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*J Immunol* 2005; 175:1540-1550; doi: 10.4049/jimmunol.175.3.1540
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α-Galactosylceramide-Induced Liver Injury in Mice Is Mediated by TNF-α but Independent of Kupffer Cells

Markus Biburger and Gisa Tiets

NKT cells expressing phenotypic markers of both T and NK cells seem to be pivotal in murine models of immune-mediated liver injury, e.g., in Con A-induced hepatitis. Also α-galactosylceramide (α-GalCer), a specific ligand for invariant V\(\alpha_{14}\) NKT cells, induces hepatic injury. To improve the comprehension of NKT-cell mediated liver injury, we investigated concomitants and prerequisites of α-GalCer-induced hepatitis in mice. Liver injury induced by α-GalCer injection into C57BL/6 mice was accompanied by intrahepatic caspase-3 activity but appeared independent thereof. α-GalCer injection also induces pronounced cytokine responses, including TNF-α, IFN-γ, IL-2, IL-4, and IL-6. We provide a detailed time course for the expression of these cytokines, both in liver and plasma. Cytokine neutralization revealed that, unlike Con A-induced hepatitis, IFN-γ is not only dispensable for α-GalCer-induced hepatotoxicity but even appears to exert protective effects. In contrast, TNF-α was clearly identified as an important mediator for hepatic injury in this model that increased Fas ligand expression on NKT cells. Whereas intrahepatic Kupffer cells are known as a pivotal source for TNF-α, they were nonessential for α-GalCer-mediated hepatotoxicity. In α-GalCer-treated mice, TNF-α was produced by intrahepatic lymphocytes, in particular NKT cells. BALB/c mice were significantly less susceptible to α-GalCer-induced liver injury than C57BL/6 mice, in particular upon pretreatment with α-galactosamine, a hepatocyte-specific sensitizer to TNF-α-mediated injury. Finally, we demonstrate resemblance of murine α-GalCer-induced hepatitis to human autoimmune-like liver disorders. The particular features of this model compared with other immune-mediated hepatitis models may enhance comprehension of basic mechanisms in the etiopathogenesis of NKT cell-comprising liver disorders. The Journal of Immunology, 2005, 175: 1540–1550.

The liver appears to be particularly susceptible for injury by exceeding immune responses, mainly mediated by T lymphocytes (e.g., in viral hepatitis) and/or emerging autoantibodies (autoimmune hepatitis). Upon viral infection of hepatocytes, the cytopathic effect of the virus per se is only moderate, and liver damage is caused rather by cellular immune responses to infected cells (1, 2). Several attempts have been made to establish mouse models for immune-mediated liver injury focusing especially on T cell-dependent hepatitis, including experimental autoimmune hepatitis (EAH)\(^1\) induced by Ags from syngeneic liver homogenate (3), MHC class I-restricted pathogenesis in HBsAg-transgenic mice (4), liver injury induced by Propionibacterium acnes and LPS (5), Pseudomonas aeruginosa exotoxin A (PEA) (6), or the plant lectin Con A (7). Whereas CD\(8^{+}\) cytotoxic lymphocytes were identified as key mediators of the deleterious effect, e.g., in the HBsAg model, cytokine-producing CD\(4^{+}\) are the main effector cells in Con A-induced hepatitis (8–10).

The Con A model reveals several features of autoimmune hepatitis but misses the criteria of an Ag-specific T cell response (reviewed in Ref. 11). The CD\(4^{+}\) lymphocytes mediating Con A-induced intrahepatic inflammation have been identified to be NKT cells, a subpopulation of mature T cells that express NK surface markers such as NK1.1, IL-2Rβ, and to some extent Ly49A and Ly49C, and reveal the Thy1\(^{high}\)CD44\(^{high}\)CD45RB\(^{high}\) phenotype of activated T cells (defined in C57BL/6 mice; see Ref. 12). Most NKT cells bear a TCR with invariant V\(\alpha_{14}-\)Ja281 TCRα-chain and a restricted TCRβ repertoire, recognizing glycolipids presented by the MHC class I-like molecule CD1d, which is essential for development of invariant NKT cells of thymic origin. Liver and thymus are enriched with CD1d-restricted NKT cells, most of which are CD\(4^{+}\) and to a lesser extent double negative.

Several experimental results suggest NKT cells to be key effector cells in Con A-mediated liver damage. This includes the fact that the absence of NKT cells caused by depletion (13), knockout of the V\(\alpha_{14}-\)Ja281 TCRα chain (14) or knockout of CD1d (15) is associated with resistance to Con A-induced hepatitis. Con A susceptibility can be restored in V\(\alpha_{14}\)-knockout mice and CD1d\(^{-/-}\) mice by adoptive transfer of functional NKT cells (14, 15). Also, V\(\alpha_{14}/\)V\(\beta_{8.2}\)-transgenic RAG\(^{-/-}\) mice are susceptible to Con A (14) in which NKT cell populations are intact, whereas T cells, B cells, and NK cells are missing (16). (The lack of NK cells in these mice is actually surprising because in contrast to T and B cells NK cell development is not dependent on RAG-mediated recombination of receptor genes. NK cell absence was suggested to be caused by block of NK cell development by the V\(\beta_{8}\) transgene and has been observed in V\(\beta_{8}\)-transgenic TCR\(^{-/-}\) mice as well (17).)

Instead of pan-activating mitogens such as anti-CD3 mAb or Con A, specific activation of NKT cells can be achieved with the glycolipid Ag α-galactosylceramide (α-GalCer) being presented by CD1d (16). Injection of α-GalCer induces liver injury in mice in a time frame similar to Con A (18, 19). As with Con A (15),
α-GalCer (18) induces a rapid reduction of cells expressing NKT cell surface markers from the liver, followed by repopulation within several days.

