Inflammatory response in obstructive jaundice and peritonitis
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Endogenous interferon-gamma protects against cholestatic liver injury in mice

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ABSTRACT

Cholestatic patients suffer from high perioperative morbidity and mortality, but the pathophysiology is still unknown. Interferon-gamma (IFN-γ) may play a role during cholestasis. Therefore, bile duct ligation (bdl) was induced in IFN-γ α-chain receptor-deficient (IFN-γR-/-) and wild type (IFN-γR+/+) mice. Bdl elicited increased IFN-γ mRNA and protein levels in the liver. One week after bdl, IFN-γR+/- mice showed less severe jaundice and liver injury than IFN-γR-/- mice as reflected by lower bilirubin and liver enzyme levels. In accordance, livers of IFN-γR+/- mice displayed smaller areas of necrosis by 2 3 than IFN-γR-/- mice upon histopathologic examination (p < 0.05), whereas mitotic activity and PCNA labeling index was more than twice higher in IFN-γR+/- mice (p = 0.05). Livers of IFN-γR+/- mice displayed higher rates of apoptosis as indicated by DNA fragmentation rate, the number of apoptotic bodies, and PARP immunostaining. Bdl was not associated with lethality in IFN-γR+/- mice; IFN-γR-/- mice, however, died from 10 days onwards and survival after 2 weeks was 62% (10/16). In conclusion, these data suggest that IFN-γ protects against liver injury during extrahepatic cholestasis by stimulation of apoptosis and subsequent proliferation of hepatocytes leading to elegant removal of damaged hepatocytes thus preventing necrosis and concomitant inflammatory responses.

INTRODUCTION

The pathophysiology of high perioperative morbidity and mortality of cholestatic patients is largely unknown. Surgical mortality rates are higher than expected independently of the cause of the biliary obstruction either benign or malignant. A variety of physiological abnormalities occurs during obstructive cholestasis, including delayed wound healing with increased rates of wound infection, increased propensity to renal failure, increased plasma endotoxin levels, and cholangitis.

Cholestasis is a main feature of many human liver diseases, including primary biliary cirrhosis, primary sclerosing cholangitis, and chronic allograft rejection following liver transplantation. In many of these cholestatic diseases, the initial insult is directed towards the bile ducts and often mediated by T lymphocytes and cytokines.

IFN-γ is a dimeric glycoprotein that has many immune regulatory functions and induces cell proliferation and apoptosis. IFN-γ is mainly synthesized by natural killer cells and activated T cells. The cell surface receptor complex for IFN-γ is composed of two chains, IFN-γR1 and IFN-γR2. The IFN-γ homodimer binds two molecules of the IFN-γR1 chain. Although the IFN-γR2 chain cannot bind IFN-γ by itself, it is required for IFN-γ signaling and receptor complex formation.

The technique of targeted gene deletion has been shown useful for the elucidation of functions of cytokines. In particular, generation of mice with a homozygous disruption of the IFN-γR1 chain gene (IFN-γR1-/- mice) enables the evaluation of the tissue protective role of IFN-γ in immune response and host defense during cholestasis. IFN-γR1-/- mice are normal, healthy, and fertile without obvious phenotypic differences when compared with wild type mice.
In the present study, we used IFN-γR−/− and wild type mice to determine the role of IFN-γ in hepatic responses to acute biliary obstruction induced by bile duct ligation (bdl).

MATERIALS AND METHODS

Animals
Breeders of 129 Sv/By wild type (IFN-γR−/−) mice and 129 Sv interferon-γ receptor I (γ-chain)-deficient mice (16-20 g) were kindly provided by Dr. Manfred Kopp (Basel Institute for Immunology, Basel, Switzerland). Male mice at the age of 8-10 weeks were used. They were all housed in one animal room, maintained under a 12-hour light/dark cycle with regular mouse chow (SRM-A: Hope Farms, Woerden, the Netherlands) and water ad libitum.

Surgical procedures
The Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, the Netherlands approved all experiments.

Bdl and sham surgery was performed exactly as described previously. Briefly, after 5 days of acclimatization, surgery was performed under sterile conditions (t = 0). Mice were divided randomly into sham-operated control groups and bdl groups. In total, 184 IFN-γR−/− mice and 184 IFN-γR+/− mice were used. Half of the mice in both groups were sham operated and half underwent bdl.

Organ and blood sampling
Mortality was monitored during the first 14 days after sham operation and bdl (n = 16 in each group). In another experiment, animals were sacrificed daily during the first week and at 14 days after sham operation and bdl (n = 8 per group per time point). Since IFN-γR−/− mice started to die from hepatic failure from 10 days onwards (see results), data obtained at 14 days were obtained from animals that were still alive. Blood samples were transferred to tubes containing heparin, and immediately placed on ice; plasma was prepared by centrifugation (3000 x g, 10 min, 4°C), after which aliquots were stored at -20°C. Samples from all liver lobes were removed rapidly after killing the animals, dissected in small portions and either stored at -20°C for biochemistry and histochemistry or fixed for histology.

