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# Activating Immunity in the Liver. II. IFN-$\beta$ Attenuates NK Cell-Dependent Liver Injury Triggered by Liver NKT Cell Activation[1]

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**Dendritic cell (DC)-dependent activation of liver NKT cells triggered by a single i.v. injection of a low dose (10–100 ng/mouse) of $\alpha$-galactosyl ceramide ($\alpha$GalCer) into mice induces liver injury. This response is particularly evident in HBs-tg B6 mice that express a transgene-encoded hepatitis B surface Ag in the liver. Liver injury following $\alpha$GalCer injection is suppressed in mice depleted of NK cells, indicating that NK cells play a role in NK T cell-initiated liver injury. In vitro, liver NKT cells provide a CD80/86-dependent signal to $\alpha$GalCer-pulsed liver DC to release IL-12 p70 that stimulates the IFN-$\gamma$ response of NKT and NK cells. Adoptive transfer of NKT cell-activated liver DC into the liver of nontreated, normal (immunocompetent), or immunodeficient (RAG$^{-/-}$ or HBs-tg/RAG$^{-/-}$) hosts via the portal vein elicited IFN-$\gamma$ responses of liver NK cells in situ. IFN-$\beta$ down-regulates the pathogenic IL-12/IFN-$\gamma$ cytokine cascade triggered by NKT cell/DC/NK cell interactions in the liver. Pretreating liver DC in vitro with IFN-$\beta$ suppressed their IL-12 (but not IL-10) release in response to CD40 ligation or specific ($\alpha$GalCer-dependent) interaction with liver NKT cells and down-regulated the IFN-$\gamma$ response of the specifically activated liver NKT cells. In vivo, IFN-$\beta$ attenuated the NKT cell-triggered induction of liver immunopathology. This study identifies interacting subsets of the hepatic innate immune system (and cytokines that up- and down-regulate these interactions) activated early in immune-mediated liver pathology.** ***The Journal of Immunology,*** **2002, 168: 3763–3770.**

Injection of low doses of $\alpha$-galactosyl ceramide ($\alpha$GalCer)[3] into mice activates NKT cells and elicits liver immunopathology. Liver dendritic cells (DC) (but not hepatocytes) that present $\alpha$GalCer to CD1d-restricted liver NKT cells are activated and stimulate proinflammatory cytokine release. The liver DC express an immunostimulatory phenotype after the NKT cell population has been eliminated following $\alpha$GalCer stimulation (1). The NKT cell-triggered liver injury thus involves two phases. The initiating NKT cell/DC interaction activates DC and leads to IL-12/IFN-$\gamma$/IL-4 release. Subsequently, activated liver DC seem to recruit additional cell subsets into the response that release proinflammatory cytokines. Two important questions are: which cell subsets propagate the response, and which regulation can control the inflammatory response in situ? NKT cell activation rapidly induces NK cell proliferation, cytotoxicity, and cytokine release in vivo that depend on DC-derived IL-12 and NKT cell-derived IFN-$\gamma$ (2–4). We tested whether NK cells are involved in the manifestation of NKT cell-stimulated liver immunopathology.

TCR/CD1d-dependent activation of NKT cells is initiated when they recognize glycolipid-presenting cells (APC). $\alpha$GalCer-pulsed DC activate human and murine NKT cells (5–8). This TCR/CD1d-dependent NKT cell stimulation induces CD40/CD40 ligand-dependent IL-12 production by DC and IFN-$\gamma$ release by NKT cells (6). The type and maturity of stimulating APC and responding NKT cells as well as the cytokine/costimulation signals operating during their interaction determine the phenotype of the response. Neonatal and adult NKT cells differ in the cytokine profile they express after expansion with DC (9). Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways is suggested by the observation that blocking B7.2 (CD86) costimulation during $\alpha$GalCer stimulation of NKT cells by DC biases their cytokine profile toward Th2, whereas presentation of $\alpha$GalCer by CD40-activated DC primes a Th1 response (10, 11). In addition, NKT cell activation can be induced by cytokines (independent from TCR/CD1d engagement). IL-18-, IL-18- plus IL-12-, and IL-12-induced pathways to fully activate NKT cells have been reported (12–14). The study of NKT cell/DC interactions in the liver may help to understand liver immunopathology, because tissue-specific activation of DC by NKT cells induces strikingly different immune responses.

The liver contains a large compartment of cells of the innate immune system (NK cells, NKT cells, DC, and macrophages) that have the potential for rapid release of proinflammatory cytokines with potent synergistic actions (e.g., TNF, IL-12, IL-18, and IFN-$\gamma$). Dysregulated release of these cytokines can trigger extensive liver injury. It is of interest to identify (constitutively present or rapidly inducible) regulatory cells or cytokines in the hepatic microenvironment that can limit the action of proinflammatory cytokines. In this study, we have identified the type I IFN-$\beta$ as a factor that can suppress CD40-dependent IL-12 (but not IL-10) release by $\alpha$GalCer-pulsed DC interacting with NKT cells. Thereby,

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[3] Abbreviations used in this paper: $\alpha$GalCer, $\alpha$-galactosyl ceramide; ALT, alanine transaminase; DC, dendritic cell; FCM, flow cytometry; KO, knockout; tg, transgene; MNC, mononuclear cell; MHC-II, MHC class II.



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IFN-γ (but not IL-4) release by NKT cells was suppressed. In vivo, the activation of NK cells in the response was inhibited. Through these mechanisms, IFN-β strikingly attenuated the liver injury response triggered by NKT cell stimulation.

## Materials and Methods

### *Mice*

C57BL/6J mice (H-2$^b$) (B6), MHC class II (MHC-II)$^{-/-}$ (A$\beta^{-/-}$ knockout (KO)) B6 mice, and RAG1$^{-/-}$ mice were kept under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). C57BL/6J-TgN(Alb1HBV)44Bri transgenic (HBs-tg) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). RAG1$^{-/-}$ HBs-tg mice were generated in the animal colony of Ulm University by cross-breeding and backcrossing RAG1$^{-/-}$ and HBs-tg mice. Mice were used at 10–16 wk of age.

### *Injection of αGalCer and IFN-β*

αGalCer dissolved in 0.5-ml PBS was injected i.v. into mice (100 ng/mouse). IFN-β was injected i.v. ($10^4$ U/mouse). The IFN-β preparation was obtained from BHK21 cells expressing the murine IFN-β under control of the constitutive myeloproliferative syndrome virus promoter. A confluent culture of stably expressing cells was incubated in serum-free DMEM for 24 h and cleared by centrifugation. IFN-β activity was determined to be $2 \times 10^4$ U/ml by an anti-viral test. As control supernatants, nonexpressing BHK21 master cell supernatants were used.