To further deepen the insights into mechanisms underlying liver injury mediated by this remarkable class of lymphocytes, we investigated concomitants and prerequisites of α-GalCer-induced liver injury and compare characteristics of Con A- and Aldrich and administered i.v. at the indicated doses in pyrogen-free saline.

Within several days, inhibition of caspase-3-like caspases did not protect from liver injury in this model. However, in contrast to Con A hepatitis (see Ref. 11), neither IFN-γ nor Kupffer cells (KCs) seem to be required for α-GalCer-mediated hepatotoxicity. We demonstrate that BALB/c and C57BL/6 mice reveal pronounced strain differences in susceptibility to α-GalCer-induced liver injury with C57BL/6 mice showing higher TNF-α production and FasL expression on NKT cells in accordance to their higher susceptibility. Finally, the suitability of α-GalCer-induced liver injury as a murine model for autoimmune hepatitis is discussed.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were obtained from Charles River Breeding Laboratories, Harlan-Winkelmann, Elevation Janvier, or from the animal facilities of the Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg. They were maintained under controlled conditions (22°C, 55% humidity, 12-h day/night rhythm) and fed standard laboratory chow. For strain differences, only male C57BL/6 mice at 8–12 wk of age were used in this study. All mice received human care according to the guidelines of the National Institute of Health and the legal requirements in Germany.

Animal treatments

α-GalCer was kindly provided by Kirin Brewery, stored as a stock solution of 200 µg/ml in vehicle (0.5% w/v polysorbate-20), and diluted in pyrogen-free saline to the indicated doses directly before i.v. injection in a total volume of 200 µl. Con A (type IV) was purchased from Sigma-Aldrich and administered i.v. at the indicated doses in pyrogen-free saline at 200 µl/20 g of mouse. For KC depletion, mice were injected i.v. with 100 µl of liposome-encapsulated dichloromethylene-bisphosphonate (Cl2MBP) (clodronate liposomes) 48 h before challenge, as described previously (21, 22). Clodronate liposomes were kindly provided by Dr. N. van Rooijen (Vrije Universiteit, Amsterdam, The Netherlands). Cl2MBP that was used for preparation of clodronate liposomes was a gift of Roche (Mannheim, Germany). In initial control experiments, mice were pretreated with PBS liposomes. In the main experiments, saline rather than PBS liposomes was used as a negative control because liposomes themselves interfere with macrophage phagocytosis (23). For sensitization of mice with p-galactosamine (GalN), galactosamine hydrochloride (Carl Roth) was administered i.p. in pyrogen-free saline (70 mg/ml) at 200 µl/20 g of mouse 15 min before i.v. injection of 200 ng of α-GalCer to weight- and age-matching BALB/c and C57BL/6 mice (20–22 g; 7–8 wk of age). For inhibition of caspase-3-like caspases, the irreversible inhibitors zVAD(OMe).fmk (R&D Systems) or zVAD.fmk (Bachem) were reconstituted in DMSO and diluted in pyrogen-free saline to the indicated doses directly before injection in a total volume of 200 µl/20 g mouse with a final concentration of <5% DMSO. Application regimens were 5 mg/kg Z-VAD(OMe).fmk i.p. 20 min before treatment with 1 µg of α-GalCer or alternatively 10 µg/kg z-VAD.fmk i.v. 20 min before α-GalCer treatment (200 ng) with an additional i.p. injection of 5 mg/kg 6 h thereafter. Activity of z-VAD.fmk was verified by protection of mice from apoptotic liver damage induced by anti-Fas Ab (clone Jo2, 120 µg/kg; BD Pharmingen) with 10 mg/kg inhibitor i.v. For in vivo neutralization of TNF-α or IFN-γ, mice were injected i.v. with 300 µg of IgG purified from sheep anti-mouse TNF-α polyclonal antiserum (8), with 75 µl of polyclonal rabbit anti-TNF-α Ab IP-400 (Genzyme) or with 200 µl of rabbit anti-mouse IFN-γ serum (9) 20–30 min before treatment. Corresponding amounts of normal rabbit serum (Sigma-Aldrich) or purified total IgG from normal sheep serum (Sigma-Aldrich) were used as negative controls.

Sampling of material

Mice were anesthetized lethally (150 mg/kg i.v. pentobarbital + 15 mg/kg heparin). Blood was withdrawn by cardiac puncture for analysis of plasma transaminases and cytokines. Livers were excised and divided into two parts, one embedded in tissue-embedding medium (Slee) and frozen at −75°C for immunofluorescent staining and confocal laser imaging and the other part frozen in liquid nitrogen and stored at −20°C for preparation of RNA and subsequent real-time RT-PCR.

Analysis of liver transaminase

Hepatocyte damage was assessed at the indicated time points after α-GalCer or Con A treatment by measuring plasma enzyme activities of alanine aminotransferase (ALT) (24) using an automated procedure. Unless otherwise noted, liver injury was quantified 8 h after Con A treatment and 16–17 h after α-GalCer treatment.

Cytokine quantification by ELISA

Sandwich ELISAs for murine plasma TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10, and TGF-β were performed using Nunc-Immuno 96-well flat-bottom high-binding Maxisorp polystyrene microtiter plates (Nalge Nunc International). Abs were purchased from BD Biosciences/Pharmingen for IL-2, IL-4, IL-6, and IL-10. Streptavidin-peroxidase was purchased from Roche Diagnostic Systems. IFN-γ, TNF-α, and TGF-β were quantified using DuoSet ELISA Development Systems from R&D Systems. As peroxidase chromogen, we used the TMB Substrate Reagent Set (BD Pharmingen). All components were used according to the manufacturer’s instructions.