Histology and histochemistry
Small pieces of liver tissue from left and right liver lobes were fixed in 4% buffered freshly-prepared paraformaldehyde, embedded in paraffin and stained for routine histology (haematoxylin and eosin). Histological examination was performed on coded samples by 2 independent investigators, blinded for treatment groups and biochemical and histochemical parameters. Four histopathologic features were scored semiquantitatively in 4 grades of severity (0-3): portal inflammation, necrosis, ductular proliferation, and fibrosis. The 4 features were equally weighed and summed. This scoring system has been described previously.

To detect proliferating cells, liver specimens were stained for proliferating cell nuclear antigen (PCNA) using a monoclonal antibody (Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked with 0.1% NaN3 (w/v) H2O2 dissolved in 5 ml dimethylformamide and 95 ml acetate buffer (pH 4.9, 50 mM) and 0.03% (v/v) H2O2. As secondary antibody, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Dako) was used. Finally, slides
were stained for HRP activity by incubating the sections with 5 mg/ml 3-amino-9-ethyl carbazole (Sigma, St. Louis, MO) and 0.01% H2O2. To control for background staining, incubations without primary antibody were performed. From each mouse, 4 sections of different liver lobes were stained and the number of PCNA-expressing hepatocytes was determined in 20 randomly selected fields. An average number of positive cells per field was calculated and expressed as PCNA labeling index (PCNA-LI).

Periportal and pericentral zones of liver lobules were compared. Pericentral zones were defined as areas of 5 layers of hepatocytes surrounding terminal branches of hepatic veins. For each liver lobe, 12 sections were used and 3 periportal and pericentral zones were randomly selected in each section.

Double staining immunohistochemistry to localize IFN-γ in relation with CD4-positive cells or macrophages was performed as described previously with minor modifications. Between each step, sections were rinsed 3 times in 0.01 mol/L phosphate-buffered saline (PBS), pH 7.4, unless specified otherwise. Serial sections (6 μm thick) were cut on a cryostat, mounted on gelatin-coated glass slides, and fixed in ice-cold acetone for 10 minutes. Sections were then incubated in a moist dark chamber with 10% normal rabbit serum for 10 minutes and then incubated overnight at 4°C with the primary monoclonal rat anti-mouse IFN- γ antibody (lgG1, clone XMG1.2; Pharmingen, San Diego, CA) diluted in PBS. HRP activity was visualized by incubating sections in a medium containing 50 mg 3,3′-diaminobenzidine tetracloride (Sigma) dissolved in 100 ml Tris-HCl buffer (50 mM at pH 7.6) containing H2O2 in a final concentration of 0.01%. Subsequently, sections were incubated with the second primary antibody, either a monoclonal rat anti-mouse CD4 (clone GK1.5; Pharmingen) antibody or a monoclonal rat anti-mouse pan macrophage (mO) (clone F4-80; Pharmingen) for 1 hour at room temperature. Then, sections were incubated again with a HRP-conjugated secondary rabbit anti-mouse IgG. The second peroxidase activity was visualized with the use of 40 mg 4-chloro-1-naphthol (Sigma) dissolved in 0.2 ml dimethylformamide and 0.3 ml ethanol and added to 100 ml of 50 mM Tris-HCl buffer at pH 7.6 containing 0.01% H2O2. Finally, sections were rinsed in distilled water and mounted in glycerin-gelatin. In control incubations, one of each of the primary antibodies was omitted.

To evaluate hepatocyte proliferation, mitotic figures in 100 hepatocytes were counted in 12 different fields at x 400 magnification in liver sections of 6 mice per group at each time point and were expressed as percentage.

Assays
Total plasma bilirubin, alkaline phosphatase, γ-glutamyl transferase, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were determined with commercially available kits (Sigma) using a Hitachi analyzer (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. Cytokines were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (with detection limits in pg/ml: IFN-γ (15.9), tumor necrosis factor-α (TNF-α) (34.3) (both from R&D Systems, Abingdon, United Kingdom) and interleukin-6 (IL-6) (9.8) (Pharmingen)).