### *In vivo suppression of NK cells*

NK cells were eliminated by i.p. injections of 15 μl anti-asialoGM1 (αAsGM1) Ab (Wako Pure Chemical Industries, Osaka, Japan) twice daily for 5 days. Flow cytometric (FCM) analyses of PBMC populations demonstrated that >95% of the NK1$^{high}$ CD3$^-$ NK cells were depleted 24 h after initiation of the treatment.

### *Serum alanine transaminase (ALT) determination*

Blood was collected from the tails of mice and centrifuged at 5000 × *g* for 10 min, and serum was collected. Serum ALT levels were determined within 1 h after obtaining the blood using the Reflotron test (catalog no. 745138; Roche, Mannheim, Germany) following the manufacturer's instructions.

### *Isolation of liver mononuclear cell (MNC) populations*

Mice were anesthetized by methoxyfluran (Metofane; Janssen-Cilag, Neuss, Germany), and the abdomen was opened. A needle was inserted into the portal vein. The inferior caval vein was cut to enable blood outflow. The liver was perfused with 20-ml liver perfusion medium (catalog no. 17701-038; Life Technologies, Eggenstein, Germany), followed by an injection of 5-ml liver digestion medium (catalog no. 17703-034; Life Technologies). The liver was removed and gently pressed through a mesh. The liver cell suspension was collected, and parenchymal cells (pellet) were separated from MNC (supernatant) by centrifugation at 50 × *g* for 5 min. MNC populations were purified by centrifugation through a Percoll gradient. Cells were collected, washed in PBS, and resuspended in 40% Percoll (catalog no. L6145; Biochrom, Berlin, Germany) in complete RPMI 1640 medium. The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 min at 750 × *g*. MNC were collected from the interface, washed twice in PBS, and resuspended in medium.

### *Splenic DC*

Spleens were cut into small pieces and incubated for 45 min in RPMI 1640 medium containing collagenase I (0.5 mg/ml) and DNase (0.1 mg/ml). EDTA (at a final concentration of 10 mM) was added for the last 3 min of the incubation period. The tissue pieces were minced through a nylon mesh. Single cells were harvested, washed, resuspended in NycoPrep (Nycomed, Oslo, Norway) overlaid with RPMI 1640 medium, and centrifuged at 4°C at 9500 × *g* for 20 min. Cells on the interface were collected and washed twice. CD11c$^+$ DC were purified by MACS.

### *FCM analyses of the surface phenotype and intracellular cytokine expression*

Cells were suspended in PBS/0.3% (w/v) BSA supplemented with 0.1% (w/v) sodium azide. Nonspecific binding of Abs to FcR was blocked by preincubating cells with the mAb 2.4G2 (catalog no. 01241D) directed against the FcγRIII/II CD16/CD32 (1 μg mAb/$10^6$ cells/100 μl). Cells were incubated with 0.5 μg/$10^6$ cells of the relevant mAb for 30 min at 4°C and washed. In most experiments, cells were subsequently incubated with a second-step reagent for 10 min at 4°C. The following reagents and mAb were obtained from BD PharMingen (Hamburg, Germany): FITC-conjugated and biotinylated anti-CD3ε mAb 145-2C11 (catalog no. 553062 and 01082D, respectively). PE-conjugated anti-NK1.1 mAb PK136 (catalog no. 553165) and PE-conjugated anti-CD1d mAb 1B1 (catalog no. 553846), biotinylated anti-CD44 (Pgp-1) mAb IM7 (catalog no. 01222D), biotinylated anti-CD69 mAb Kay-10 (catalog no. 01502D), biotinylated anti-CD95 ligand mAb H1.2F3 (catalog no. 09932D), and biotinylated anti-CD28 mAb 37.51 (catalog no. 01672D). Streptavidin-Red670 was obtained from Life Technologies (Berlin, Germany; catalog no. 19543-024).

MNC from normal B6 mice ($10^6$ cells/ml) were stimulated with 20 ng/ml IL-12 and/or IL-18 in RPMI 1640/10% FCS for 12 h at 37°C with 5% $CO_2$ with 10 μg/ml brefeldin A added for the last 5 h. Cells were harvested, washed twice in staining buffer (PBS without $Mg^{2+}$/$Ca^{2+}$, 0.3% (w/v) BSA, and 0.1% (w/v) sodium azide), incubated (15 min, 4°C) with purified 2.4G2 Ab to block nonspecific binding of Ab to FcRs, washed with staining buffer, resuspended in staining buffer, and surface-stained with the relevant Abs. Cells were washed with staining buffer, labeled with the second-step reagent, and washed twice. Cells were then resuspended in 100 μl Cytofix/Cytoperm solution (BD PharMingen; catalog no. 2090KZ) for 20 min at 4°C and washed twice in 1 ml 1× Perm/Wash solution (BD PharMingen; catalog no. 554723). Fixed and permeabilized cells were resuspended in 100 μl 1× Perm/Wash solution. Cells were stained for 30 min at 4°C with 1 μg mAb/$10^6$ cells of FITC-conjugated anti-IFN-γ mAb XMG1.2 (BD PharMingen; catalog no. 18114A) or appropriate negative control Ab (FITC-conjugated rat IgG1 mAb R3-34; BD PharMingen; catalog no. 20614A). Cells were washed twice in 1× Perm/Wash solution (250 × *g*) and resuspended in staining buffer; $10^4$ cells were analyzed by flow cytometry using a FACScan equipped with a 15-mW argon laser (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences). The forward narrow angle light scatter was used as an additional parameter to facilitate exclusion of dead cells and aggregated cell clumps.

### *Purification of CD4$^+$ and CD11c$^+$ cells*

Spleen and liver MNC cells were obtained from normal B6, HBs-tg, or MHC-II-deficient A$\beta^{-/-}$ B6 mice. CD8$^+$ T cells were depleted from these spleen cells by treatment with anti-CD8 Ab and low toxicity rabbit complement (catalog no. CL3051; Cedarlane Laboratories, Hornby, Ontario, Canada) following the manufacturer's instructions. CD11c$^+$ cells were isolated from NK cell-depleted mice (pretreated with two injections of αAsGM1 antiserum) to avoid NK cell contamination in the liver CD11c$^+$ DC fraction. CD4$^+$ T cells and CD11c$^+$ DC were enriched to >98% purity by positive selection using MACS (Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, $10^7$ cells were incubated with mouse CD4 (L3T4) microbeads (catalog no. 130-049-201; Miltenyi Biotec) or mouse CD11c (N418) microbeads (catalog no. 130-052-001; Miltenyi Biotec) in 100 μl PBS buffer supplemented with 2 mM EDTA and 0.5% BSA for 30 min at 4°C. Cells were washed twice in buffer, resuspended in 500 μl buffer, and transferred onto a prerinsed LS separation column (catalog no. 130-042-401; Miltenyi Biotec) attached to a MidiMACS separation unit (catalog no. 130-042-302; Miltenyi Biotec). After three washes with 3-ml buffer, columns were removed from the separation unit. To elute bound CD4$^+$ or CD11c$^+$ cells, 6-ml buffer was passed through the columns using a plunger supplied with the columns.