Isolation of RNA and real-time RT-PCR for cytokine mRNAs

Isolation of RNA from liver tissue was conducted using the Total RNA Isolation Kit (Macherey-Nagel). mRNA was transcribed into cDNA using SuperScript II RNase H− Reverse Transcriptase, oligonucleotides and oligo(dT) primers from Invitrogen Life Technologies. Real-time RT-PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostic Systems) and the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostic Systems), according to manufacturer’s instructions. Primer pairs were used as previously described (25), with the exception of pairs for 5′-TNF (GAATGGGTGTCATACCTCTCT-3′); 5′-TNF (GAATGGGTGTCATACCTCTCT-3′); 5′-IL-2 (GTTATCGGTCTCTCTC-3′); 5′-IL-4 (GTTATCGGTCTCTCTC-3′); 5′-IL-6 (GTTATCGGTCTCTCTC-3′); 5′-IL-10 (GTTATCGGTCTCTCTC-3′); and 5′-TGF-β (GTTATCGGTCTCTCTC-3′). To confirm amplification specificity, melting curves of PCR products were analyzed. Relative mRNA levels were calculated by means of 2ΔΔCt = difference of crossing points of test samples—and respective control samples as extracted from amplification curves by the LightCycler software after normalization with respect to β-actin levels.

Detection of caspase-3 activity

Presence of activated caspase-3 was assessed in lysates from liver tissue by measuring its activity using the Colorimetric caspase-3 assay kit (Sigma-Aldrich) in the presence or absence of caspase-3 inhibitor, according to the manufacturer’s instruction.

Immunofluorescent staining and confocal laser imaging

Kupffer cells and CD11c+ dendritic cells (DCs) in liver tissue were analyzed by immunofluorescent staining and confocal laser imaging. Ten-micrometer cryostat sections of livers were thawed on glass slides, air dried, and fixed in 1:1 acetone-methanol (4°C, 10 min). After washing in PBS, the sections were blocked with 3% BSA/PBS (room temperature, 30 min). For analysis of Kupffer cells, incubation was continued with rat anti-mouse macrophage mAb (clone BM8; Dianova) or rat anti-mouse-F4/80 mAb (clone BM8; Dianova) and rat anti-mouse-CD11c Ab (clone HL3; BD Pharmingen) in 5% BSA/PBS overnight at 4°C. After rinsing with PBS, binding sites were detected using FITC-labeled goat anti-rat IgG Ab (Sigma-Aldrich) diluted in 3% BSA/PBS (room temperature, 1 h). For analysis of CD11c+ DCs, Armenian hamster anti-mouse-CD11c Ab (clone HL3; BD Pharmingen) was used as primary Ab and Texas Red-labeled goat anti-hamster Ab (Jackson Immunoresearch Laboratories) as secondary Ab following the procedures described. Sections were washed with PBS, coverslipped with 10% glycerol/PBS (pH 8.6), and examined by confocal laser scanning microscopy (Axiovert 100M; Carl Zeiss).
Isolation and flow cytometric analysis of liver leukocytes

Leukocytes were isolated essentially as described previously (26). Briefly, livers were passed through 100-μm nylon meshes, hepatocytes were separated from leukocytes and RBCs using isotonic 37% Percoll solution (Amersham Biosciences) containing 100 U/ml heparin, and RBCs were removed using lysis solution containing 139 mM NH₄Cl and 19 mM Tris. For flow cytometry, 5 × 10⁵ liver leukocytes were stained using a standard protocol. For intracellular cytokine staining, the Cytofix/Cytoperm Plus kit (BD Pharmingen) was used according to the manufacturer’s instructions. The following Abs were used: FITC-labeled mouse anti-mouse NK1.1 mAb (clone PK136; BD Pharmingen), FITC-labeled rat anti-mouse CD49b mAb (clone DX5; BD Pharmingen), phycoerythrin-labeled anti-mouse TNF-α (clone IP-400; Genzyme), PE-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories), CyChrome-labeled Armenian hamster anti-mouse CD3ε mAb (clone 145-2C11; BD Pharmingen), PE-labeled Armenian hamster anti-mouse CD178 Ab (= anti-FasL Ab, clone MFL-3; BD Pharmingen) and PE-labeled Armenian hamster isotype control (clone G235-2356; BD Pharmingen). Data were recorded and analyzed using a FACScan Flow Cytometer (BD Biosciences), BD CellQuest software provided with the flow cytometer, and WinMDI 2.8 software (J. Trotter, Scripps Research Institute, La Jolla, CA).

Histology

For histological analysis of tissue structure livers were incubated for 24 h in 2% Zamboni solution (27) for fixation and subsequently embedded in paraffin. Sections were stained with H&E using a standard procedure and analyzed by light microscopy.

Statistical analysis

Except from survival curves, all data are expressed as mean ± SEM. For calculation of statistical significance, data were transformed (log) before analysis using Student’s t test if two groups were compared, the Dunnett’s test if more groups were tested against a control group, or the Bonferroni test if several groups were tested against one another. A value of p ≤ 0.05 was considered significant. Survival curves are presented as Kaplan-Meier plots, and significance upon comparison of survival curves was calculated using the log-rank test.

Results

Time course of α-GalCer-induced liver injury and caspase-3 activation

To assess the potential involvement of apoptotic processes and of single cytokines on hepatitis induction by comparing the time-dependent onset of liver injury upon injection of α-GalCer with the course of caspase-3 activation and cytokine expression, a time course experiment was performed. ALT activity was used as a measure for liver damage (Fig. 1A). A significant transaminase response was detected 12–20 h after injection, peaking 16 h. Thus, in most experiments thereafter, ALT was measured 16–17 h after α-GalCer injection. To analyze the influence of apoptotic processes in α-GalCer-induced hepatotoxicity, we investigated caspase-3 activation in the course of onset of liver injury. As demonstrated in Fig. 1B, we found clear signs of intrahepatic caspase-3 activation by measuring its activity in tissue samples. To investigate the mechanistic importance of caspase activation in the disease process of liver injury, we used two forms of the zVAD.fmk irreversible inhibitor of caspase-3-like caspases, zVAD.fmk and zVAD(Ome).fmk, which, according to the manufacturer’s information, differ in their cellular permeability. We used different application routes (i.v. or i.p.), application schedules (20-min pretreatment and pretreatment combined with additional injection 6 h after α-GalCer treatment), and doses (5 or 10 + 5 mg/kg) for these compounds. However, no protective effect was achieved with zVAD(Ome).fmk (Fig. 1C) nor with zVAD.fmk (data not shown) at a dose that has been shown to protect mice from liver injury induced by anti-Fas Ab (Ref. 28 and own tests—data not shown—with the same lot used for α-GalCer-treated mice).

