Endoplasmic reticulum stress, hepatocyte CD1d and NKT cell abnormalities in murine fatty livers

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The liver regulates lipid homeostasis and is enriched with natural killer T (NKT) cells that respond to lipid antigens. Optimal maturation and activation of NKT cells requires their interaction with lipid antigens that are presented by cluster of differentiation-1 (CD-1) molecules on antigen-presenting cells. Hepatocytes express CD1d and present lipid antigens to NKT cells. Depletion and dysregulation of hepatic NKT cells occurs in mice with fatty livers. Herein, we assess whether reduced CD1d content on steatotic hepatocytes contributes to fatty liver-associated NKT cell abnormalities. We show that despite expressing normal levels of CD1d mRNA, fatty hepatocytes from ob/ob mice have significantly less CD1d on their plasma membranes than normal hepatocytes. This has functional significance because ob/ob hepatocytes are less able to activate CD1d-restricted T-cell responses *in vitro*, and CD1d-reactive NKT cells are reduced in ob/ob livers. Events in the endoplasmic reticulum (ER) normally regulate CD1d trafficking to plasma membranes. Hepatic steatosis has been associated with ER stress. To determine if ER stress reduces CD-1 accumulation on hepatocytes, we evaluated hepatic ER stress in ob/ob mice and treated cultured hepatocytes and lean mice with tunicamycin to induce ER stress. Lipid accumulation and ER stress occurred in the livers of both ob/ob and tunicamycin-treated mice. Tunicamycin caused dose-dependent decreases in hepatocyte CD1d, inhibited hepatocyte activation of CD1d-restricted T-cell responses, depleted liver populations of CD1d-reactive NKT cells and promoted Th-1 polarization of hepatic cytokine production. In conclusion, ER stress-related decreases in hepatocyte CD1d contribute to NKT cell dysregulation in fatty livers.

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The healthy liver houses many types of immune cells and the steady-state profile of immune cells in the liver differs markedly from that of secondary lymphoid organs and other non-lymphoid tissues. For example, the healthy liver contains large resident populations of macrophages and various types of T lymphocytes, natural killer (NK) cells and dendritic cells, but few, if any B cells.^{1–3} The liver is particularly enriched with natural killer T (NKT) cells, which are present at trace levels in blood and other organs.^{4,5} During development most NKT cells are generated in the thymus^{6–8} and migrate predominately into the liver, with lesser migration into certain other sites, such as bone marrow and spleen.⁹ In adults, resident NKT cells may self-renew.¹⁰ Circulating NKT cells also accumulate intrahepatically because they express chemokine receptor 6 (CXCR6) and receive viability signals from the CXCR6 ligand, chemokine receptor ligand 16, which is robustly expressed by hepatic sinusoidal endothelial cells.³ Intravital microscopy studies demonstrated that NKT cells,

which account for most CXCR6+ cells in liver, crawl along hepatic sinusoids and stop upon activation of their T-cell receptor (TCR).³

Unlike the widely diversified TCRs of major histocompatibility complex (MHC) class I- and class II-restricted conventional T cells, which are activated by distinct peptide antigens, the TCR repertoire of NKT cells is fairly limited and responds exclusively to lipid antigens.¹¹ In both mice and humans, the largest subset of NKT cells express a semi-invariant TCR, and a restricted V β -repertoire. Such cells are commonly referred to as classical, or TCR α -invariant, NKT cells (iNKT).¹² Nearly, all iNKT recognize the marine-sponge-derived glycolipid α -galactosyl ceramide (α GalCer), presented in CD1d.¹³ Thus, CD1d tetramers loaded with α GalCer are often utilized to harvest iNKT cells from mice and humans.^{4,14}

Native CD1d is a member of a family of MHC-like integral membrane proteins that are encoded by a locus of tightly

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clustered genes located on chromosome 1q23 in humans and on chromosome 3 in mice.^{15,16} Five cluster of differentiation-1 (CD-1) genes, CD1A-E, have been identified in man,^{17–20} whereas mice contain only CD1D orthologs.^{15,16} CD-1 genes have been reported in many species, suggesting their preservation as a gene family in evolution. Various CD-1 isoforms are expanded or deleted in particular species, leading to speculation that each species has evolved CD-1 isoforms that follow distinct intracellular trafficking routes to sample antigens, even if it is not the same isoform in each species.^{21,22} In both mice and humans, CD1d is thought to present endogenous glycolipid self-antigens to the iNKT cell TCR.^{23,24} Recent studies of mouse and human iNKT, TCRs reveal a contiguous conserved hot spot that provides the basis for the cross-species reactivity of NKT cells to CD1d-presented glycolipids.²⁵

The fact that CD-1-restricted NKT cells respond to lipid, as opposed to peptide, antigens suggests that such cells may have been configured to accumulate preferentially in liver because hepatocytes are pivotal regulators of lipid intermediary metabolism. Indeed, although several types of liver cells express CD1d, the strongest CD1d expression in liver has been demonstrated on hepatocytes themselves.³ Intravital microscopy data suggest that hepatocytes are probably visited by four NKT cells/h,³ demonstrating that patrolling NKT cells regularly survey CD1d-bound lipid antigens on hepatocyte plasma membranes. Despite recent work by Bendelac co-workers²⁶ demonstrating that an endogenous lysosomal glycosphingolipid, iGb3, can activate NKT cells, the characterization of all of the endogenous glycolipid antigens remains incomplete.