Liver homogenate preparations
For measurements of cytokines, liver homogenates were suspended in 2 volumes of lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 1% Triton X-100, and pepstatin A, leupeptin and aprotinin (all 20 ng/ml; pH 7.4: Sigma) and incubated at 4°C for 30 min. Homogenates were centrifuged at 3,000 x g at 4°C for 10 min to remove cell debris, and supernatants were stored at -20°C until assays were performed.
RNA preparation and reverse transcriptase PCR

Tissue samples were homogenized in 1 ml of TRIzol Reagent (Gibco, Berlin, Germany) according to the manufacturer’s instructions and total RNA was isolated using chloroform extraction and isopropanol precipitation. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and quantified by spectrophotometry. cDNA was synthesized by mixing 2 μg of RNA with 0.5 μg oligo(dT) (Gibco), and by incubating the solution (total volume 12 μl) for 10 minutes at 72 °C. Subsequently, 8 μl of a solution containing 5 × Superscript First Strand Synthesis System for RT-PCR (Gibco), 1.25 mM of each of dNTPs (Amersham, Amersham, United Kingdom), 10 mM dithiothreitol (DTT; Gibco), and the Superscript Pre-amplification system (Gibco) were added. The final solution was incubated for 60 min at 37 °C. For RT-PCR of IFN-γ, equivalent amounts of cDNA (5 μl) were amplified in a solution (20 μl) containing 4% DMSO (Merck, München, Germany), 12.5 μg BSA (Biolabs, Carle Place, NY), 1.25 mM of each of dNTPs, 10× PCR buffer (0.67 M Tris-HCl (pH 8.8), 67 mM MgCl2, 0.1 M β-mercaptoethanol, 67 μM EDTA, 0.166 M (NH4)2SO4), 0.5 U of AmpliTag DNA polymerase (Perkin Elmer, Branchburg, NJ), and the forward (F) and the reverse (R) primers (100 mM each).

The PCR reactions were carried out in the thermocycler Gene AMP® PCR System 9700 (Perkin Elmer, Branchburg, NJ). Cycling conditions for PCR amplification of IFN-γ and β-actin were: at 94 °C for 5 minutes (1 cycle followed by 33 (IFN-γ) or 24 (β-actin) cycles at 95 °C for 60 seconds, at 58 °C for 60 seconds and 72 °C for 60 seconds, followed by a final extension phase at 72 °C for 10 min. The primers used were: IFN-γ (F): 5′-GAAGTGAAGTGGAAGGGXXXAGAAX-.V .. (R): 5′-AGGGAACTGGGAGAGGAGAAATAT-3′; and β-actin (F): 5′-GTCAGAAGGACTCCTATGTG-3′ .. <R: 5′-GCTCGTTGCCAATAGTGATG-3′ . The PCR products were separated on 1.5% agarose gel containing 0.5 x TBE (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH 8.3) with 0.5 μg/ml ethidium bromide. For IFN-γ and β-actin, the number of cycles that resulted in linear amplification was determined (data not shown). Single band intensities of IFN-γ and β-actin were analyzed using the Eagle Eye II video system (Eagle® Software System, Stratagene, La Jolla, CA) using the negative image as described previously. 5

Apoptosis

Morphologic determination of apoptotic bodies

Apoptotic bodies were scored as defined by Kerr and co-workers. 4 The number of apoptotic bodies per 100 hepatocytes was counted in 12 different fields at x 400 magnification in liver sections of 6 mice per group at each time point and expressed as percentage.

DNA fragmentation assay

To quantify apoptosis as characterized by DNA fragmentation, cytoplasmic oligonucleosome-bound DNA was determined using an ELISA kit according to the manufacturer’s instructions (Boehringer). In brief, cytoplasmic fractions of homogenized liver tissues after centrifugation (angle rotor, 4 °C, 15 min, 13,000 x g supernatant) were used as antigen sources in ELISA’s. Percentages of fragmentation in comparison to controls were calculated from the absorbance values as previously described. 3

Immunodetection of poly (ADP-ribose) polymerase (PARP) p85 fragment-positive cells

To rule out false-positivity in the determination of apoptosis based on DNA fragmentation rates due to apoptosis of non parenchymal cells and/or hepatoocyte necrosis, PARP immunohistochemistry was performed as additional methodology.

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Sections of paraffin-embedded liver were deparaaffinized in xylene, rehydrated in a descending series of alcohols, and endogenous peroxidase was blocked by incubation with 0.3% H~2~O~2~ in methanol for 20 min. Liver sections were then incubated in a microwave oven at 100 °C for 10 min in citrate buffer (0.01 M, pH 6). Sections were washed in PBS and incubated with 10% normal goat serum for 15 min. The primary anti-PARP p85 fragment polyclonal antibody (Promega, Leiden, The Netherlands) was diluted in PBS and sections were incubated overnight at 4 °C. After a brief rinse in PBS, sections were incubated for 30 min at room temp with biotinylated-swine anti-rabbit antibody (Dako) in PBS containing 5% normal mouse serum and subsequently with streptavidin-biotin peroxidase complex (Dako) for 30 minutes at room temp. Peroxidase activity was detected by incubation in a solution of 1 mg/ml 3,3-diaminobenzidine tetrahydrochloride (Sigma) and 0.01% H~2~O~2~ in 50 mM Tris-HCl (pH 7.6). Sections were counterstained with haematoxylin. In control incubations, the primary antibody was replaced by PBS. Apoptosis was estimated by counting the number of PARP-positive cells per unit area.