α-GalCer-induced cytokine expression

As known from several publications, NKT cells are able to produce a broad range of immunostimulatory or immunoregulatory cytokines upon activation, such as IL-2, IL-4, IL-10, IFN-γ, or TNF-α, depending on the respective microenvironment, cytokine milieu, and presence or absence of costimulation (reviewed in Refs. 29 and 30). Also, the principal ability of α-GalCer to induce production of several of these cytokines by NKT cells has been reported by some groups. In this work, we wanted to provide a detailed time course analysis of intrahepatic cytokine expression and plasma concentrations upon α-GalCer injection, inter alia to correlate these with the onset of liver injury. Thus, in accordance to the time course of α-GalCer-induced liver injury, plasma cytokine levels up to 24 h after administration of 2 μg of α-GalCer to C57BL/6 mice were measured by ELISA (Fig. 2A). The transaminase response as described above was preceded by significant expression of IL-2, IL-6, TNF-α and—particularly pronounced—IFN-γ and IL-4. Except for IFN-γ, these cytokines revealed peak
levels ~2–4 h after α-GalCer application. IFN-γ peak expression was delayed and resembled the course of plasma ALT. No significant induction of plasma IL-10 was observed with only single mice showing detectable amounts of IL-10 in this, as well as in other experiments (data not shown). To analyze the impact of α-GalCer treatment locally within the liver, regulation of cytokine mRNA was investigated by real-time RT-PCR (Fig. 2B). Except from IL-10, hepatic mRNA induction in principle matched the course of plasma cytokine concentrations, with IFN-γ mRNA expression preceding the plasma peak ~10 h. In contrast to the marginal plasma IL-10 concentration, its mRNA expression is significantly increased 1 h after α-GalCer treatment, returning almost to background levels soon after. Corresponding to a lack of significant amounts of bioactive plasma TGF-β within 24 h after α-GalCer treatment, no relevant induction of TGF-β mRNA was observed (data not shown).

Upon comparison of Con A- or α-GalCer-treated C57BL/6 mice, we found a significantly stronger bias to IL-4 and IFN-γ expression upon α-GalCer treatment and to IL-6 upon Con A application, whereas expression levels of IL-2, TNF-α, and IL-10 appeared comparable for both models (data not shown).

Relevance of α-GalCer-induced cytokines for liver injury

Con A-mediated hepatitis has been proven to be dependent on both TNF-α and IFN-γ (Refs. 8, 9, 31 and others as summarized in Ref. 11). Because both cytokines are induced upon α-GalCer treatment, with the time course of α-GalCer-induced IFN-γ expression resembling that of ALT release, we analyzed the effect of neutralizing Abs against these cytokines in the α-GalCer model. Surprisingly, neutralization of IFN-γ not only failed to block α-GalCer-mediated hepatic injury, as had already been previously described (32), but even caused an increased ALT release (Fig. 3A). In contrast, neutralization of TNF-α significantly reduced hepatic injury induced by α-GalCer (Fig. 3B). Use of the polyclonal anti-TNF-α Ab IP-400 (Genzyme) even revealed a somewhat more pronounced effect than sheep anti-TNF-α IgG. It should be mentioned that the α-GalCer dose used in the experiments depicted in Fig. 3 was lower than the one used in experiments for determination of cytokine expression: upon the finding that, in contrast to the Con A model (reviewed in Ref. 11) neutralization of IFN-γ was not protective here, we used a dose of 200 ng of α-GalCer per mouse, which is sufficient to evoke significant liver injury, to minimize the chance that the observed lack of protection might probably be caused by an “oversaturated” cytokine boost, which potentially could not be efficiently blocked by neutralizing Abs. However, we also found that neutralization of TNF-α protected from liver injury induced by 2 μg of α-GalCer (data not shown).

Neutralization of TNF-α also counterthrew the aggravating effect of IFN-γ neutralization (Fig. 3C). It is worth mentioning that in three of four experiments TNF-α neutralization significantly interfered with the α-GalCer-induced plasma IFN-γ boost (data not shown). Our results demonstrate that, whereas in contrast to Con A hepatitis, IFN-γ is not essential for α-GalCer-induced hepatotoxicity but rather appears to exert protective effects, TNF-α is an important mediator of liver injury in both models of immune-mediated hepatitis. Flow cytometric analysis of vehicle-treated, α-GalCer-treated, and anti-TNF-α/α-GalCer-treated mice 2 h after treatment revealed a significant influence of α-GalCer-induced TNF-α on the expression of Fasl (CD178 and CD95L) by NKT cells and thereby probably on the Fas/FasL-mediated cellular cytotoxicity of these cells: α-GalCer injection within 2 h induced a significant increase of the constitutive Fasl-specific surface staining of intrahepatic NKT cells (mean increase in Fasl-specific staining: 1.8-fold, p < 0.01). However, neutralization of TNF-α partially but significantly (p < 0.05) reduced the α-GalCer-induced Fasl up-regulation (mean increase in Fasl-specific staining: 1.5-fold, p < 0.05; Fig. 3D).