### *Cell cultures*

Cells were cultured in 200-μl flat-bottom microwells in RPMI 1640 medium supplemented with 5% FCS. MACS-purified CD11c$^+$ liver DC were pulsed in vitro with 100 ng/ml αGalCer for 2 h. DC were washed, and $2 \times 10^4$ DC/well were cocultured for 48 h with liver CD4$^+$ NKT cells ($1 \times 10^5$/well) from MHC-II$^{-/-}$ (A$\beta^{-/-}$ KO) B6. Supernatants were collected after 24 and 48 h of culture for cytokine determination. Costimulatory signals in DC/NKT cocultures were blocked by anti-CD80 mAb 16-10A1 (catalog no. 09601D; BD PharMingen) and anti-CD86 mAb GL-1 (catalog no. 09271D; BD PharMingen). Isotype control mAb A110-2 (catalog no. 11191D; BD PharMingen) and B81-3 (catalog no. 11221D; BD PharMingen) were used for the negative control. For the CD40 stimulation, supernatant of the FGK 45.5 hybridoma (anti-mouse CD40) was used. Liver CD11c$^+$ DC cells were pretreated with 500 U/ml IFN-β in the RPMI 1640/10% FCS for 2 h at 37°C with 5% $CO_2$ and washed twice. IFN-γ production from liver NK cells was stimulated with 20 ng/ml IL-12 and/or 20 ng/ml IL-18.

### *Cytokine detection by ELISA*

Cytokines released into culture supernatants were detected by a double-sandwich ELISA. For detection and capture, the following mAbs (from BD PharMingen) were used: mAb R4-6A2 (catalog no. 18181D) and biotinylated mAb XMG1.2 (catalog no. 18112D) were used for IFN-γ, mAb BVD4-1D11 (catalog no. 18031D) and biotinylated mAb BVD6-24G2 (catalog no. 18042D) were used for IL-4, mAb 9A5 (catalog no. 20011D) and biotinylated mAb C17.8 (catalog no. 18482D) were used for IL-12 p70 detection, and mAb JES5-2A5 (catalog no. 554422) and biotinylated mAb SXC-1 (catalog no.554423) were used for IL-10 detection. Extinction was analyzed at 405/490 nm on a TECAN microplate ELISA reader (TECAN, Crailsheim, Germany) using EasyWin software (TECAN).

### *CTL assays*

Liver MNC were harvested and washed, and serial dilutions of effector cells were cultured with $2 \times 10^3$ $^{51}$Cr-labeled YAC-1 targets in 200-μl round-bottom wells. Specific cytolytic activity of cells was tested in a $^{51}$Cr release assay. After 4-h incubation at 37°C, 50-μl supernatant was collected for gamma-radiation counting. The percentage of specific release was calculated as ((experimental release − spontaneous release)/(total release − spontaneous release)) × 100. Total counts were measured by resuspending target cells. Spontaneous release was <10% of the total counts. Data shown are the mean of triplicate cultures. The SD of triplicate data was always <5% of the mean.

## Results

### *NKT cell-induced activation of liver NK cells*

Livers of normal B6 mice contain ~$2 \times 10^5$ (NK1$^+$ CD3$^-$) NK cells that represent 10–20% of the liver MNC population (1). Hepatic lymphoid cell populations from RAG1$^{-/-}$ and RAG1$^{-/-}$ HBs-tg B6 mice contained a higher fraction of NK cells, but similar total numbers of NK cells. Liver NK cells were CD1d$^{low}$ but CD44$^{high}$ and expressed low, but readily detectable, levels of CD95 ligand and CD28 (Fig. 1). About 30% of liver NK cells were CD11c$^+$. A similar surface phenotype was found in liver NK cells from normal and RAG1$^{-/-}$ B6 mice. No (or low) expression of the activation marker CD69 was detected on the surface of liver NK cells from nontreated, normal, or immunodeficient B6 mice. NK cells from the liver and spleen of B6 mice from our standard pathogen-free colony showed no spontaneous IFN-γ release and only low cytolytic activity against the NK cell target YAC-1 (Fig. 1 and data not shown).

The i.v. injection of 10 or 100 ng αGalCer activated splenic and hepatic NK cells, confirming previous reports (2–4, 15, 16). After injection of αGalCer, liver and spleen NK cells from normal, but not NKT cell-deficient, RAG1$^{-/-}$ B6 mice up-regulated CD69 surface expression, released IFN-γ, and displayed high cytolytic activity against YAC-1 targets (Fig. 1 and data not shown). Specific stimulation of NKT cells is thus a potent activator of NK cells in liver and spleen.

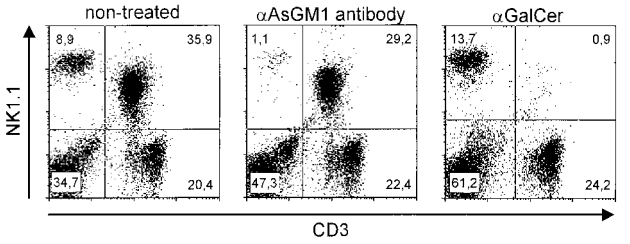


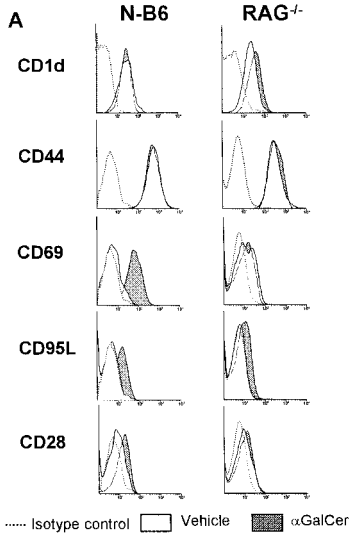

**FIGURE 2.** Selective elimination of NK and NKT cells from the murine liver. Liver (NK1$^{high}$ CD3$^-$) NK cells and (NK1$^{int}$ CD3$^+$) NKT cells from αAsGM1 Ab, αGalCer-treated, or control B6 mice. Mice were injected 18 h previously with either 100 ng αGalCer (in 500 μl) i.v. or two injections of 15 μl αAsGM1 Ab i.p. (according to the manufacturer's instructions) or 500 μl vehicle solution i.v. Surface expression of CD3 and NK1.1 was analyzed by FCM. A representative example (from three independent experiments) is shown.