**Statistical analysis**

Statistics were performed using the SPSS Base 10.0 for Windows - Statistical Software Package (SPSS, Chicago, IL). All results are given as mean ± SEM. Survival curves were compared with the log-rank test. Differences in biochemical, immunological, immunohistochemical and histological data were analyzed using the Mann-Whitney U test. Changes in time were analyzed by one-way analysis of variance. Differences at indicated time points were assessed by Bonferroni's test for multiple comparisons where appropriate. A two-tailed p value < 0.05 was considered to indicate significant differences.

**RESULTS**

**Biochemical evidence for increased cholestasis and liver injury in IFN-γR1/-/- mice**

Plasma levels of total bilirubin, alkaline phosphatase, and γ-glutamyl transferase (data not shown for γ-glutamyl transferase) were comparable in all 4 experimental groups before surgery (t = 0), and significant changes did not occur thereafter in both sham groups. In bdl IFN-γR1+/+ and IFN-γR1/-/- mice, these biochemical cholestatic parameters increased comparably during the first 7 days (Fig 1). At 14 days after bdl, when the surviving IFN-γR1/-/- mice were critically ill, these cholestatic parameters were higher in bdl IFN-γR1/-/- mice than in IFN-γR1+/+ mice. Sham and bdl mice exhibited comparable levels of plasma ALT and AST at t = 0. Whereas in sham mice plasma levels of these liver enzymes did not change as compared to preoperative values, bdl was associated with progressive rises in ALT and AST plasma levels with significantly higher concentrations in IFN-γR1/-/- mice from day 2 onwards (p < 0.05). These data suggest that although bdl induced the same extent of cholestasis, IFN-γR1/-/- mice were more prone for cholestasis-induced liver injury.
Chapter 7

Figure 1. Mean (±SE) plasma levels of total bilirubin, alkaline phosphatase (alk. phosph.), AST and ALT in IFN-γR1+/+ and IFN-γR1-/− after bile duct ligation (bdl) or sham operation (n = 8-10 per group at each time point).

Mortality in cholestatic IFN-γR1-/− mice
Sham surgery was not associated with morbidity or mortality during the 2-week follow-up. Bdl IFN-γR1+/+ mice became progressively ill from day 14 onwards, but mortality did not occur up to 4 weeks after bdl. (Fig. 2) In contrast, bdl IFN-γR1-/− mice showed severe signs of illness from day 7 onwards, as reflected by lethargy, anorexia, ruffled fur, and huddling behavior. Mortality in cholestatic IFN-γR1-/− mice occurred from day 10 onwards. At 14 days after bile duct ligation, survival in IFN-γR1-/− mice was 62% (10/16) as compared to 100% in IFN-γR1+/+ mice (p < 0.01).

Figure 2. Mortality in cholestatic IFN-γR1+/+ and IFN-γR1-/− mice. Mortality did not occur in the sham groups (not shown in this figure; n = 16 per group).
Interferon-γ protects against cholestatic liver injury

Production of IFN-γ during cholestasis

After bdl, plasma and liver levels of IFN-γ showed a profound increase in time, reaching a plateau after 3 days (Fig. 3A, B; \( p < 0.01 \)), but were 4 to 8-fold lower in IFN-γR\(_1\)/-/- mice than in IFN-γR\(_1\)/+/+ mice. In addition, RT-PCR confirmed expression of IFN-γ mRNA in livers of both IFN-γR\(_1\)/+/+ and IFN-γR\(_1\)/-/- mice after bdl (Fig. 3C).

Cytokine response to bdl in IFN-γR\(_1\)/-/- mice

Plasma TNF and IL-6 levels in both bdl IFN-γR\(_1\)/+/+ and IFN-γR\(_1\)/-/- mice increased significantly from day 2 after surgery compared to sham mice (Fig. 4). From day 3, plasma TNF and IL-6 levels were significantly higher in bdl IFN-γR\(_1\)/-/- mice than in bdl IFN-γR\(_1\)/+/+ mice. Plasma TNF and IL-6 levels remained undetectable in all sham mice at all time points.

Figure 3. Mean (± SEM) levels of IFN-γ protein in plasma (A) and livers (B) of IFN-γR\(_1\)/+/+ and IFN-γR\(_1\)/-/- mice after bdl or sham operation (\( n = 8 \) per group at each time point, except for bdl IFN-γR\(_1\)/-/- at 14 days, \( n = 5 \)) and IFN-γ and β-actin mRNA levels in livers of IFN-γR\(_1\)/+/+ mice at 4 and 7 days after bdl or sham (\( n = 8 \) per group) (C).