FIGURE 2. Time-dependent systemic and intrahepatic cytokine expression upon α-GalCer treatment. Two micrograms of α-GalCer were injected into the tail vein of C57BL/6 mice. At the indicated time points, animals were sacrificed by terminal narcosis, blood was drawn by cardiac puncture, and liver was excised for mRNA preparation. A, Plasma cytokine concentrations were measured by ELISA. The summary of two independent experiments is shown. “0 h” corresponds to vehicle-treated control mice. B, Intrahepatic cytokine mRNA expression was measured by real-time RT-PCR. β-actin mRNA from each sample was used as an internal standard to normalize for equal levels of total mRNA. x-fold induction was calculated referring to mRNA levels of the respective cytokines in mock-treated mice (“0 h”) (mean ± SEM; n = 3; *, p ≤ 0.05 vs vehicle control).
Role of Kupffer cells in α-GalCer hepatitis

KCs, the resident macrophages of the liver, are major producers of proinflammatory cytokines such as TNF-α upon activation and are pivotal in murine models of T cell- and TNF-α-dependent liver injury (22). Because we could reveal TNF-α to be involved in α-GalCer hepatitis as well, we analyzed the relevance of KCs in this model by KC depletion upon pretreatment with clodronate liposomes. α-GalCer liver damage was not reduced in KC-depleted mice but was in fact slightly aggravated (Fig. 4A). In initial experiments using PBS-containing liposomes as negative control also, no protective effect of KC depletion was observed (data not shown). Effective KC depletion was verified by immunohistology using macrophage-specific Ab F4/80 (Fig. 4B) and supported by the largely abolished expression of IL-6 in clodronate-pretreated mice, a cytokine for which KC are a major source in T cell populations that conduct TNF-α secretion in the liver upon α-GalCer treatment (Fig. 4C). These results indicate that KCs are not essential for α-GalCer-induced liver damage. This, together with the observation that α-GalCer-induced TNF-α expression within the peak period was unaffected by KC depletion (data not shown), suggested that the amount of TNF-α produced by other cell types upon α-GalCer treatment may be sufficient to accomplish its function in hepatic injury. Because some reports also demonstrated depletion of DC by clodronate liposomes as discussed below, we additionally analyzed a potential influence of clodronate treatment on this population in the liver. Whereas Kupffer cells were efficiently eliminated by clodronate liposomes as verified using macrophage-specific Ab BM8, no obvious depletion of CD11c+ DCs was observed in clodronate-pretreated mice (Fig. 4D).

α-GalCer-induced TNF-α production by NKT cells

Because α-GalCer-induced, TNF-α-mediated liver injury is not compromised in KC-depleted mice, we wanted to test whether TNF-α can be expressed by intrahepatic lymphocytes. To identify cell populations that conduct TNF-α secretion in the liver upon α-GalCer treatment, we stained both cell surface TNF-α and FasL expression on liver NKT cells within 2 h and TNF-α neutralization by anti-TNF-α injection partially counteracts this effect. The figure depicts the relative CD95L-labeling index, i.e., FasL-specific ΔMFI (difference of median fluorescence intensities of the PE-conjugated anti-FasL Ab and respective isotype control) of each probe in relation to the ΔMFI of the vehicle-treated control in the same experiment (i.e., ΔMFIcontrol, which represents the constitutive FasL expression, is set to “1”). Liver mononuclear cells were isolated 2 h after treatment, and NKT cells were gated electronically by lymphocyte-typical light scatter characteristics and the coexpression of CD3ε and NK1.1. The figure summarizes all five experimental groups, each of which revealed α-GalCer-induced FasL up-regulation that is counterbalanced by TNF-α neutralization.

FIGURE 3. TNF-α but not IFN-γ neutralization alleviates α-GalCer-induced liver injury in mice. Twenty to 30 min before injection of 200 ng of α-GalCer, C57BL/6 mice were pretreated with the indicated neutralizing Abs or alternatively with saline, sheep IgG, or rabbit serum as negative controls. ALT levels were measured in plasma gathered 17 h after α-GalCer treatment. A, IFN-γ neutralization by pretreatment with 200 μl of anti-IFN-γ serum aggravates hepatic injury by α-GalCer. The summary of four independent experiments is depicted with a total of n = 7, 13, and 15 for the saline (not included in all experiments), serum, or anti-IFN-γ groups, respectively. B, TNF-α neutralization by either polyclonal sheep anti-mouse TNF-α Ab IP-400 (b) (75 μg/mouse) significantly ameliorates α-GalCer-induced liver injury. The protective effect of TNF-α neutralization was observed in four independent experiments (n ≥ 3). C, Neutralization of TNF-α counteracts the aggravating effect of IFN-γ neutralization. Neutralizing rabbit anti-IFN-γ serum and sheep anti-TNF-α IgG were used in the same amounts as above. The figure shows the results of one of two independent experiments with corresponding outcome (n ≥ 3). (A–C, Mean values ± SEM; * p ≤ 0.05 vs the respective control represented in the left column in each graph, respectively; #, p ≤ 0.05 vs the experimental group depicted in the second left column in each graph, respectively). D, α-GalCer treatment (200 ng) up-regulates FasL expression on liver NKT cells within 2 h and TNF-α neutralization by anti-TNF-α injection partially counteracts this effect. The graph depicts the relative CD95L-labeling index, i.e., FasL-specific ΔMFI (difference of median fluorescence intensities of the PE-conjugated anti-FasL Ab and respective isotype control) of each probe in relation to the ΔMFI of the untreated control in the same experiment (i.e., ΔMFIcontrol, which represents the constitutive FasL expression, is set to “1”). Liver mononuclear cells were isolated 2 h after treatment, and NKT cells were gated electronically by lymphocyte-typical light scatter characteristics and the coexpression of CD3ε and NK1.1. The figure summarizes all five experimental groups, each of which revealed α-GalCer-induced FasL up-regulation that is counterbalanced by TNF-α neutralization.

