAT1 activation were detected in syngeneic grafts, with peak activation on day 5. In contrast, stronger STAT1 activation was detected in the allogeneic grafts (L-DA or DA-L), with peak effect occurring three days after surgery. Accordingly, expression of STAT1 protein, which is known to be induced by activated STAT1 (24), was induced and expressed more in the allogeneic grafts (L-DA or DA-L) vs. the syngeneic grafts (L-L).

In contrast to high levels of STAT1 activation, STAT3 activation (pSTAT3) was lower in the allogeneic grafts (L-DA and DA-L) vs. the syngeneic grafts (L-L). As shown in Fig. 3, A–B, peak expression of pSTAT3 was detected in the L-L group on day 2 posttransplant, consistent with previous reports (7, 43). Expression of pSTAT3 was much lower in the L-DA or DA-L allogeneic grafts compared with the L-L syngeneic grafts.

Next, we also examined the expression of cyclin-dependent kinase inhibitor p21^CDKN1A (p21) and IFN regulatory factor (IRF-1), the genes downstream of STAT1, in the transplanted livers. As shown in Fig. 3C, expression of IRF-1 and p21 was significantly higher in the allogeneic grafts than the syngeneic grafts.

Upregulation of hepatic IFN-γ mRNA and serum IFN-γ levels in the allogeneic grafts vs. the syngeneic grafts. To understand the molecular mechanisms by which activation of STAT1 was enhanced while STAT3 was decreased in the allogeneic grafts, we examined the expression of several cytokines in the livers. As shown in Fig. 4A, expression of IFN-γ was much higher in the allogeneic grafts compared with the syngeneic grafts. In the L-L group, IFN-γ mRNA was only significantly induced five days after the surgery, whereas significant elevation of IFN-γ mRNA expression was observed two days after surgery and detected at high levels up to day 7 posttransplant in both L-DA and DA-L groups. Hepatic IL-6 mRNA expression was also induced after OLT, with peak induction on days 2 and 3 postsurgery in the syngeneic grafts, consistent with a previous report (7). Induction of hepatic IL-6 mRNA was greater and prolonged in the L-DA and DA-L groups. Moreover, expression of IL-2 and IL-15 was slightly induced in all three groups, with slightly higher expression in the allogeneic grafts than the syngeneic grafts. Hepatic suppressor of cytokine signaling 1 (SOCS1) and SOCS3 mRNA expression were also significantly induced posttransplant in all groups. This expression was slightly higher in the allogeneic grafts vs. the syngeneic grafts, which is likely due to higher levels of IFN-γ and IL-6 expression in the liver (Fig. 4A) since IFN-γ and IL-6 are responsible for induction of SOCS1 and SOCS3, respectively (19).
Serum levels of IFN-γ were elevated five days post-OLT in the L-L group, which correlated with peak expression of hepatic IFN-γ mRNA (Fig. 4A) and peak activation of STAT1 (Fig. 3A) in this group. Interestingly, serum IFN-γ levels were elevated much earlier and higher in the allogeneic groups compared with the syngeneic groups (Fig. 4B), consistent with a previous report (32). Higher serum IFN-γ levels in the allogeneic groups also correlated with higher levels of hepatic IFN-γ mRNA expression (Fig. 4A) and STAT1 activation (Fig. 3A).

Depletion of NK cells decreases STAT1 activation but enhances liver regeneration in the allografts. It is known that NK cells are activated after OLT and are the major cells responsible for producing IFN-γ (32). To define the role of NK cells in liver regeneration, the NKRPI antibody was used to deplete NK cells in both donor and recipient rats. Injection of the NKRPI antibody for 24 h depleted completely liver NKRPI\textsuperscript{high}CD3\textsuperscript{−} NK cells and depleted 90% NKRPI\textsuperscript{medium}CD3\textsuperscript{−} NKT cells in both donor and recipient rats (only recipient DA rat flow cytometric data are shown in Fig. 5A). Immunohistochemistry analyses showed that the percentages of Ki-67 or PCNA-labeled hepatocytes were higher in the allografts treated with NKRPI antibodies compared with those treated with IgG (Fig. 5, B–C). STAT1 activation and expression were lower in the NKRPI antibody-treated groups than in the IgG-treated groups (Fig. 5D). Similarly, expression of IFN-γ, IRF-1, and p21 decreased in the NKRPI antibody-treated groups compared with the IgG-treated groups (Fig. 5E).