However, it is known that CD1d traffics through cellular endolysosomal compartments and is likely loaded with its lipid antigen in the endoplasmic reticulum (ER) via a process that is mediated by microsomal triglyceride transfer protein (MTP).²⁷

Lipid loading of CD1d influences subsequent trafficking of CD1d to the cell surface, because hepatocytes from MTP-deficient mice have a significantly reduced content of CD1d in their plasma membranes.²⁷ Reduced cell surface expression of CD1d has functional implications, as evidenced by the diminished capacity of CD1d-deficient cells to activate TCR on NKT cell hybridomas.²⁷ Other studies confirm that CD1d is a major regulator of NKT cell viability and function throughout life. For example, mice that are genetically deficient in CD1d are deficient in NKT cells because they cannot accomplish positive selection of NKT cell precursors by CD1d-expressing thymocytes during development.²⁸ CD1d is also important in maintaining NKT cell homeostasis in adults, as demonstrated by evidence that immature NKT cells harvested from adults fail to fully mature when transferred to a CD1d-deficient environment.²⁹ In addition, the level of CD1d expression correlates with the ability to activate NKT cells. Antigen-presenting cells expressing higher CD1d levels more

efficiently activate NKT cell hybridomas and primary NKT cells.³⁰

Using methods that rely on NK1.1-immunoselection, we and others have reported that NKT cells are selectively depleted in the fatty livers of mice with genetic- or diet-induced obesity.^{31–33} These findings have important implications because liver is the primary reservoir for NKT cells in adults, and these cells regulate both innate and adaptive immune responses.^{4,5} Hence, depletion of hepatic NKT cells is predicted to have many, potentially deleterious consequences,^{31,32,34} including some that might contribute to the genesis of obesity-associated diseases, such as non-alcoholic fatty liver disease, autoimmunity and cancer. Because it has become evident that NK1.1 expression by NKT cells fluctuates according to their level of maturation and activation,^{35,36} interpretation of the initial finding of reduced NK1.1 (+) lymphocytes in obesity-associated fatty livers requires additional studies with tetramer-based methodologies that identify CD-1-restricted cells regardless of their level of NK1.1 expression. Also, it is important to determine if, and how, hepatic steatosis might influence the level of CD1d expressed by hepatocytes, since these cells function as important as antigen-presenting cells for CD1d-restricted iNKT cells.

Herein, we demonstrate that cell surface expression of CD1d is reduced in fatty hepatocytes and present evidence that an ER-stress-related mechanism is involved. Down-regulation of CD1d expression in fatty hepatocytes is accompanied by functional consequences, including impaired activation of NKT cell hybridomas, reduced numbers of hepatic tetramer-reactive NKT cells, and Th-1 polarization of hepatic cytokine production. These findings complement and extend earlier evidence for NKT cell defects in fatty livers^{31–33} and have potential implications for the pathogenesis of other diseases that are strongly associated with hepatic steatosis, including diabetes and the metabolic syndrome.

MATERIALS AND METHODS

Animal Experiments

Adult (age 10–12 weeks), male C57Bl-6 ob/ob and lean mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), maintained in a temperature- and light-controlled facility, and permitted *ad libitum* consumption of water and standard pellet chow. In initial acute studies, healthy lean C57Bl-6 mice were injected intraperitoneally (i.p.) with either tunicamycin (Calbiochem, San Diego, CA, USA) (400 ng/g body weight/day, $n = 6$ mice) or phosphate-buffered saline (PBS) ($n = 4$ mice) for 4 days. Mice were killed and liver samples were collected on day 5. Because treatment with 400 ng tunicamycin/g body weight for 4 days resulted 33% lethality, in subsequent longer-term studies mice ($n = 4$ /group) were treated with either PBS or a reduced dose of tunicamycin (200 ng/g body weight) by i.p. injection two times a week for 4 weeks. One day after the last dose of tunicamycin, the mice were injected i.p. with a single dose of

Escherichia coli lipopolysaccharide (LPS) (Sigma Chemical, St Louis, MO, USA) (20 µg/mouse) or PBS and then killed after 6 h to obtain serum and liver tissue. Animal care and procedures were approved by the Duke University Medical Center Institutional Animal Care and Use Committee as set forth in the 'Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health.

Cell Isolation and Cell Surface Labeling

Hepatocytes were isolated by collagenase perfusion of the liver and centrifugal elutriation as described.³⁷ The viability of all cells was verified by phase-contrast microscopy and the ability to exclude propidium iodide. The viability of all cell cultures utilized for study was >90%. For non-parenchymal cells, after *in situ* perfusion of the liver with 10 mg pronase (Boehringer Mannheim, Indianapolis, IN, USA) followed by collagenase (Crescent Chemical, Hauppauge, NY, USA) as described,³⁸ dispersed cell suspensions were layered on a discontinuous density gradient of 8.2 and 15.6% Accudenz (Accurate Chemical and Scientific, Westbury, NY, USA). Non-parenchymal cells containing endothelial cells and Kupffer cells in the lower layer were collected.