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Strain differences in susceptibility to hepatitis induction

α-GalCer- and Con A-induced liver injury both reveal some features resembling those of autoimmune hepatitis in men. Genetic prevalence is one characteristic of autoimmune hepatitis, finding its analog in strain differences in mice. We wanted to test whether there is a genetic prevalence for disease development upon α-GalCer treatment, further supporting its consideration as a murine model for this disorder. Therefore, BALB/c mice and C57BL/6 mice were both treated in parallel either with α-GalCer doses of 1 μg/mouse (Fig. 6A) or 200 ng/mouse (data not shown), and liver injury was assessed by measuring plasma ALT activity. In both treatment regimes, BALB/c mice were less susceptible to hepatitis induction than C57BL/6 mice. Consistent with published results (33, 34), we also found distinct strain differences in susceptibility to Con A, with C57BL/6 mice showing a significantly higher release of liver transaminases than BALB/c mice in response to Con A injection (data not shown). Histological analyses of H&E-stained livers of α-GalCer-treated mice from both strains (Fig. 6B) substantiated the interpretation of the difference in ALT responses to α-GalCer treatment to actually reflect differences in severity of liver injury rather than nonliver-associated factors such as potential variations of ALT activity or clearance: spacious necroinflammatory areas were found in livers of α-GalCer-treated C57BL/6 mice but not in those of BALB/c mice. The strain difference in susceptibility to α-GalCer was further emphasized by an additional survival experiment with sensitized mice. BALB/c mice and C57BL/6 mice were both pretreated with the hepatocyte-specific transcription inhibitor GalN, which sensitizes hepatocytes to TNF-α-mediated injury (35) and were subsequently treated with α-GalCer. Whereas all C57BL/6 mice died in between 7 and 8 h, only one-third of the BALB/c mice died at ~10 h after treatment. The others survived the entire duration of the experiment (Fig. 6C) and recovered within 1.5 days as judged by means of amelioration of disease signs such as scaly fur and modest lethargy, clearly demonstrating the less pronounced sensitivity of BALB/c mice to α-GalCer. To investigate potential mechanistic reasons for this strain difference, we analyzed both TNF-α expression and FasL expression in α-GalCer-treated mice of both strains 2 h after...
liver injury with C57BL/6 mice becoming significantly more sensitive to significantly higher concentration of plasma TNF-α. Cells of C57BL/6 mice revealed higher levels of cell surface FasL, whereas intrahepatic NKT cell populations within each strain, FasL-specific staining of NK cells of both strains was found to be four to five times greater and staining of T cells of both strains than BALB/c mice. Hence, FasL was not involved but, as reported earlier (22), spacious necrotic areas were found in livers of BALB/c mice prepared 17 h after Con A- and α-GalCer-induced liver injury, with 200 ng of α-GalCer mediating lethal toxicity for all C57BL/6 mice, whereas BALB/c mice revealed a significantly better survival (p < 0.001; n = 6). D, Upon α-GalCer treatment, C57BL/6 mice produced significantly higher amounts of systemic TNF-α than BALB/c mice, as measured by ELISA with blood drawn 2 h after α-GalCer injection (1 μg). Higher TNF-α production by C57BL/6 mice was verified in two independent experiments (p < 0.05; n = 3). E, FasL expression on intrahepatic CD3+DX5+ NKT cells, from α-GalCer-treated C57BL/6 mice was elevated in comparison to those from BALB/c mice identically treated in parallel (1 μg of α-GalCer injected 2 h before cell preparation; n = 4). To enable interstrain comparison, relative CD95L-labeling indices are shown with the CD95L-specific difference of median fluorescence intensities being set in relation to the value for the respective lymphocyte subpopulation in BALB/c mice (i.e., all BALB/c values were defined as “1”). With respect to differences in FasL expression on the different lymphocyte populations within each strain, FasL-specific staining of NK cells of both strains was found to be four to five times greater and staining of T cells of both strains about three-fourths of that of BALB/c NKT cells (data not shown).

Discussion
In view of the corresponding idiopathic disorders in men, Con A-induced hepatitis in mice served as a model for the analysis of basic mechanisms of immune-mediated liver injury in vivo and has been used for the development of novel immunosuppressive strategies for more than a decade. Involvement of intrahepatic and infiltrating cell types and important cytokines has been elucidated to a large extent (reviewed in Ref. 11). It is well known that administration of α-GalCer to mice also induces immune-mediated liver injury (18, 19, 32), but basic mechanisms and the involvement of cytokines in this process are not completely elucidated. Therefore, we wanted to further characterize mediators of α-GalCer hepatitis and to compare features of both models.

We could show that the onset of α-GalCer-induced liver injury is accompanied by pronounced caspase-3 activation in the liver, but treatment with the broad-range caspase inhibitor zVAD.fmk failed to prevent α-GalCer-induced liver injury. Distinct caspase-3 activation upon Con A injection has also been shown in some recent publications (36, 37), but there are also conflicting data where no significant increase of caspase activity could be detected (20). Also, in contrast to the GalN/TNF, GalN/LPS (20), and anti-Fas models (28), Con A-induced liver injury could not be prevented by zVAD.fmk (20), thereby revealing an analogy to our findings in the α-GalCer model. To examine the presence of apoptotic processes in this model, we performed our own histological analyses of livers from Con A-treated mice and actually found some hepatocytes revealing signs of apoptotic cell death (data not shown). However, only few of such apoptotic hepatocytes could be detected but, as reported earlier (22), spacious necrotic areas were
found. It is worth mentioning that also in the histological analysis regarding strain differences in susceptibility to α-GalCer-induced liver injury, practically no apoptotic hepatocytes were found (see Fig. 6B). These results suggest that caspase-3-mediated apoptosis may indeed accompany the onset of liver injury in both the α-GalCer and Con A model but may be not essential for hepatocyte damage. Possibly, a considerable fraction of the observed caspase-3 activity might be related to nonhepatocyte cell types such as activated lymphocytes dying by apoptotic activation-induced cell death.