**DISCUSSION**

In this investigation, we demonstrated that liver injury and apoptosis were higher, but hepatocyte proliferation was lower in the allogeneic transplant rats compared with the syngeneic transplant rats. Furthermore, we provided evidence suggesting 1) that upregulation of STAT1 activation and downregulation of STAT3 activation likely contribute to enhanced liver injury but suppressed hepatocyte proliferation in the allogeneic grafts compared with the syngeneic grafts and 2) upregulation of IFN-γ production is responsible, at least in part, for stronger STAT1 activation in the allogeneic groups vs. the syngeneic group. NK cells likely contribute to IFN-γ production.

**Downregulation of STAT3 and upregulation of STAT1 contribute to enhanced liver injury but decreased hepatocyte proliferation in allografts.** Hepatic STAT3 is activated in all forms of liver injury, including liver transplantation (7, 8, 14, 43). STAT3 activation likely contributes to hepatocyte proliferation, as ablation of the hepatic STAT3 gene diminished hepatocyte proliferation after partial hepartectomy or administration of CCl4 (17, 27, 31). Upregulation of IL-6 and STAT3 activation has been detected in liver transplants after prolonged cold ischemia, correlating with increased hepatocyte proliferation (7). Moreover, overexpression of constitutively activated STAT3 via adenoviral gene transfer attenuates liver injury and promotes hepatocyte proliferation in a rat model of 20% partial liver transplantation (20). In contrast to STAT3, STAT1 activation has been shown to inhibit hepatocyte proliferation induced by partial hepatectomy in mice and contribute to IFN-γ inhibition of mouse hepatocyte proliferation (6, 42). The inhibitory effects of STAT1 in hepatocyte proliferation are mediated partly via induction of IRF-1 and p21 gene expression, followed by induction of cell cycle arrest and apoptosis in hepatocytes (42). Interestingly, compared with the syngeneic grafts, STAT3 activation was significantly lower, whereas STAT1 was higher in the allogeneic grafts (L-DA or DA-L) (Fig. 3). Accordingly, the STAT1 downstream genes, IRF-1 and p21, were also upregulated in the allogeneic grafts (Fig. 3).
Here we demonstrated that serum levels of IFN-γ, one of the major cytokines that activate STAT1 in the liver, were higher in DA-to-PVG than in DA-to-DA, but hepatic IFN-γ protein expression was similar in both groups after transplantation (28). The reason for higher levels of hepatic IFN-γ mRNA, but not hepatic IFN-γ protein, in the syngeneic group may be due to the rapid secretion of hepatic IFN-γ protein into the blood stream.

The next obvious question for our laboratory was what kinds of factors are responsible for IFN-γ production in the allogeneic transplantation groups. The findings that treatment with anti-NKRP1 antibodies reduced hepatic IFN-γ mRNA expression in allogeneic transplanted rats (32) suggest that both NK (NKRP1highCD3 antibody) and NKT (NKRP1mediumCD3 antibody) cells seem not involved (Ref. 32 and Dr. Lord et al. 28). Interestingly, Lord et al. (28) reported that hepatic IFN-γ mRNA expression was lower in a model of allogeneic transplantation (DA-to-DA) than in the syngeneic transplantation group (DA-to-DA), but hepatic IFN-γ protein expression was similar in both groups after transplantation (28). The reason for higher levels of hepatic IFN-γ mRNA, but not hepatic IFN-γ protein, in the syngeneic group may be due to the rapid secretion of hepatic IFN-γ protein into the blood stream.

Upregulation of IFN-γ likely contributes to increased STAT1 activation and apoptosis in the allogeneic grafts. IFN-γ is one of the major cytokines that activate STAT1 in the liver (14). Here we demonstrated that serum levels of IFN-γ protein and hepatic IFN-γ mRNA are much greater in the allogeneic groups versus the syngeneic group (Fig. 4) and correlate with STAT1 activation and apoptosis (TUNEL) in the allogeneic grafts. This strongly suggests that higher levels of IFN-γ are responsible for inducing stronger STAT1 activation and apoptosis in the allogeneic group. Interestingly, Lord et al. (28) reported that hepatic IFN-γ mRNA expression was lower in a model of allogeneic transplantation (DA-to-DA) than in the syngeneic transplantation group (DA-to-DA), but hepatic IFN-γ protein expression was similar in both groups after transplantation (28). The reason for higher levels of hepatic IFN-γ mRNA, but not hepatic IFN-γ protein, in the syngeneic group may be due to the rapid secretion of hepatic IFN-γ protein into the blood stream.

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