Hepatic mononuclear cells (HMNC) were isolated and labeled, using a minor modification of the method we described previously.³¹ Anti-mouse H2-D-PE, CD-1-FITC, CD3-FITC and CD4-APC were obtained from Pharmingen (San Diego, CA, USA). NKT cells were stained using PE-mCDd/PBS57 ligand tetramer (generously provided by the National Institute of Allergy and Infectious Disease MHC Tetramer Core Facility, Atlanta, GA, USA).³⁹ Endothelial cells and Kupffer cells were labeled with anti-mouse CD31-PE (eBioscience, San Diego, CA, USA), F4/80-PE (Serotec, Oxford, UK) respectively. After surface labeling, cells were evaluated by flow cytometry (Becton Dickinson, Palo Alto, CA, USA). Data are analyzed by Cell Quest software (Becton Dickinson). To ascertain reproducibility of our findings, all flow cytometry studies were repeated at least three times. Data are shown as mean ± s.e. percentage of positive cells from all of the experiments.

Immunoblot

Frozen liver samples and cells were homogenized in 150 µl of Dignam C buffer⁴⁰ containing protease and phosphatase inhibitors as described.⁴¹ The homogenized samples were rotated on a tumbler for 30 min at 4°C, and then centrifuged at 14 000 r.p.m. for 5 min at 4°C. The protein concentration of samples was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (50 µg from whole liver) were separated by SDS-PAGE and then transferred to nitrocellulose (Schleicher and Schuell, Keene, NH, USA) in a buffer containing 20 mmol/l Tris, pH 8.3, 150 mmol/l glycine, 0.01% SDS and 20% methanol. Equal loading of the gel was confirmed by staining nitrocellulose membranes with Ponceau S. After blocking with 5% nonfat milk (Carnation, Swampscott, MA, USA) in Tris-buffered

saline (20 mmol/l Tris, pH 7.5, 150 mmol/l NaCl) containing 0.1% Tween-20 (TBS-T) for 1 h, nitrocellulose membranes were incubated with primary antibody anti-glucose regulated/binding immunoglobulin protein-78 (Grp-78) (Stressgen, Victoria, Canada) or β -actin antibody (Sigma, St Louis, MO, USA), all diluted 1:1000 in 5% nonfat milk for 1 h (or in the case of immunoblotting involving whole liver extracts, for 12 h), and then washed three times in TBS-T. Secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit from Amersham, UK), was incubated with nitrocellulose membranes at a dilution of 1:1000 in 5% nonfat milk for 30 min. After four washes in TBS-T, antibody complexes were detected using the Amersham ECL system in linear range.

Cell Culture and Antigen Presentation Assays

AML-12 cells were grown in 1:1 mixture of DME/Ham's F-12 medium supplemented with 10% fetal bovine serum and antibiotics. DN32.IF3 cell were kindly provided by Dr Albert Bendelac. AML-12 hepatocytes or primary hepatocytes in 96-well plate (1 × 10⁴/well) were loaded overnight with either vehicle or 100 ng/ml α -GalCer (AXXORA, San Diego, CA, USA), washed three times in PBS followed by the addition of 5 × 10⁴ DN32.IF3 cells/well. Mouse interleukin-2 (IL-2) and interferon- γ (IFN- γ) production were assessed by ELISA (OptEIA, BD Pharmingen) after 24 h.

Tissue Triglyceride Assay

Extraction of liver triglycerides with chloroform/methanol was modified by Burant *et al.*⁴² The triglyceride content was determined using Triglyceride Detection Kit Sigma.

mRNA Quantification by Real-Time PCR and Semiquantitative PCR

Total RNA was extracted from different type cells or whole livers using TRIzol (Invitrogen, Carlsbad, CA, USA). One microgram of RNA was reverse-transcribed using random primer and Superscript RNase H-reverse transcriptase (Invitrogen). Reverse transcription reactions contained 1 µg of total RNA in a reaction mixture containing 25 mmol/l Tris-HCl, pH 8.3, 75 mmol/l KCl, 3 mmol/l MgCl₂, 10 mmol/l DTT, 1.25 µg oligo dT, 400 µmol/l each dNTP, and 20 U RNase inhibitor (Gibco/BRRL, Rockville, MD). The reaction mixture was incubated at 68°C for 2 min, and then placed on ice. Afterwards, 200 U Moloney murine leukemia virus reverse transcriptase (RT) (GIBCO/BRRL, Rockville, MD) was added and the reaction incubated at 42°C for 1 h.