Upon α-GalCer injection a strong induction of both the Th1 and Th2 cytokines IFN-γ and IL-4 was found in plasma and on mRNA level in liver tissue, with this two-edged cytokine response being a well-known effect of NKT cell activation (16, 29, 38). Except for IL-10 and IFN-γ, cytokines revealed a coherence of intrahepatic α-GalCer-induced mRNA induction and subsequent increase of plasma concentrations. IL-10 revealed a strong but momentary induction of mRNA expression in the liver that did not result in a significant plasma response. IFN-γ revealed a reasonable and persistent expression with the plasma peak being 10 h delayed compared with the intrahepatic mRNA expression peak. This may be related to secondary expression of IFN-γ by NK cells, possibly activated by IFN-γ produced by NKT cells and IL-12 produced by DCs upon NKT activation by α-GalCer, as has been shown previously (39–41). Compared with Con A, α-GalCer induced a cytokine profile that was more biased toward IL-4 and IFN-γ, presumably reflecting the more specific activation of NKT cells by α-GalCer. Because KCs are known as a major source of IL-6 in the liver, the weaker IL-6 response to α-GalCer than to Con A may indicate a minor KC involvement in the α-GalCer-induced immune response. This interpretation suits our finding that KCs are dispensable for α-GalCer-mediated liver injury.

Immune-mediated hepatitis induced by Con A is strictly dependent on the activity of both IFN-γ and TNF-α (reviewed in Ref. 11). However, in this work, we could clearly show that IFN-γ is not pivotal for α-GalCer-induced hepatotoxicity, which is consistent with recently published results (32). Unexpectedly, IFN-γ neutralization even aggravated liver injury in this model. The mechanistic basis for the apparently protective effect of IFN-γ is not yet clear. Because IFN-γ is known as an inductor of MHC class I up-regulation, its expression may enhance the density of these surface molecules that in turn may extenuate effector functions of NKT and NK cells by killer inhibitory receptors expressed on both cell types. The importance of Ly49 inhibitory receptors on NKT cells has been elucidated quite recently (42). It is worth mentioning that IFN-γ has also been shown to exert apparent hepatoprotective effects in other models of liver injury such as cholestasis (43) or liver allograft rejection (44). In contrast to IFN-γ, we could clearly identify TNF-α as an important mediator of α-GalCer-induced hepatotoxicity because its neutralization was not only protective against α-GalCer alone but even protected against aggravated α-GalCer hepatotoxicity upon IFN-γ neutralization. The importance of TNF-α in this model is emphasized by work on a murine model of NKT cell-mediated liver injury during alcohol consumption, where a TNF-αR-1 knockout mutation interferes with α-GalCer-induced hepatitis (45). Previously, Fas-FasL signaling has also been suggested to be involved in the onset of α-GalCer hepatitis (19, 45). We could demonstrate that α-GalCer—similar to Con A (15)—induces an increase in FasL expression on intrahepatic NKT cells and additionally that this increase is partially blocked upon TNF-α neutralization. This clearly shows a link between the TNF-α-dependent liver injury investigated in this work and Fas/FasL-mediated mechanisms in this model of α-GalCer-induced liver injury. Thus, injection of α-GalCer induces TNF-α secretion by NKT cells and probably other cell types. TNF-α may then induce hepatocyte damage by direct cytotoxic mechanisms, as well as autocrine and paracrine induction of FasL on NKT cells.

In BALB/c mice, both α-GalCer-induced TNF-α expression, as well as FasL expression on NKT cells, are less pronounced than in C57BL/6 mice, and both—probably causally linked—effects may constitute the mechanistic basis for higher susceptibility of C57BL6 mice to α-GalCer-induced liver injury.

KCs have been found to be crucially involved in several models of immune-mediated liver injury, e.g., induced either by ischemia/reperfusion, by injection of Con A or PEA, or by a combination of subtoxic doses of PEA with Staphylococcus enterotoxin B as an important source for chemo- and/or cytokine production (22, 46–48). In the aforementioned models fulminant liver injury is prevented by KC inactivation. In an early study (49), Con A-induced hepatitis was not suppressed upon injection of gadolinium chloride (GdCl3), a potent inhibitor of macrophages; however, GdCl3 is known to be capable of rather activating macrophages upon application in vivo if not all target cells are reached with a sufficiently high local concentration to be fully blocked (50). In contrast, protection was achieved in subsequent studies using GdCl3 (46, 47), as well as in studies using alternative KC inhibitors such as carrageenan (51) or clodronate liposomes (22), thus convincingly indicating that KCs are essential also in Con A hepatitis.

KC depletion experiments in this work demonstrated that KCs are dispensable for α-GalCer-induced liver injury. Probably they do not constitute a pivotal source for TNF-α, which can be produced by intrahepatic lymphocytes, especially NKT cells, upon α-GalCer treatment as shown here and, thus, may reach the critical concentration locally in the liver. Production of TNF-α by Ab-stimulated “intermediate T cells” (with regard to the TCRint phenotype) prepared from the liver of C57BL/6 mice has been recently demonstrated (52), thereby supporting our results. Its independence from TNF-α-production by KCs distinguishes the α-GalCer model from the Con A model, as well as from other models of TNF- and T cell-mediated liver injury such as PEA and PEA + Staphylococcus enterotoxin B.