### *Efficient and selective in vivo elimination of NK and NKT cell populations*

A single injection of a low dose of αGalCer into mice eliminates the CD1d-dependent NKT cell population within hours (17–19). This is confirmed by the data shown in Fig. 2: a >30-fold reduction is observed in the number of liver (NK1$^+$ CD3$^+$) NKT cells after a single i.v. injection of 100 ng αGalCer into normal B6 mice. This treatment has no effect on the number of liver (NK1$^+$ CD3$^-$) NK cells. Repeated injections of anti-asialoGM1 (αAsGM1) antiserum into normal or RAG1$^{-/-}$ B6 mice eliminated >90% of the (NK1$^+$ CD3$^-$) NK cells (Fig. 2). Treatment of mice with injections of αGalCer and αAsGM1 antiserum eliminated both NK and NKT cell populations from liver and spleen. Hence, effective treatments are available to selectively eliminate in vivo the NK cell or the NKT cell population, or both.

### *NKT cell-induced, NK cell-dependent liver injury*

Injection of αGalCer into HBs-tg B6 mice that are hypersensitive to IFN-γ induces severe, transient liver injury, which is apparent by a striking rise in serum transaminase levels and histopathology



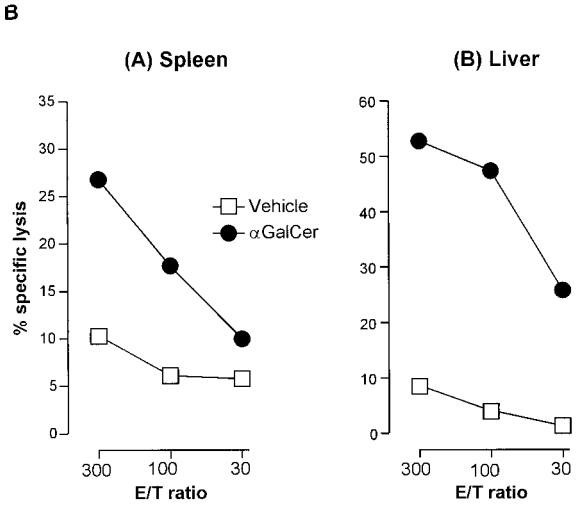




**FIGURE 1.** *A*, Surface phenotype of liver NK cells. MNC were obtained from the livers of B6 mice 18 h after the i.v. injection of either 100 ng αGalCer or control vehicle. Cells were stained with Abs and analyzed by three-color FCM. NK1.1$^{high}$ CD3$^-$ NK cells were gated; histograms of marker expression of the gated cells are shown. Representative examples of an individual mouse per group (from four mice analyzed) are shown. *B*, NKT cell activation enhances spleen and liver NK cell cytotoxicity against YAC-1 targets. Liver and spleen MNC were isolated from B6 mice injected 18 h previously with 100 ng αGalCer (or vehicle). These cells were cocultured with $^{51}$Cr-labeled YAC-1 targets for 4 h at the indicated E:T cell ratios. Results are mean values of triplicate determinations of the percent-specific $^{51}$Cr release.

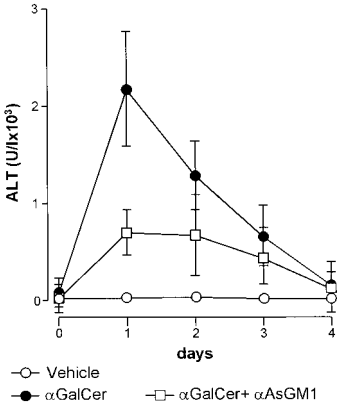



**FIGURE 3.** NKT cell-induced liver injury is NK cell-dependent. HBs-tg mice were injected i.p. with either 15 μl αAsGM1 antiserum or 15 μl control antisera twice daily for 2 days before and 4 days after the i.v. injection of 100 ng αGalCer or vehicle. Mean serum ALT values (±SEM) from groups of four mice were measured daily for 4 days postinjection.

manifested 24–72 h postinjection (1, 20). We injected αGalCer into HBs-tg B6 mice that either contained NK cells or were depleted of NK cells by repeated injections of αAsGM1 antiserum (Fig. 3). The liver injury of HBs-tg B6 mice depleted of NK cells was reduced 2- to 8-fold in six independent experiments, as evident from the serum ALT levels of the mice. NKT cell-stimulated NK cells are thus involved in triggering liver immunopathology.

The histology of the liver of untreated HBs-tg mice was characterized by diffusely enlarged hepatocytes with a pale eosinophilic cytoplasm, reminiscent of the "ground glass" appearance found in HBV-associated hepatitis in humans. Inflammatory infiltrates were minimal and mostly confined to periportal areas. Isolated apoptotic hepatocytes were scarce. Injection of αGalCer resulted in profound changes in the liver histology of HBs-tg mice. Large, occasionally confluent areas of necrotic parenchyma were striking. Necrotic cells showed enhanced eosinophilic staining of the cytoplasm combined with a complete loss of nuclei. In addition, high numbers of apoptotic cells were diffusely scattered throughout the liver. Periportal fields, especially the lobular parenchyma, displayed dense inflammatory infiltrates of mononuclear cells and neutrophils. Portal venules and central veins showed severe endothelialitis resulting in extensive desquamations of the endothelium and focal formation of small fibrin plugs. When animals were depleted of NK cells before the αGalCer injection, extensive necrosis of liver parenchyma was not observed, while the other histopathological alterations found in NK cell-competent, αGalCer-treated HBs-tg mice were unaffected (data not shown). The histopathological findings thus correlated with the serum transaminase responses.

### *CD80/86-dependent interaction of αGalCer-pulsed DC with NKT cells stimulates their IL-12 p70 release*

Injection of rIL-12 p70 triggers liver injury in immunocompetent and immunodeficient HBs-tg B6 mice (1) (data not shown). IFN-γ is a key effector molecule that triggers liver cell injury in HBs-tg mice (21). Although low levels of CD1d are detectable on their surface (Fig. 1), αGalCer-pulsed liver NK cells stimulated neither IFN-γ nor IL-4 release by cocultured, purified liver NKT cells in vitro (data not shown). In contrast, αGalCer-pulsed DC (but not hepatocytes) stimulated IFN-γ release by cocultured liver NKT cells (1). Therefore, DC-derived IL-12 could play a role in initiating IFN-γ-dependent liver injury.