For quantitative real time PCR, 1.5% of the first-strand reaction was amplified using iQ-SYBR Green Supermix (Bio-Rad), an iCycler iQ Real-Time Detection System (Bio-Rad), and specific oligonucleotide primers for target sequences, as well as the 18S housekeeping gene. The sequences of primers for mouse IL-4, tumor necrosis factor- α (TNF- α), IFN- γ , IL-12 and 18S are as follows: IL-4: sense: 5'-TCCTGCTCTCTTTCTCG-3', antisense: 5'-CTTCTCCTGTGACCTCGT

T-3', product size: 102. IL-12 sense: 5'-GGAAGCACGGCAG CAGAATA-3', antisense: 5'-AACTTGAGGGAGAAGTAGGA ATGG-3', product size: 180. IFN- γ sense: 5'-CATCAGCAA CAACATAAGCGTCA-3', antisense: 5'-CTCCTTTTCCGCTT CCTGA-3', product size: 202. TNF- α sense: 5'-TCGTAGCA AACCACTAAGTG-3' anti-sense: 5'-AGATAGCAAATCGG CTGACG-3', product size: 207. 18S sense: 5'-TTGACG GAAGGGCACCAACCAG-3', anti-sense: 5'-GCACCACCA CCCACGGAATCG-3', product size: 130. The PCR parameters were as follows: denaturing at 95°C for 3 min followed by 40 cycles of denaturing at 95°C for 15 s and annealing-extension at the optimal primer temperatures for 45–60 s. Threshold cycles (C_t) were automatically calculated by the iCycler iQ Real-Time Detection System. Target gene levels in the treated cells or tissues are presented as a ratio to levels detected in the corresponding control cells or tissues, respectively, according to the $\Delta\Delta C_t$ method. These fold changes were determined using point and interval estimates.

For semiquantitative PCR, an aliquot of the reverse transcription reaction was used for PCR amplification using the reaction was cycled as follows: 94°C for 3 min, then 94°C for 30 s, 55°C for 30 s and 72°C for 30 s for 25 cycles. PCR products were separated by electrophoresis on a 2.0% agarose gel. The PCR for both CD1d and CCAAT enhancer binding protein homologous protein (CHOP) were determined to be within the linear range of amplification (data not shown). The sequences of primers for mouse CD1d, CHOP and glucuronidase (GUS) are as follows: CD1d sense: 5'-AATTA CACCTTCCGCTGCC-3', antisense: 5'-CAGTAGAGGATGA TATCCTG-3', product size: 818; GUS sense: 5'-GCAGTTG TGTGGGTGAATGG-3', antisense: 5'-GGGTCAGTGTGTTG TTGATGG-3', product size: 142, CHOP sense: 5'-CCCTGCCT TTCACCTTGG3', Antisense: 5'-CCGCTCGTTCTCCTGCTC3' product size: 371.

Statistical Analysis

Results are expressed as mean $\pm$ s.e.m. Significance was established using the Student's *t*-test. Differences were considered significant when $P < 0.05$.

RESULTS

Selective Hepatic CD4⁺ NKT Cell Depletion in ob/ob Mice

We initially evaluated HMNC subtypes using cell surface markers and CD1d tetramers. Ob/ob mice had a significant decrease in tetramer-reactive hepatic NKT cells compared to lean mice (Figure 1a and b). The decrease in liver NKT cells was predominately attributable to reduced numbers of CD4⁺ NKT cells. This result is similar to our previous data acquired using CD3 and NK1.1 as NKT cell markers.^{31–33}

Decreased CD1d Expression on the Surface of ob/ob Hepatocytes

CD1d expression on the surfaces of hepatocytes from ob/ob mice and their lean littermates was studied. We found that

virtually all hepatocytes from lean mice expressed high surface levels of CD1d. In contrast, surface expression of CD1d was generally much lower on most ob/ob hepatocytes (Figure 2a). Also, the overall percentage of hepatocytes that were CD1d⁺ was significantly reduced in ob/ob mice compared to lean mice (Figure 2b). We next tried to identify the reason why CD1d expression was decreased on ob/ob hepatocytes. RT-PCR was used to detect the mRNA level of CD1d in ob/ob and lean hepatocytes. CD1d mRNA levels in ob/ob hepatocytes were not significantly different than in hepatocytes from lean controls (Figure 2c).

No Significant Difference in CD1d Expression on the Surface of Endothelial and KUPFFER Cells of ob/ob and Lean Mice

To determine if defects in surface CD1d expression were restricted to ob/ob hepatocytes, we investigated the CD1d expression on endothelial cells and Kupffer cells from both groups of mice. Endothelial cells and Kupffer cells were labeled with anti-mouse CD31-PE and F4/80-PE respectively. As reported previously, both cell types expressed CD1d.³ However, we found no significant difference in endothelial or Kupffer cell CD1d expression between lean and ob/ob mice (Figure 2d and e).