Figure 4. Mean (± SEM) TNF (A) and IL-6 (B) plasma levels in IFN-γR\(_1\)/+/+ or IFN-γR\(_1\)/-/- mice after bdl or sham operation (\( n = 8-12 \) per group at each time point).
Increased liver pathology in cholestatic IFN-γR1-/- mice

Liver histopathology

In both sham IFN-γR1+/+ and IFN-γR1-/- mice, liver tissue appeared to be normal at all time points. IFN-γR1-/- mice sacrificed from 3 days after bdl onwards showed overt lesions in the liver that were macroscopically visible. In all bdl IFN-γR1-/- mice from 4 days after bdl onwards, extensive necrosis of hepatocytes was observed (Fig. 5B), whereas at this time point only slight degenerative changes were observed in hepatocytes without necrosis in sections of bdl IFN-γR1+/+ mice (Fig. 5A). The size of these necrotic lesions in bdl IFN-γR1-/- mice increased dramatically in time, occupying up to 40% of the total area of the liver sections at 14 days after bdl in mice that were still alive (Fig. 5F). Furthermore, these IFN-γR1-/- mice showed significantly more inflammatory cells in portal tracts, predominantly consisting of polymorphonuclear cells (PMN), than bdl IFN-γR1+/+ mice. Moreover, infiltrations of PMN were prominent in liver parenchyma, often causing occlusion of sinusoidal lumens (data not shown). In contrast, IFN-γR1+/+ mice that underwent bdl only showed mild forms of hepatic lesions (Fig. 5A, C, E). Parenchymal necrosis and portal inflammation were observed in livers of these bdl mice from day 3 onwards (Fig. 5A, C), and becoming more severe only after 2 weeks (Fig. 5E).

Liver tissue of bdl IFN-γR1-/-mice showed significantly more damage than liver tissue of bdl IFN-γR1+/+ mice at 7 days after bdl (Table 1). Scores for fibrosis and necrosis were significantly higher, but not for portal inflammation and ductular proliferation. The total score of liver damage after bdl as plotted on a time-scale, shows that liver damage was larger in bdl IFN-γR1-/- mice than in bdl IFN-γR1+/+ mice (Fig. 6).

Immunohistochemistry

Cells invading portal areas included both PMN and mononuclear cells. To define the phenotype of these mononuclear cells, serial liver sections at 7 days after operation were stained for CD4+ T cells and (resident) mΦs (Table 2).

Cholestasis caused a significant rise in the number of infiltrating CD4+ T cells in portal tracts of bdl mice as compared to sham mice, but differences were not found between IFN-γR1+/+ and IFN-γR1-/- mice. In cholestatic livers, the number of CD4+ T cells that were positive for IFN-γ was similar in livers of bdl IFN-γR1+/+ (Fig. 7A) and bdl IFN-γR1-/- (Fig. 7B) mice. The number of (resident) mΦs was significantly increased in livers of bdl mice as compared to sham mice. The number of mΦs in livers of IFN-γR1+/+ and IFN-γR1-/- sham mice was similar; however, the number of mΦs was significantly higher in livers of bdl IFN-γR1-/- mice as compared with IFN-γR1+/+ (p < 0.05). These cells were further characterized in a double staining analysis using the IFN-γ-specific antibody XMG1.2.
Figure 5. Representative H&E-stained liver sections from cholestatic mice \((n = 8-10)\) at 4 (A, B), 7 (C, D), and 14 (E, F) days after bdl. Note the larger size and number of necrotic lesions (arrowheads) in cholestatic IFN-\(\gamma R_1^{-/-}\) mice (B, D, and F) as compared to cholestatic IFN-\(\gamma R_1^{+/+}\) mice (A, C, E). PV = portal vein, CV = central vein. Bar = 100 \(\mu\)m.

Figure 6. Mean \((\pm SEM)\) total histological scores of liver damage in IFN-\(\gamma R_1^{+/+}\) and IFN-\(\gamma R_1^{-/-}\) mice during 14 days after bdl or sham operation \((n = 6-8\) per group at each time point). The total histological score is composed as in Table 1.
Table 1. Histological scores of liver damage in livers of IFN-γR_{1+/-} and IFN-γR_{1/-} mice at 7 days after bdl or sham surgery

<table>
<thead>
<tr>
<th></th>
<th>Mean scores per mouse (n = 6-8 mice per group)^a</th>
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<tbody>
<tr>
<td></td>
<td>Portal inflammation</td>
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<tr>
<td>Sham IFN-γR_{1+/-}</td>
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</tr>
<tr>
<td>Sham IFN-γR_{1/-}</td>
<td>0.0</td>
</tr>
<tr>
<td>Bdl IFN-γR_{1+/-}</td>
<td>1.8</td>
</tr>
<tr>
<td>Bdl IFN-γR_{1/-}</td>
<td>2.0</td>
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</table>

Mean scores in all groups for each of the 4 parameters and the total mean scores (plus range) at 7 days after surgery.

^a Scores with a minimum of 0 and a maximum of 3 on a semiquantitative scale for each parameter.

^b p < 0.001, bile duct ligation (bdl) vs. sham groups.