We tested whether αGalCer-pulsed DC release IL-12 p70 when cocultured with liver NKT cells. Purified liver CD11c$^+$ DC were pulsed with αGalCer. IL-12 p70, IL-4, and IFN-γ were detected in the supernatants of cocultures of these pulsed (but not nonpulsed) DC with purified liver NKT cells (Fig. 4, *groups 1* and *5*). CD80/86-dependent costimulation was required to support IL-12 release by αGalCer-presenting stimulator DC as well as IL-4 and IFN-γ release by NKT responder cells (Fig. 4, *group 6*). Adding a neutralizing anti-IL-12 mAb to the cultures strikingly reduced the IFN-γ response of liver NKT cells stimulated by αGalCer-pulsed liver DC (data not shown). CD40 ligation stimulated IL-12 release by DC (*group 3*) and enhanced IL-12 release in cocultures of pulsed DC with NKT cells (*group 7*). The induced or enhanced IL-12 release by CD40-stimulated DC was strictly dependent on CD80(86) costimulation (*groups 4* and *8*). Hence, liver DC interacting with liver NKT cells require CD80(86)-dependent signals to release bioactive IL-12 p70.

IL-12 stimulated (alone or in synergy with IL-18) IFN-γ release by freshly isolated, nonstimulated liver NKT cells and NK cells (Fig. 5). These NKT cells and NK cells released IFN-γ in response to IL-12 or IL-18 alone, but a larger fraction of cells from these lymphoid liver cell subsets could be induced to express IFN-γ by stimulation with both IL-12 and IL-18. Synergistic IL-12/IL-18 stimulation of IFN-γ release by NK cell was particularly striking, as almost every cell is inducible to cytokine expression under these conditions. More NK cells than NKT expressed IFN-γ after IL-12/IL-18 stimulation; expression of IFN-γ per cell was higher in

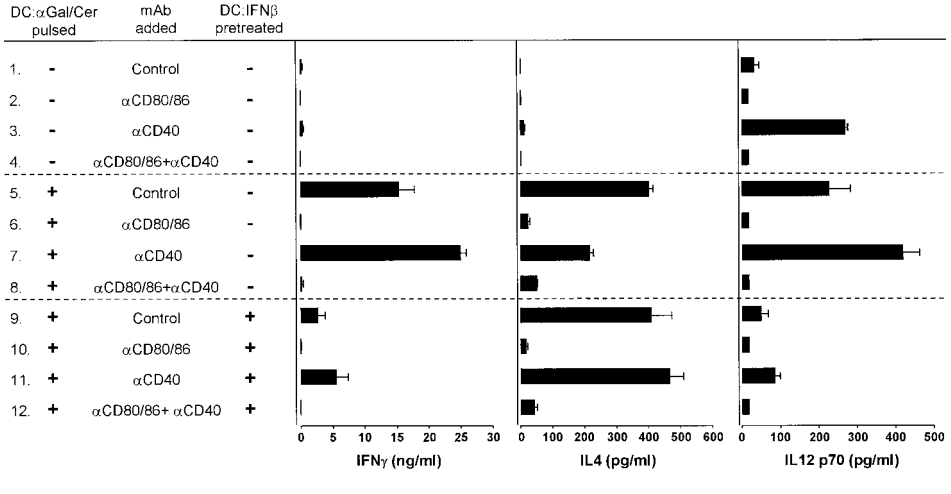



**FIGURE 4.** IFN-β down-regulates CD80/86- and CD40-dependent IL-12 release by αGalCer-pulsed DC cocultured with NKT cells. CD11c$^+$ liver DC from αAsGM1-pretreated B6 mice were purified by MACS and pulsed in vitro with 100 ng/ml αGalCer and/or 500 U/ml IFN-β for 2 h. DC were washed and cocultured for 48 h with liver CD4$^+$ NKT cells from MHC-II$^{-/-}$ (Aβ$^{-/-}$ KO) B6 mice in the presence of αCD80/αCD86 blocking Ab, αCD40 stimulating Ab, or control Ab. IFN-γ, IL-4, and IL-12 p70 release into 48-h supernatants was measured by ELISA. Results are the mean values (±SEM) of triplicate cultures. Representative data from one (of three independent) experiment is shown.

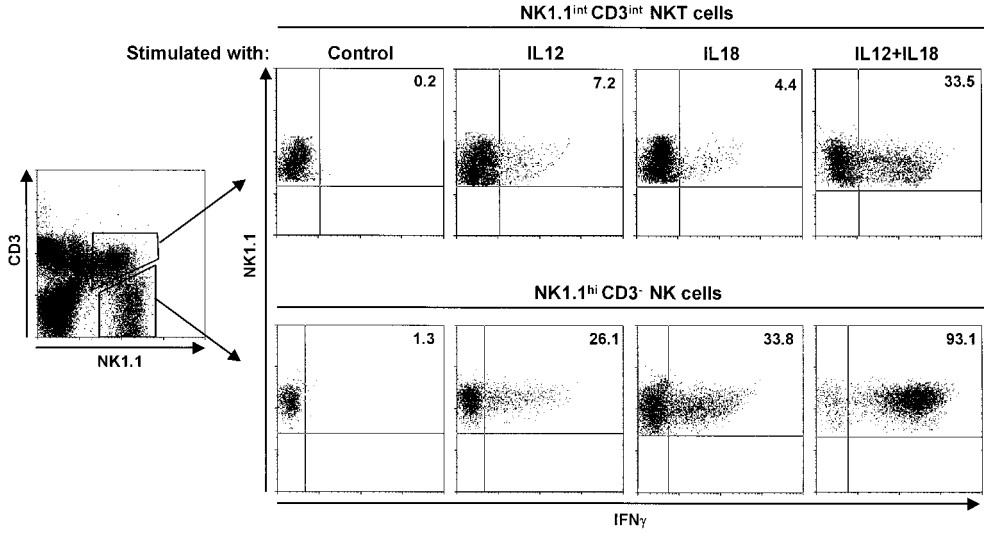



**FIGURE 5.** Liver NK cells stimulated by IL-12/IL-18 express abundant IFN-γ. The IFN-γ expression by liver NK and NKT cells stimulated with IL-12 and/or IL-18 is shown. Liver MNC from normal B6 mice were cultured with 20 ng/ml IL-12 and/or IL-18 for 12 h. Brefeldin A was added for the last 5 h of the culture. Cells were stained and analyzed by three-color FCM. NK1.1$^{high}$ CD3$^{-}$ NK cells and NK1.1$^{int}$ CD3$^{int}$ NKT cells were gated, and the dot plots of IFN-γ expression of the gated cells are shown. A representative example of one experiment of three independent experiments is shown.

the NK cell than in the NKT cell population (Fig. 5), and stimulated NK cells survived longer in culture than NKT cells. These data confirm that costimulation of T, NK, DC, and B cells by IL-12 and IL-18 enhances their IFN-γ release (reviewed in Ref. 22).