Although liver CD11c+ DCs display lower CD1d surface expression than hepatocytes, they are potent stimulators of IFN-γ and IL-4 release by liver NKT cells when pulsed with α-GalCer in vitro or in vivo (53). In addition, they can contribute to TNF-α production upon α-GalCer exposure, as has been shown for splenic DCs with CD11c+ DC-enriched cells isolated from spleen 2 h after α-GalCer injection (54). DCs pulsed with α-GalCer effectively induce potent antitumor cytotoxic activity by specific activation of NKT cells in mice (55) and have recently gained clinical importance with a phase I study analyzing the effects of α-GalCer-pulsed DCs in lung cancer patients (56). Elimination induced by clodronate liposomes is described mainly for monocytes/macrophages. However, because some reports suggest that also DCs are eliminated by clodronate liposomes in vitro (57) or—at least some subpopulations—also in vivo (58, 59), we examined the presence of CD11c+ DCs in the livers of clodronate-treated mice. If the intrahepatic DC population would have been eliminated in these mice without any effect on liver injury, a major impact of DCs on α-GalCer-induced liver injury would have been questionable. However, whereas Kupffer cells had been depleted efficiently, no obvious elimination of intrahepatic CD11c+ cells was observed under our experimental conditions of clodronate treatment. So, with respect to their suggested function as an α-GalCer-presenting and TNF-α-producing population, the hypothesis of an important role of DCs in this model remains plausible.
Con A-induced experimental hepatitis matches several criteria of autoimmune hepatitis (AIH) and had been discussed as a murine model for this idiopathic disorder. However, in contrast to the EAH model, which is inducible by (auto)antigens in syngeneic liver homogenate (3), Con A hepatitis is not induced by an (auto)antigen and, thus, lacks one central hallmark of autoimmunity. In contrast, α-GalCer can be considered as a surrogate Ag for natural autoantigens that may be presented to NKT cells by CD1d. This represents a very important difference between Con A and α-GalCer with respect to their mode of lymphocyte activation. Whereas Con A binds mannose residues of many different glycoproteins and, thus, pan-activates lymphocyte populations irrespective of their Ag specificity, α-GalCer is strictly dependent on the presentation of the MHC-homologous CD1d molecule and exerts its activating function via Ag-specific TCR recognition. The HLA-associated differences in genetic prevalence to AIH development in humans might be regarded as a hint for the importance of MHC-mediated Ag presentation at least in some forms of AIH. All three models match other criteria of AIH (Table I). The criteria of a prevalent role of CD4+ lymphocytes in pathogenesis is fulfilled in the CD4+ T cell-dependent Con A model, as well as in EAH, where passive transfer of the disease is mediated by CD4+ T cells (60). Also α-GalCer hepatitis matches this criteria because intrahepatic NKT cells, activated by this surrogate Ag, are predominantly CD4+ or, to a lesser extent, double negative. Passive transfer of disease with mononuclear cells from the liver has been shown for EAH (3, 60) and for Con A hepatitis (51) but to our knowledge not yet for α-GalCer hepatitis. Genetic prevalences that are typical for autoimmune disorders may be reflected by strain differences regarding susceptibility to disease induction in murine models. Such differences have been identified for EAH (3), as well as Con A (Refs. 33 and 34 and this work), and are shown for α-GalCer hepatitis in this work by means of significant differences in ALT response, as well as survival upon GalN sensitization and histological analysis. Specific production of autoantibodies, a characteristic of humoral mediated autoimmunity, has been shown for EAH where it appeared not to be disease relevant (61) but not yet in the course of Con A or α-GalCer hepatitis. It has been shown recently that α-GalCer administration also caused activation of B cells in mice, but serum levels of Igs were not changed significantly within 48 h (62). It is worth mentioning that α-GalCer-induced autoantibody production has been observed in a murine model of lupus, another autoimmune disorder (63). The induction of an immunosuppressive state in the phase of remission is another feature of AIH and was also demonstrated during EAH remission (64) and—as to thymus-dependent Ab response—in the Con A model as well (65). α-GalCer treatment also induced a state of immunosuppression because C57BL/6 mice remained largely unresponsive to α-GalCer rechallenge (Ref. 19 and our unpublished observations). Autoimmune disease typically responds to immunosuppressive treatment. Protective effects of such approaches have been shown in the EAH model (60) and Con A-induced experimental hepatitis (reviewed in Ref. 11). Because anti-TNF-α treatment can certainly be regarded as an anti-inflammatory approach, the present work clearly demonstrates that α-GalCer hepatitis responds to immunosuppressive treatment as well. This analysis shows that besides EAH and Con A hepatitis α-GalCer-induced liver injury also matches important criteria of AIH. Whereas one particular hallmark of autoimmune hepatitis, activation by an autoantigen, is clearly missing in the Con A model, α-GalCer can be considered as a surrogate for a physiologic Ag and—upon presentation by MHC-like CD1d to Ag-specific TCRs—induces onset of liver injury in an Ag-specific manner. Thus, in our opinion, this experimental system can be regarded as a murine model for autoimmune hepatitis more validly than Con A-induced hepatitis.

In summary, the present work describes prerequisites and concomitants of α-GalCer-induced liver injury and demonstrates that to some degree there is a mechanistic resemblance to, e.g., Con A hepatitis as another classical model of immune-mediated liver injury. This partial analogy is not unexpected, because a pan-T cell activating effect like that of Con A also affects NKT cells. However, there is also a considerable divergence between α-GalCer liver injury and other murine models of immune hepatitis, e.g., with respect to dependence on IFN-γ or KCs as essential source of TNF-α. Considering the fact that α-GalCer hepatitis is associated with moderate liver injury, it might be considered as a model describing basic and fundamental mechanisms of immune-mediated hepatitis. Enclosing such basic events and building on them, additional effects, caused by, e.g., the less specific pan-activating properties of Con A, may establish a more complex process culminating in fulminant hepatitis. Taken together, this model may enable the analysis of initial and fundamental events in the etiopathogenesis of (NKT)-comprising liver disorders and may, thus, be used for development and investigation of novel therapeutic approaches against diseases of that kind, including some forms of AIH.

Acknowledgments
We thank Kirin Brewery for providing α-GalCer, Dr. Nico van Rooijen (Vrije Universiteit) for providing cladronate liposomes, Dr. Neuhuber (Institute of Anatomy I, University of Erlangen-Nuremberg) for histological analysis, and Annette Erhardt, Katarzyna Bera and Thomas Postler for supporting work, as well as Andrea Agli and Sonja Heinlein for perfect technical assistance.

Disclosures
The authors have no financial conflict of interest.
References