^c p < 0.05, bdl IFN-γR_{1/-} vs. bdl IFN-γR_{1+/-} mice.

Table 2. Immunohistochemical scores of lymphocytes (CD4+ T cells) and macrophages (mΦs) and IFN-γ producing cells (IFN-γ+) in livers of IFN-γR_{1+/-} and IFN-γR_{1/-} mice at 7 days after bdl or sham surgery

<table>
<thead>
<tr>
<th></th>
<th>CD4+ T cells</th>
<th>MΦs</th>
<th>IFN-γ+ CD4+ T cells</th>
<th>IFN-γ+ mΦs</th>
<th>% IFN-γ producing mΦs</th>
<th>^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham IFN-γR_{1+/-}</td>
<td>4 (2-5)</td>
<td>18 (16-21)</td>
<td>2 (1-3)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sham IFN-γR_{1/-}</td>
<td>3 (1-5)</td>
<td>17 (14-20)</td>
<td>1 (1-2)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bdl IFN-γR_{1+/-}</td>
<td>14 (11-18)</td>
<td>^b 28 (23-30)</td>
<td>12 (9-15) ^b</td>
<td>14 (10-16) ^b</td>
<td>50 ^b</td>
<td></td>
</tr>
<tr>
<td>Bdl IFN-γR_{1/-}</td>
<td>12 (10-17)</td>
<td>^b 35 (32-39)</td>
<td>9 (5-12) ^b</td>
<td>4 (2-8) ^b</td>
<td>11 ^b</td>
<td></td>
</tr>
</tbody>
</table>

CD4+ lymphocytes and mΦs were demonstrated with antibodies F4-80 and GK1.5 respectively, and IFN-γ- producing cells were demonstrated by using antibody clone XMG1.2.

^a Percentage of IFN-γ-producing (resident) mΦs was calculated as the ratio of the number of cells positive with both GK1.5 and XMG1.2 antibodies.

^b p < 0.001, bdl vs. sham groups.

^c p < 0.05, bdl IFN-γR_{1/-} vs. bdl IFN-γR_{1+/-} mice.
In sham mice, no mΦs were found that were positive for IFN-γ. In contrast, mΦs positive for IFN-γ were found both in bdl IFN-γR₁/⁺/⁺ (Fig. 7C) and in bdl IFN-γR₁/-/- (Fig. 7D) mice, indicating that in mice IFN-γ is produced by activated mΦs under cholestatic circumstances. Note that the number of mΦs positive for IFN-γ were significantly lower in IFN-γR₁/-/- mice as compared to IFN-γR₁/⁺/⁺ mice.

![Image](image_url)

Figure 7. Representative liver sections from IFN-γR₁/⁺/⁺ (A, C) and IFN-γR₁/-/- (B, D) mice at 7 days after bdl (n = 8 mice per group) that were double stained for CD4⁺ T cells (arrows) and IFN-γ (A, B) or mΦs (arrow heads) and IFN-γ (C, D). The number of CD4⁺ T cells that were positive for IFN-γ were comparable in bdl IFN-γR₁/⁺/⁺ (A) and bdl IFN-γR₁/-/- (B) mice. However, the number of mΦs that were positive for IFN-γ was larger in livers of bdl IFN-γR₁/⁺/⁺ mice (C) than in bdl IFN-γR₁/-/- mice (D). Bar = 100 μm.

Decreased liver regeneration capacity in cholestatic IFN-γR₁/-/- mice

Liver damage induces increased mitotic activity as assessed by PCNA expression. PCNA staining was virtually absent in sham mice, with an average PCNA-LI of 2 ± 1 at 7 days after operation (Fig. 8A, B). In contrast, proliferative activity was high at 7 days after induction of cholestasis, particularly in periportal zones (Fig. 8C, D). PCNA expression in hepatocytes was significantly higher in bdl IFN-γR₁/⁺/⁺ (Fig. 8C) mice than in bdl IFN-γR₁/-/- (Fig. 8D) mice. The average PCNA-LI in liver sections of bdl IFN-γR₁/⁺/⁺ vs. IFN-γR₁/-/- mice was 42 ± 9 and 22 ± 7, respectively, at 7 days after bdl (p < 0.05).

The number of mitoses in sham mice did not exceed baseline levels (0.43 ± 0.13%) (Fig. 9A). The number of mitoses in bdl IFN-γR₁/⁺/⁺ mice was higher at each time point than in bdl IFN-
γR1-/− mice, reaching peak levels after 4 days and slowly decreasing afterwards. At 7 days of cholestasis, mitotic activity in bdl IFN-γR1+/+ mice had decreased from a peak level of 3.6 ± 0.6% (data not shown) to 2.4 ± 0.4%; this was still significantly higher than 1.6 ± 0.3% in bdl IFN-γR1-/− mice (Fig. 9A; p = 0.01).