### *Adoptive transfer of NKT cell-activated liver DC stimulates IFN-γ response of liver NK cells*

In an adoptive transfer system, we confirmed in vivo the role of NKT cell-activated DC in triggering IFN-γ release by liver NK cells. CD11c$^{+}$ liver DC were isolated from normal, NK cell-depleted B6 donor mice that were either not treated or injected i.v. 18 h previously with 100 ng αGalCer. We injected $2 \times 10^5$ purified DC that were either activated by NKT cells or nonactivated into the portal vein of nontreated (immunocompetent) B6 hosts, (immunodeficient) RAG1$^{-/-}$ B6 hosts, or HBs-tg RAG1$^{-/-}$ B6 hosts. Liver MNC populations were harvested from adoptive hosts 18 h posttransfer, and their IFN-γ release was tested in vitro. Cell fractionation and FCM analyses showed that liver (NK1$^{+}$ CD3$^{-}$) NK cells were the main IFN-γ producers in the liver MNC populations of RAG1$^{-/-}$ mice. Transfer of NKT cell-activated DC into the livers of normal, RAG1$^{-/-}$, or HBs-tg RAG1$^{-/-}$ hosts stimulated IFN-γ release by liver MNC (Fig. 6, groups 2, 4, and 6). IFN-γ release of liver MNC was completely abrogated when the host was depleted of NK cells by treatment with anti-AsGM1 Ab, identifying NK cells as the main source of IFN-γ in the system (Fig. 6, group 7). Transfers of nonactivated liver DC triggered only low levels of IFN-γ release in the three groups (Fig. 6, groups 1, 3, and 5). The most striking IFN-γ responses of liver NK cells were detected in transfers of activated DC into HBs-tg RAG1$^{-/-}$ hosts (Fig. 6, group 6). NKT cell-activated liver DC can thus stimulate in situ an IFN-γ response of liver NK cells.

### *IFN-β suppresses in vitro the IL-12 response of liver DC activated by specifically stimulated NKT cells*

DC-derived IL-12 triggers a cytokine cascade in the NKT cell/DC/NK cell interaction that leads to liver injury. Type I IFNs are known to suppress the CD40-dependent IL-12 response of DC (23, 24). We tested whether IFN-β can suppress IL-12 release by αGalCer-pulsed, splenic, or hepatic DC cocultured with liver NKT cells in vitro. In these cocultures, abundant amounts of IL-10 and

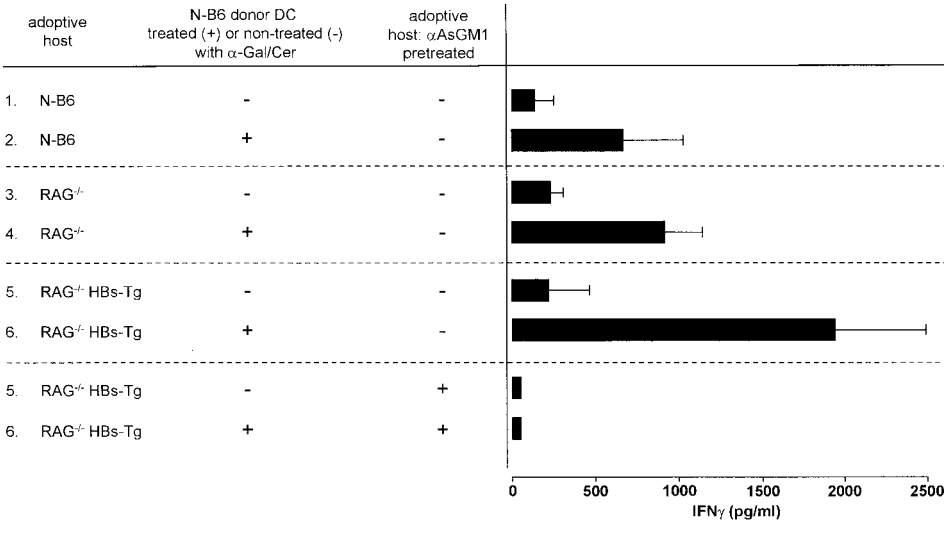



**FIGURE 6.** Activation of liver NK cells by adoptive transfer of NKT cell-activated liver DC. B6 mice were injected i.v. with 100 ng αGalCer or vehicle solution. Liver CD11c$^{+}$ DC were isolated from these mice 18 h postinjection. The purified DC were injected into the portal vein of either nontreated or NK cell-depleted normal, RAG1$^{-/-}$, or RAG1$^{-/-}$ HBs-tg adoptive hosts ($2 \times 10^5$ cells/mouse in 50 μl). Liver MNC were isolated from transplanted hosts 18 h posttransfer and cultured for 24 h. IFN-γ was measured in supernatants by ELISA. Results are the mean values (±SEM) of triplicate cultures. Representative data from one (of two independent) experiment is shown.

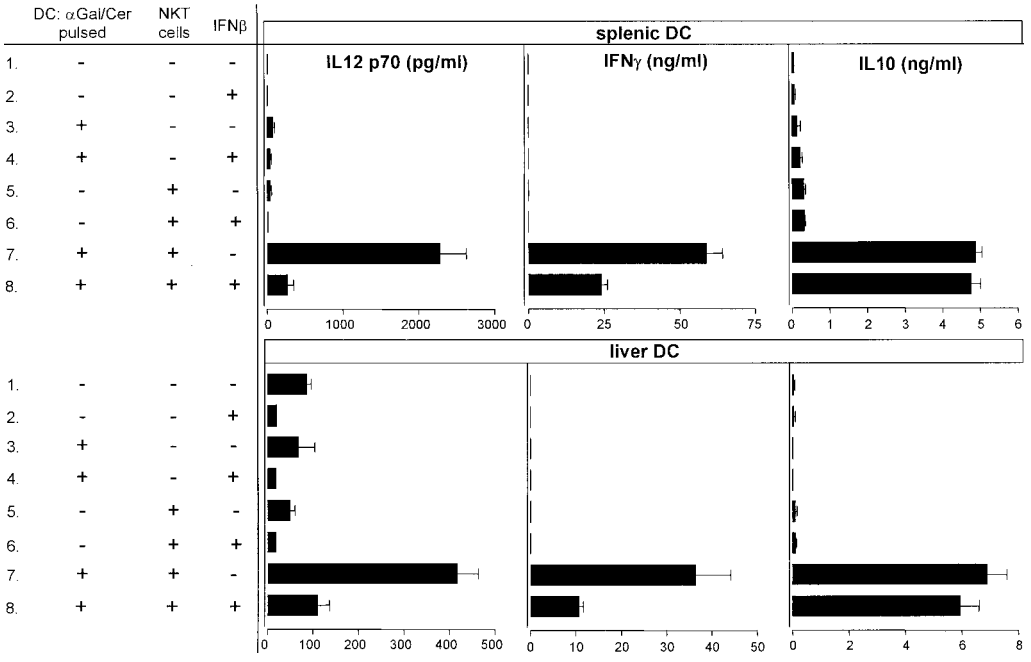



**FIGURE 7.** IFN-β suppresses IL-12 p70 and IFN-γ release in αGalCer-activated NKT/DC cocultures. Liver CD11c$^+$ DC were isolated from B6 mice by MACS. Splenic DC were enriched by centrifugation on NycoPrep and isolated by MACS selection. Nonpulsed or in vitro αGalCer-pulsed CD11c$^+$ DC ($2 \times 10^4$ cells/well) were cocultured with purified liver CD4$^+$ NKT cells ($10^5$ cells/well) from A$\beta^{-/-}$ B6 mice for 48 h. Supernatants (0.2 ml) containing either $10^4$ U murine IFN-β or no IFN-β were added to some cultures as indicated. IL-12 p70, IL-10, and IFN-γ release was determined in supernatants by ELISA. The mean IFN-γ, IL-12 p70, and IL-10 release (of triplicates) ± SEM of a representative experiment is shown.