Decreased ob/ob Hepatocyte Presentation of α GalCer to DN32.IF3 Cells

To determine whether decreased CD1d influenced the ability of hepatocytes to activate invariant NKT cells, hepatocytes obtained from ob/ob or lean mice were co-cultured with DN32.IF3, a mouse CD1d-restricted invariant NKT cell hybridoma. In the absence of α GalCer, neither hepatocytes from lean nor ob/ob mice stimulated IL-2 release from the NKT hybridoma cells. However, IL-2 production increased substantially when α GalCer was added to the co-cultures. Hepatocytes from lean mice stimulated DN32.IF3 cells to secrete IL-2 significantly more than hepatocytes from ob/ob mice (Figure 3), providing evidence that decreased CD1d on hepatocytes impairs regulation of CD1d-restricted T-cell responses. Under our assay conditions, neither hepatocytes from lean mice nor hepatocytes from ob/ob mice stimulated DN32.IF3 cell production of INF γ .

Increased Endoplasmic Reticulum Stress in Livers of ob/ob Mice and Tunicamycin-Treated Mice

Decreased hepatocyte CD1d surface expression and antigen-presenting function in ob/ob mice with fatty livers resemble findings in mice that are genetically deficient in MTP;²⁷ the latter strain also has fatty livers, reduced expression of CD1d on hepatocyte surfaces, and impaired activation of CD1d-restricted T-cell responses. To evaluate the possibility that MTP deficiency might underlie these abnormalities in ob/ob mice, we compared MTP activity in ob/ob and lean hepatocytes. Our results verified a previous report⁴³ that MTP activity is actually increased in ob/ob mice (data not shown).

Figure 1 Distribution of CD1d tetramer-reactive liver mononuclear cells in ob/ob and lean C57BL6 mice. Mononuclear cells were isolated from the livers of ob/ob mice and lean mice. Purified mononuclear cell fractions were incubated with antibody to CD3, CD4 and CD1d tetramer. Fluorescence-activated cell sorting (FACS) analysis was used to determine the relative distribution of CD4 + CD1d tetramer-reactive (ie, NKT) cells in the two groups of mice. The FACS result shown is representative of three independent experiments (a). Cumulative data are graphed (results expressed relative to number of CD1d tetramer-reactive cells that were also CD4 + in lean control livers. * $P < 0.05$ compared to lean mice (b).

Thus, we explored alternative explanations for the CD1d defects in ob/ob mice. Given evidence that MTP-mediated events in the ER regulate trafficking of CD1d to hepatocyte plasma membranes as well as secretion of lipoproteins, we wondered if other types of ER dysfunction, such as ER stress, might dysregulate CD1d.

First, we examined whether hepatic ER stress was increased in ob/ob mice. To do this, we investigated the expression patterns of CHOP and GRP-78 (Bip) by RT-PCR and western blot, respectively. CHOP mRNA levels were higher in ob/ob mice than in lean mice (Figure 4a). The 78-kDa GRP-78 is an ER chaperone whose expression is increased upon ER stress.⁴⁴ As others have reported,⁴⁵ GRP-78 (Bip) protein levels were elevated in livers of the obese mice compared with lean controls (Figure 4b). For comparison, we examined these ER stress markers in tunicamycin-treated animals. Tunicamycin treatment has been well validated as an approach for inducing ER stress in hepatocytes. As expected,⁴⁵ CHOP and GRP-78 (Bip) were significantly increased after treatment of lean mice with tunicamycin (Figure 4c and d).

Decreased CD1d Expression and Antigen Presentation of AML-12 Hepatocytes after Treatment with Tunicamycin

Given the correlation between fatty liver, ER stress and reduced surface expression of CD1d on hepatocytes in ob/ob

mice, we next determined if a pharmacologic inducer of ER stress (eg, tunicamycin) decreased CD1d expression on the surface of non-steatotic hepatocytes. AML-12 hepatocytes were treated with vehicle or tunicamycin (5.0 $\mu\text{g/ml}$) for 48 h. After discarding the medium, plates were washed to remove dead (non-adherent) cells, and then adherent (viable) cells were harvested and analyzed by flow cytometry to determine the percentage that were CD1d+. Tunicamycin treatment significantly decreased the percentage of CD1d+ AML-12 hepatocytes (Figure 5a). In contrast, growing AML-12 cells in high insulin medium with dexamethasone for 48 h to induce triglyceride accumulation (Figure 5b and c) had no significant effect on surface CD1d expression (Figure 5a). Therefore, ER stress, rather than triglyceride accumulation *per se*, acutely inhibited surface CD1d expression on cultured hepatocytes.

We next examined whether ER stress influenced the ability of hepatocytes to activate invariant NKT cells. AML-12 hepatocytes were treated with a range of tunicamycin doses (0, 0.5, 1 or 5 $\mu\text{g/ml}$) for 24 h, tunicamycin-containing medium was removed, the cultures were washed three times to remove non-adherent (dead) cells, and then the remaining adherent (viable) cells were co-cultured with DN32.IF3 hybridoma cells in the presence of α -galactosylceramide (α Galcer) to evaluate CD1d antigen presentation. Tunicamycin pre-treatment resulted in a dose-dependent decrease in the ability of