Apoptotic body counts at 7 days after operation are shown in Fig. 9B. At this time point, there were hardly any apoptotic bodies in both sham groups, whereas liver sections from bdl IFN-γR1+/+ mice showed significantly more apoptotic bodies than bdl IFN-γR1-/− mice.

Quantification of fragmented DNA revealed that the rate of apoptosis in liver homogenates after 7 days of bdl in IFN-γR1-/− mice was significantly lower than that in IFN-γR1+/+ mice (Fig. 9C). The apoptotic rate had decreased more strongly in IFN-γR1+/+ mice than in IFN-γR1-/− mice at 14 days after bdl but the difference between both bdl groups was still significant (p < 0.05).

![Image of liver sections](image)

**Figure 8.** PCNA staining of liver sections from sham (A, B) and bdl (C, D) animals (n = 8 mice per group) at 7 days after surgery. Arrows indicate PCNA-positive hepatocytes, arrow heads necrotic areas. The number of PCNA-positive cells was decreased and the number of necrotic areas was increased in cholestatic IFN-γR1-/− mice. PV = portal vein, CV = central vein. Bar = 100 μm.

Liver sections were stained with the anti-PARP-p85 fragment antibody that binds to the p85-kDa
caspase 3-cleaved fragment of PARP. BDL mice exhibited significantly more PARP-positive cells as compared with sham mice. This difference was more overt in bdl IFN-γR1/- mice (Fig. 10), confirming both the results of the DNA fragmentation rate analysis and the morphological determination of apoptotic bodies.

**Figure 9.** (A) Mitotic index is presented as mean number (± SEM) of mitotic bodies/100 hepatocytes in livers of sham and bdl animals at 7 days after surgery (n = 8 mice per group). Mitotic index is decreased in IFN-γR1/- mice. (B) Apoptotic activity in livers of bdl IFN-γR1/- mice is decreased as compared to bdl IFN-γR1+/+ mice at 7 days after operation. (C) Mean (± SEM) absorbance values as a measure of DNA fragmentation in liver homogenates from bdl IFN-γR1+/+ and bdl IFN-γR1/- mice at 7 and 14 days of cholestasis (n = 8 per group, except for bdl IFN-

**DISCUSSION**

In the present study, we show that murine cholestasis induced by bdl is associated with enhanced production of IFN-γ in the liver, confirming and extending previous reports. More importantly, this endogenous IFN-γ appeared to play a key role in controlling early cholestatic liver injury. The increased liver injury in bdl IFN-γR1/- mice, as reflected by both clinical chemistry and histopathology, was associated with mortality within 2 weeks after induction of cholestasis, whereas bdl IFN-γR1+/+ mice survived easily up to 4 weeks of cholestasis. Furthermore, liver damage in bdl IFN-γR1+/+ mice was associated with increased apoptosis and liver regeneration, whereas liver damage in bdl IFN-γR1/- mice was characterized by necrosis, large inflammatory infiltrates, and a diminished hepatic regenerative capacity. In addition, we show that not only CD4+ T cells but also mΦs produce IFN-γ during cholestasis in mice. It has been previously reported that murine mΦs can be induced to secrete IFN-γ after combined stimulation with IL-12 and IL-18 or in a TNBS (2,4,6-trinitrobenzene sulfonic acid)-induced
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colitis model.\textsuperscript{23,24} However, as far as we know this is the first study that reports \textit{in vivo} IFN-\(\gamma\) production by mouse liver m\(\Phi\)s. The fact that IFN-\(\gamma\) concentrations were higher in bdl IFN-\(\gamma\)R\(_{1}/+\) mice than in bdl IFN-\(\gamma\)R\(_{1}/-\) mice is in line with earlier studies with IFN-\(\gamma\)R\(_{1}/-\) mice and suggests that the presence of an intact IFN-\(\gamma\)R\(_{1}\) provides an autoregulatory loop to regulate the amount of IFN-\(\gamma\) expressed both locally at the site of inflammation and at remote sites.\textsuperscript{25-27} These data suggest that IFN-\(\gamma\) is an important regulator of apoptosis and regeneration in the liver in response to extrahepatic cholestasis, and that apoptosis protects the host against excessive liver injury in this condition.

Mortality rates due to bile duct obstruction in wild type mice were similar as in previous reports,\textsuperscript{15,28} while Ezure \textit{et al.} reported mortality in cholestatic mice only after more than 9 weeks after bdl.\textsuperscript{29} In this respect, the 100\% survival after bdl in wild type mice that we found is not surprising although mice are less tolerant to bdl than rats.\textsuperscript{30-32}

The extent of TNF production during cholestasis is controversial. Some studies report elevated serum levels of TNF during cholestasis,\textsuperscript{33-36} whereas others report no alterations due to cholestasis.\textsuperscript{2,37,38} In the present study, TNF concentrations increased after induction of cholestasis and were significantly higher in IFN-\(\gamma\)R\(_{1}/-\) mice, likely due to the ongoing necrosis and inflammation, rather than directly caused by the absence of a functional IFN-\(\gamma\)R\(_{1}\) considering that IFN-\(\gamma\) enhances rather than inhibits cytokine production.\textsuperscript{8,39}