IL-12 were released by αGalCer-pulsed, splenic, or hepatic DC, and IFN-γ was released by cocultured liver NKT cells (Fig. 7, group 7). IFN-β efficiently suppressed the IL-12 response (but not the IL-10 response) of αGalCer-pulsed DC cocultured with NKT cells (Fig. 7, group 8). IFN-β also suppressed IFN-γ release by NKT cells stimulated with αGalCer-pulsed DC (Fig. 7, group 8). IFN-β did not stimulate IL-10, IL-12, or IFN-γ release by nonpulsed or αGalCer-pulsed (splenic or hepatic) DC (Fig. 7, groups 1–4) or by liver NKT cells cultured with nonpulsed DC (Fig. 7, group 6). These in vitro data show that type I IFNs down-regulate IL-12-dependent responses in NKT cell/DC cocultures.

A short pretreatment of DC with IFN-β inhibits their ability to release IL-12 after coculture with NKT cells (Fig. 4, group 9) or after additional CD40 ligation (group 11). Concomitantly, IFN-γ release by NKT cells was also inhibited. These data point to an early and rapid effect of IFN-β on DC that strikingly affects their ability to efficiently prime Th1-biased T cell responses.

IFN-β did not inhibit IFN-γ release by NKT or NK cells when it was added 12 h after the initiation of the coculture of pulsed DC with either NKT cells or NK cells (data not shown).

### *IFN-β can attenuate the NKT cell-triggered induction of liver immunopathology*

In the last set of experiments, we tested whether IFN-β can attenuate the IL-12/IFN-γ-dependent induction of liver immunopathology by NKT cells. Activation of liver NKT cells by the i.v. injection of 100 ng αGalCer triggers severe liver immunopathology in immunocompetent HBs-tg B6 mice (1), as shown in Fig. 8. When mice were treated by $10^4$ U murine IFN-β 1 h before the i.v. injection of 100 ng αGalCer, the liver injury response, evident by the rise in serum transaminase levels, was strikingly suppressed (Fig. 8). The rise in serum ALT levels was 2- to 4-fold lower under IFN-β treatment (in four independent experiments). Hence, type I IFNs counteracts in vivo the deleterious effects elicited by dysregulated IL-12/IFN-γ-driven immune responses.

## Discussion

For the in vivo studies described in this report, we used immunocompetent or immunodeficient (RAG1$^{-/-}$) HBs-tg B6 mice that overproduce abnormally large amounts of the large envelope protein of hepatitis B virus in the endoplasmic reticulum of the hepatocytes and develop ground glass cells and spontaneous liver disease (25–27). Liver injury induced by αGalCer in HBs-tg mice is more severe than liver injury induced in normal mice, because these mice are extremely hypersensitive to the toxic effects of IFN-γ (1, 20, 28, 29). Therefore, this is a sensitive in vivo model to monitor the induction and regulation of IFN-mediated liver immunopathology.

We demonstrate that activation of liver NKT cells stimulates activation of liver NK cells as evidenced by up-regulated surface

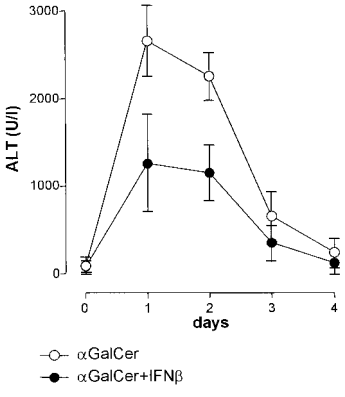



**FIGURE 8.** IFN-β attenuate liver injury induced in HBs-tg mice injected with αGalCer. HBs-tg mice (four per group) were injected i.v. with 100 ng/mouse αGalCer; mice were also injected i.v. with 0.5 ml supernatant either containing $10^4$ U murine IFN-β or not containing IFN-β. Serum ALT levels were determined daily for 4 days postinjection. Data are the mean ± SEM of three individual mice per group.

expression of CD69, increased cytotoxicity (Fig. 1), and increased IFN-γ release. The selective depletion of either NKT cells (by treatment with αGalCer) or NK cells (by treatment with αAsGM1 antisera) from the liver (Fig. 2) allowed us to test whether NKT cell-induced liver injury is NK cell-dependent. The data in Fig. 3 demonstrate that this is, in fact, the case and confirm that NKT cells rapidly activate NK cells (2). NK cells have been shown to display signs of activation in vivo (including IFN-γ production and CD69 induction) as early as 90 min after αGalCer injection. NK cell activation was not observed in RAG1$^{-/-}$ mice injected with αGalCer. NK cell activation depends on IFN-γ release by NKT cells because it is blocked by pretreating mice before the αGalCer injection with anti-IFN-γ Ab (2). Most data on NKT cell/NK cell cross-talk have been generated in murine tumor models. The antimetastatic effect of αGalCer-stimulated NKT cells was impaired in NK cell-depleted or IFN-γ-deficient mice (3). Similarly, NK cells and NKT cells collaborated in host protection from fibrosarcoma (16). Activated NKT cells efficiently induce NK cell proliferation and cytotoxicity (4) and bystander proliferation of memory CD4$^+$ and CD8$^+$ T cells (30). NK cell activation depends on NKT cell-derived IFN-γ and DC-derived IL-12 (4). In this study, we show that the NK cell response is an essential step in the manifestation of NKT cell-stimulated liver immunopathology (Fig. 3) and asked how NK cells are stimulated by activated NKT cells in the liver. TCR/CD1d-dependent stimulation of NKT cells by αGalCer-pulsed DC induces IL-12 release by stimulating DC as well as by up-regulation of IL-12R and IFN-γ expression by responding NKT cells (6). Production of IFN-γ by NKT cells in response to αGalCer requires CD40/CD40 ligand-dependent IL-12 release by DC. We show that neutralizing IL-12 in DC/NKT cell cocultures blocked IFN-γ release by NKT cells. As IL-12 and IFN-γ stimulate up-regulation of the IL-12R expression, further positive regulations amplify the IL-12/IFN-γ response.