Figure 2 Percentage of CD1d + cells in hepatocyte, endothelial cell and Kupffer cell fractions from ob/ob and lean mouse livers. Hepatocytes and liver non-parenchymal cells were isolated. Hepatocytes were incubated with antibody to CD-1 and H2-Db, an MHC class-1 molecule. Results of a representative FACS analysis of CD1d + hepatocytes in the two groups of mice (**a**). The percentage of hepatocytes that were CD1d + was analyzed in a total of four independent experiments. Cumulative data are graphed (* $P < 0.05$ compared to lean mice, $n = 4$) (**b**). CD1d expression was also evaluated by RT-PCR analysis of total RNA isolated from lean and ob/ob hepatocytes. GUS was detected in the same cDNA sample and used as an internal control. The RT-PCR gel shown is representative of findings in four independent experiments. Cumulative data are graphed (**c**). Liver non-parenchymal cells were incubated with anti-mouse CD1d-FITC. Endothelial cells and Kupffer cells were labeled with anti-mouse CD31-PE and F4/80-PE respectively. In each group of mice, the percentage of each cell type that was CD1d + is shown for endothelial cells (**d**) and Kupffer cells (**e**).

Figure 3 CD1d antigen presentation by ob/ob and lean hepatocytes. Hepatocytes were isolated from ob/ob and lean livers and then incubated with DN32.IF3 NKT hybridoma cells in the absence or presence of α GalCer (100 ng/ml). Efficacy of hepatocyte antigen presentation was assessed by quantifying induction of IL-2 production by the NKT hybridoma cells. DN32.IF3 cell production of IL-2 was no different than basal levels when the hybridoma cells were incubated with either ob/ob or lean hepatocytes in the absence of α GalCer. However, addition of α GalCer triggered substantial IL-2 production. Results of four independent experiments are graphed (* $P < 0.05$ compared to lean mice, $n = 4$).

AML-12 cells to stimulate DN32.IF3 cell secretion of IL-2 in the presence of α GalCer (Figure 5d). Interestingly, the 5 μ g/ml dose of tunicamycin that caused about a 40–50% decrease in the percentage of CD1d(+) hepatocytes (Figure 5a) virtually abolished hepatocyte induction of NKT hybridoma cell production of IL-2 (Figure 5d), suggesting that relatively small decrements in surface CD1d content on hepatocytes may result in profound impairment of certain NKT cell functions. Thus, pharmacologic induction of ER stress decreased CD1d on surfaces of otherwise healthy hepatocytes and this impaired hepatocyte regulation of CD1d-restricted NKT cell responses.

Tunicamycin Decreased Hepatic CD4 + NKT Cells *In Vivo*

To assess the effect of ER stress on NKT cell accumulation in intact mice, healthy lean, 6-week-old C57Bl-6 mice were injected with tunicamycin (400 ng/g body weight/day) or PBS i.p. for 5 days. PBS-treated mice had normal liver histology (Figure 6a), whereas mice treated with tunicamycin developed hepatic steatosis (Figure 6b). Biochemical assays confirmed that tunicamycin increased liver triglyceride content by about two-fold (Figure 6c). Interestingly, hepatic CD4 + NKT cells were significantly decreased in livers of tunica-

Figure 4 Markers of ER stress in ob/ob mice, tunicamycin-treated mice, and respective controls. Total liver RNA and protein were isolated from lean and ob/ob mice, and also from wild-type mice treated with saline vehicle or tunicamycin. CHOP was detected by RT-PCR; GUS was detected in the same cDNA sample and used as an internal control (a and c). Bip was detected in total liver protein by immunoblotting; β -actin was detected on the same blot and used as a loading control (b and d). The RT-PCR and immunoblots shown are representative of three independent experiments. Cumulative data are graphed.

mycin-treated mice compared to the livers of saline-treated controls (Figure 5d). In contrast, tunicamycin treatment did not decrease other T-cell populations. In fact, hepatic populations of both CD4(+) and CD4(-) conventional T cells tended to be higher in tunicamycin-treated mice than controls (Figure 6e and f). Hence, inducing ER stress in healthy lean mice caused hepatic steatosis and selectively reduced resident populations of CD1d-restricted of hepatic T cells.

Th-1 Polarization of Hepatic Cytokine Production in Tunicamycin-Treated Mice with Reduced Hepatic CD4+ NKT Cells

CD4+ NKT cells perform important immunoregulatory functions, including modulation of cytokine production by many other types of cells.^{46,47} To determine if tunicamycin-mediated alterations in hepatic NKT cells populations were accompanied by differences in hepatic cytokine profiles, real time RT-PCR analysis of liver RNA from tunicamycin-treated and control mice was performed. Tunicamycin treatment increased basal expression of Th-1 cytokines, including TNF- α and IFN- γ (Figure 7a and b), while levels of the Th-2 cytokine, IL-4, tended to decrease (Figure 7c). Such Th-1 polarization of hepatic cytokine production resulted in increased expression of pro-inflammatory cytokines, such as IL-12, when tunicamycin-treated mice were challenged with LPS (Figure 7d).