Although bdl IFN-\(\gamma\)R\(_{1}/-\) mice showed significantly higher IL-6 plasma levels than their wild

\begin{center}
\textbf{Figure 10.} Immunohistochemical staining of PARP in liver sections from sham (A, B) and bdl (C, D) animals (\(n = 8\) mice per group) at 7 days after surgery. The number of PARP-positive cells (arrows) was decreased and the number of necrotic areas (arrowheads) was increased in cholestatic IFN-\(\gamma\)R\(_{1}/-\) mice. PV = portal vein, CV = central vein. Bar = 100 \(\mu\)m.
\end{center}
type counterparts, this cytokine could not sustain the compensatory increase in liver mass commonly observed in chronic obstructive cholangiopathy. Ezure et al. demonstrated that exogenous IL-6 treatment can contribute to biliary tree integrity and maintenance of hepatocyte mass during chronic liver injury by completely reversing loss of liver mass by stimulating hepatocyte proliferation.\textsuperscript{20} The fact that we found higher IL-6 levels in bdl IFN-\(\gamma\)R\(_1\)/-/- mice than in bdl IFN-\(\gamma\)R\(_1\)+/+ mice indicates that in the acute phase of cholestatic liver injury the initial increase in liver mass during the first week is not IL-6 but IFN-\(\gamma\) dependent despite a brisk upregulation of IL-6 within hours to days after bdl (Fig. 6).\textsuperscript{41}

In accordance with previous studies,\textsuperscript{42,43} PCNA expression at 7 days after induction of cholestasis was higher in periportal and midzonal hepatocytes than in pericentral hepatocytes indicating a wave of cell regeneration particularly in these zones. Akyol et al. did not find a significant relationship between cholestasis and PCNA positivity in samples of human liver in various stages of disease.\textsuperscript{44} However, one should bear in mind that the high PCNA-LI found in the present study is a response to relatively early cholestatic changes, most likely mediated not only by unconjugated bile salts but even more so by cytokines released by inflammatory cells.\textsuperscript{43,45} Indeed, proliferative activity is increased during progression of chronic hepatitis and decreases during progression of cirrhosis.\textsuperscript{46} The significantly higher percentage of PCNA-expressing nuclei in livers from bdl IFN-\(\gamma\)R\(_1\)+/+ mice was also surprising, because TNF has been implicated to play a crucial role in liver regeneration,\textsuperscript{47,49} and TNF levels were higher in bdl IFN-\(\gamma\)R\(_1\)/-/- mice than in bdl IFN-\(\gamma\)R\(_1\)+/+ mice. It is conceivable, that spillover of toxic reagents after liver cell necrosis followed by a rapid and substantial release of pro-inflammatory mediators from Kupffer cells and circulating mononuclear cells resulted in a disturbed regenerative response. Bdl IFN-\(\gamma\)R\(_1\)+/+ mice exhibited higher rates of mitoses that were paired with higher rates of apoptosis as compared with bdl IFN-\(\gamma\)R\(_1\)/-/- mice, so that the number of hepatocytes remained constant and tissue homeostasis was maintained, which is in agreement with previous findings.\textsuperscript{50}

During chronic and subacute liver diseases, cell death and compensatory regeneration occur simultaneously, and the present study proves that IFN-\(\gamma\) plays an important role in maintaining liver homeostasis during acute liver injury. Decreased apoptosis and regeneration of hepatocytes accompanied the increased necrosis in bdl IFN-\(\gamma\)R\(_1\)/-/- mice. Apoptosis occurs in normal liver, albeit in low levels, and in various forms of liver disease. The cytotoxic effect of IFN-\(\gamma\) on hepatocytes is known to involve apoptosis,\textsuperscript{51,52} thus the lower apoptotic rate in bdl IFN-\(\gamma\)R\(_1\)/-/- mice can be explained by the lack of effects of IFN-\(\gamma\). Previous studies have shown that involvement of IFN-\(\gamma\) leads to more programmed cell death and less necrosis,\textsuperscript{53,54} further supporting this interpretation of our data.

In conclusion, our results provide evidence that bdl in mice leads to increased levels of endogenous IFN-\(\gamma\), which is able to induce the apoptotic pathway to prevent necrosis. In this way, liver damage is limited in cholestatic mice with a functional IFN-\(\gamma\) receptor, in contrast with overt necrosis in IFN-\(\gamma\)R\(_1\)/-/- mice. Further insight into the mechanisms of IFN-\(\gamma\)-induced apoptosis and subsequent proliferation of hepatocytes may help providing additional strategies for the treatment of cholestatic liver diseases.
Interferon- protects against cholestatic liver injury