Cytokine and costimulation signals modulate cytokine release of NKT cells stimulated by αGalCer-pulsed DC. Immature NKT cells preferentially express IL-4, but not IFN-γ, after expansion with DC (9). The transfer of αGalCer-pulsed DC can suppress or enhance EAE according to their ability to polarize NKT cells toward Th2 or Th1 in vitro (10). Other reports support the differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways (11). Blocking CD80/CD86-dependent costimulation during stimulation of human NKT cells by αGalCer-pulsed DC completely suppressed their IFN-γ and IL-4 production. In contrast, blocking CD40-CD154 interactions inhibited αGalCer-induced IFN-γ production, but not IL-4 production. This was confirmed by experiments in KO mice; CD28-deficient mice showed impaired IFN-γ and IL-4 production in response to αGalCer stimulation in vitro and in vivo, whereas production of IFN-γ, but not IL-4, was impaired in CD40-deficient mice. Both CD28-CD80/CD86 and CD40-CD154 costimulatory pathways thus contribute to the regulation of Th1 and Th2 functions of NKT cells. Our data (Fig. 4) demonstrate that αGalCer-pulsed liver DC produce IL-12 when cocultured with liver NKT cells, and this response requires CD28-CD80/86 costimulation. When this costimulation was blocked, the responding NKT cells produced neither IFN-γ nor IL-4.

NKT cells can be directly activated (independently from TCR engagement) by cytokines. Cytotoxic NKT cells have been induced in the liver of mice injected with IL-12 (12, 13). IL-18 can activate NKT cells (14). NKT cells stimulated with IL-18 and IL-12 proliferate, are cytotoxic, and produce high levels of IFN-γ. Following activation of NKT cells by glycolipid-presenting DC, NKT cells as well as NK cells are involved in the response in a TCR/CD1d-independent way by IL-12 and/or IL-18-dependent signals, as evident from the data in Fig. 5.

We directly tested whether NKT cell-stimulated liver DC can activate liver NK cells in situ by adoptive transfer experiments (Fig. 6). CD11c$^+$ DC were isolated from the liver of NK cell-depleted B6 donor mice after injection of αGalCer (or vehicle solution). Purified DC were injected into the portal vein of nontreated or NK cell-depleted (immunocompetent) B6 hosts, (immunodeficient) RAG1$^{-/-}$ B6 hosts, or (immunodeficient) RAG1$^{-/-}$ HBs-tg B6 hosts. Transfer of liver DC from treated (but not untreated) donor mice induced an IFN-γ response in the liver of all NK cell-competent adoptive hosts. This response was comparable in NK cell-competent (NKT cell-competent) and RAG1$^{-/-}$ (NKT cell-deficient) hosts, indicating that αGalCer presentation to NKT cells is not essential to trigger an IFN-γ response in this system. This confirms our in vitro data that αGalCer presentation of pulsed DC to NKT cells rapidly decays (data not shown). RAG1$^{-/-}$ HBs-tg hosts were most sensitive to the adoptive transfer of activated DC because they are more sensitive to IFN-γ. Hence, NKT cell-activated DC stimulate IFN-γ production in NK cells in the liver.

In view of autoamplifying cascades of proinflammatory cytokine production in the liver, it was of interest to identify cytokines that can down-regulate the NKT cell-triggered liver injury response. We found IFN-β to be a potent suppressor of NKT cell-stimulated IL-12 release of DC in vitro (Fig. 7) and to attenuate in vivo the liver injury in HBs-tg B6 mice injected with αGalCer (Fig. 8). The anti-viral role of type I IFNs (IFN-αβ) is well established, but their role as immunomodulatory cytokines is incompletely understood. IFN-β can prevent the generation of Th1 cells. IFN-β promotes in vitro the differentiation of human CD4$^+$ T cells that produce only low levels of IFN-γ and lymphotoxin compared with IL-12-derived Th1 CD4$^+$ T cells (31). IFN-β-treated human DC produce predominantly IL-10, but only low levels of IL-12 p40, and strongly suppress IFN-γ production by allogeneic T cells (23, 24, 32). Although IFN-β inhibits IL-12 release by DC by interfering with CD40 signaling, this cytokine does not inhibit all signaling emanating from CD40, as e.g., CD40/CD40 ligand-dependent IL-6 secretion by DC is augmented by IFN-β. IL-12 release by DC in response to LPS has been reported to be inhibited by IFN-β in mice (33), but not in human cells (24). IFN-αβ enhances the production of TNF-α in response to LPS, thereby causing rapid sensitization to LPS during viral infections (34). IL-10 may be required for the IFN-αβ inhibitory effect on IL-12 release by DC, as this has been shown to be IL-10 dependent (35). The suppression of Th1 immunity by type I IFNs seems to be relevant for understanding the attenuation of NKT cell-induced liver injury observed in our model.

IFN-αβ also has Th1-promoting activity. IFN-α can promote mucosal Th1 responses in the small intestine in celiac disease patients (36). Virus-induced IFN-αβ and IL-18 synergistically enhance IFN-γ gene expression in human T cells (37). IFN-α and IL-12 induce *IL-18R* gene expression in human NK and T cells, thereby enhancing innate as well as Th1 immune response (38). IFN-γ responses of CD8$^+$ CTL during early virus infection are dependent on endogenous IFN-αβ, but are IL-12 independent (39). IFN-αβ is known to induce gene expression mainly through Stat1-dependent, but also through Stat1-independent, pathways. Furthermore, IFN-αβ suppresses, in a Stat1-dependent manner, the expression of certain genes. One example is the IFN-αβ-mediated inhibition of IFN-γ in cytokine-stimulated NK and T cells through Stat1 (40). Another example is c-Myc suppression in nonlymphoid cells. Inhibition of *IL-12* gene expression is targeted to the IL-12

p40 promoter by IFN regulatory factor binding sites. The IFN consensus sequence binding protein IFN consensus sequence binding protein, a member of the IRF family, is a conditional transcriptional repressor of IFN-inducible promoters (41), and its transcription is induced by IFN-$\alpha\beta$ (42). Further studies are needed to elucidate the mechanism of suppression of IL-12 expression in IFN-$\beta$-treated DC.

Our data suggest that the attenuation of NKT cell-induced liver injury by IFN-$\beta$ operates early in the triggering phase of the response by blocking generation of DC-derived IL-12. This can lead to 1) reduced IL-12R expression by DC and NKT cells, 2) suppression of IFN-$\gamma$ production by NKT cells, 3) no TCR-independent of NKT cells into the response by IL-12/IL-18, and 4) no NK cell into the response. In view of the widespread use of type IFNs in the treatment of chronic hepatitis, these data may further our understanding of the mechanism of action of this cytokine substitution.

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