DISCUSSION

Earlier, we demonstrated reduced numbers of NK1.1(+) T cells in the fatty livers of mice with genetic- or diet-induced obesity and the metabolic syndrome.^{31,32} Although NK1.1 expression may vary during the course of NKT cell maturation and activation, virtually all NKT cells react to the glycolipid, α GalCer, presented by CD1d.⁴⁸ The present study shows that, like NK1.1(+) cells, tetramer-reactive cells are significantly reduced in the fatty livers of genetically obese, leptin-deficient ob/ob mice. Similar reductions were noted in wild-type mice with high-fat diet-induced obesity, hyperleptinemia/leptin resistance and fatty livers. Others recently reported defective *in vitro* activation of NKT cells by fatty hepatocytes from mice with an intact leptin axis but genetic deficiency of MTP, an ER protein that mediates lipoprotein assembly and secretion.²⁷ Thus, NKT cell depletion/dysfunction appears to be a common feature of fatty livers, occurring in mice with genetic obesity (eg, ob/ob mice) and diet-induced obesity (eg, high-fat diet fed mice).

This finding raises questions about the role of hepatocyte lipid accumulation in regulating NKT cells. Hepatocytes are known to express CD1d,^{3,27} the MHC-like molecule that presents lipid antigens to NKT cell TCRs. CD-1 isoforms are thought to acquire endogenous lipid antigens as they traffic through cellular endolysosomal compartments,¹ and recent studies in MTP-deficient cells and mice demonstrated that

Figure 5 Decreased CD1d expression and antigen presentation by AML-12 hepatocytes after treatment with Tunicamycin. AML-12 hepatocytes were cultured in serum supplemented DME/Ham's F-12 medium and treated with vehicle (control) or tunicamycin (5.0 µg/ml) for 48 h, or cultured in isobutylmethylxanthine, dexamethazone and insulin-containing medium (MDI) and treated with vehicle, but no tunicamycin, for 48 h. After washing the cultures to remove medium and non-adherent (dead) hepatocytes, hepatocytes were harvested, stained with oil red O stain or incubated with anti-mouse CD1d-FITC, and analyzed by FACS. Results in each treatment group are expressed as percentage of hepatocytes that were CD1d+. Data from three independent experiments are graphed (**a**). Photomicrograph of representative, vehicle-treated AML-12 cell grown in DME/Ham's F-12 medium demonstrates few lipid droplets (**b**). Photomicrograph of representative, vehicle-treated AML-12 cell grown in MDI demonstrates cellular accumulation of lipid droplets (**c**). To evaluate the effect of tunicamycin treatment on hepatocyte antigen presentation, experiments were repeated with AML-12 cells cultured in DME/Ham's F-12 medium. After treatment with a range of tunicamycin doses (0–5 µg/ml) for 48 h, cultures were washed to remove medium and dead cells. The remaining viable AML-12 hepatocytes were incubated with DN32.IF3 NKT hybridoma cells in the absence or presence of αGalCer. AML-12 hepatocyte presentation of αGalCer was assessed by measuring DN32.IF3 cell IL-2 production (**d**) (* $P < 0.05$ compared to control, ND, not detected).

events in the ER regulate subsequent delivery of lipid-loaded CD1d to hepatocyte plasma membranes.²⁷

We found that the expression of CD1d is reduced on the surface of fatty hepatocytes from ob/ob mice, but not on endothelial or Kupffer cells that were harvested from the same livers. As was reported in MTP-deficient mice with fatty livers,²⁷ reduced hepatocyte surface expression of CD1d occurred despite conserved levels of CD1d mRNA. However, MTP deficiency or dysfunction is not likely to explain decreased surface expression of CD1d or steatosis in ob/ob hepatocytes because MTP levels and very low-density lipoprotein secretion are known to be increased in these mice,⁴³ and we demonstrated that MTP activity is actually higher in ob/ob hepatocytes than in hepatocytes from their wild-type littermates.

MTP is an ER protein. CD1d is also known to interact with other ER proteins, including calnexin and calreticulin. The latter two ER proteins regulate ER quality control mechanisms by chaperoning proteins to assure proper folding.¹ Given that ER events regulate appropriate trafficking of CD1d, we, therefore, considered alternative ER-related mechanisms that might disrupt both hepatocyte lipid homeostasis and

CD1d trafficking. Fatty livers caused by alcohol consumption, high-fat diet-induced obesity and obesity from inherited leptin deficiency have been associated with ER stress.^{45,49} To test whether or not ER stress is sufficient to disrupt hepatocyte lipid homeostasis and reduce CD1d expression on hepatocyte plasma membranes, we treated mouse hepatocytes and intact healthy, lean mice with tunicamycin. As expected, tunicamycin induced classical markers of ER stress, including CHOP and BIP.⁵⁰ It also caused hepatic steatosis and produced a dose-dependent decrease in hepatocyte surface CD1d. This reduction in CD1d expression was paralleled by comparable decrements in hepatocyte activation of NKT cell hybridomas by αGalCer *in vitro*, as well as decreased numbers of CD1d-restricted cells in the livers of intact mice. Together, these findings suggest that hepatocyte ER stress contributes to both hepatic steatosis and altered homeostasis of hepatic NKT cell populations. The latter involves reduced accumulation of CD1d on hepatocyte plasma membranes.

Interactions with CD1d-presented lipid antigens regulate both the maturation and activation of NKT cells. Both processes are known to be impaired in the context of CD1d

Figure 6 Tunicamycin, an ER stress-inducing agent, causes hepatic steatosis and reduces liver CD4 + NKT cells. Six-week-old healthy lean C57BL-6 mice were injected with tunicamycin (400 ng/g body weight/day) or PBS i.p. for 5 days. Hematoxylin and eosin stained liver sections from representative from PBS-treated mice (a), and mice 5 days after tunicamycin treatment (b). Liver triglyceride content in all PBS- and tunicamycin-treated mice ($n = 4/\text{group}$), $P < 0.05$ vs PBS group (c). At the end of treatment, liver mononuclear cells were isolated from other mice in each group. Purified mononuclear cell fractions were harvested and analyzed by flow cytometry: CD4 + NKT cells (d), CD4- T cells (e) and CD4 + T cells (f) (* $P < 0.05$ compared to PBS-injected mice, $n = 4$).

deficiency.²⁸ We reported previously that NKT cell depletion resulted in Th1 polarization of hepatic cytokine production in ob/ob mice and wild type mice with diet-induced obesity.^{31,32} Both strains have also been shown to have fatty livers and hepatic ER stress.⁴⁵ It is unclear which insult (ie, steatosis or ER stress) is primary in mice with fatty livers because steatosis promotes ER stress.⁴⁵ However, in the present studies when we treated healthy, wild-type mice with tunicamycin to cause primary ER stress, the resultant outcomes included hepatic steatosis, decreased hepatocyte CD1d expression, depleted hepatic NKT cell populations and Th1 polarization of hepatic cytokines. Together, these data suggest that ER stress may trigger self-reinforcing mechanisms that contribute to both hepatic steatosis and innate immune dysfunction. Consistent with this concept, the livers of mice with tunicamycin-induced steatosis and NKT cell depletion expressed higher baseline mRNA levels of several Th-1 cytokines, including TNF- α and IFN- γ , that are known to exacerbate fatty liver damage.^{31,51} Also, similar to obese mice that are exquisitely sensitive to LPS-hepatotoxicity,³¹ tunicamycin-treated lean mice produced more pro-inflammatory cytokines, such as IL-12, when challenged with LPS. Hence, defects in the hepatic innate immune system generated a pro-inflammatory bias in local immune responses. Given the strong association between pro-inflammatory states and hepatic steatosis,³¹ the latter finding raises the intriguing possibility that hepatic NKT cell depletion *per se* might actually precipitate hepatic fat accumulation. This concept is sup-

ported by a recent publication demonstrating that adoptive transfer of small numbers of NKT cells improved steatosis and insulin sensitivity in ob/ob mice.⁵² In any case, given that iNKT cell-derived cytokines rapidly instruct other cells that participate in subsequent innate and adaptive immune responses,¹ these alterations in hepatic cytokine production are likely to have broad implications. Also, because iNKT cells are potently cytolytic, releasing perforin and granzymes and expressing membrane-bound Fas ligand like NK cells,^{53,54} decreases in hepatic iNKT populations might impair local clearance of tumor and microbial antigens.

At this point it is not clear to what extent, if any, ER stress-related alterations in hepatic NKT cell homeostasis contribute to obesity-related morbidity and mortality in humans. Lean mice that chronically consume high-calorie diets become obese, and develop fatty livers, hepatic ER stress and reduced numbers of liver NKT cells. While overeating clearly promotes obesity and hepatic steatosis in humans, whether or not this is accompanied by hepatic ER stress or NKT cell depletion has not, to our knowledge, been investigated. Relative to murine livers in which iNKT cells are one of the predominant lymphocyte populations, human livers harbor fewer iNKT cells.^{14,55,56} Nevertheless, the liver remains the predominant locus for iNKT cells in both species.^{4,55} In addition, structural analysis of human and murine iNKT TCRs demonstrates conservation of domains that interact with CD1d-presented glycolipids,⁵⁷ predicting cross-species reactivity to antigens that are presented to iNKT cells by

Figure 7 Increased hepatic TNF- α , IFN- γ and IL-12 mRNA expression in livers of mice with tunicamycin-induced ER stress. Tunicamycin (200 ng/g body weight) was injected i.p. two times a week. After 4 weeks, basal expression of the pro-inflammatory (TH1) cytokines TNF- α (a) and IFN- γ (b) and the anti-inflammatory cytokine, IL-4 (c), were measured in liver tissues by real time PCR. After 4 weeks of tunicamycin treatment, some mice were injected i.p. with a single dose of *E. coli* LPS (20 μ g/mouse) or PBS and then killed after 6 h to obtain liver tissue. Total liver RNA was extracted; mRNA expression of the pro-inflammatory cytokine IL-12 (d), was measured by real time PCR. In all experiments, * $P < 0.05$ for tunicamycin-treated mice compared to PBS-treated mice ($n = 4$ mice/group).

hepatocyte CD1d. Thus, it is plausible (albeit unproven) that mice and humans with fatty livers exhibit similar defects in CD1d-mediated responses. Consistent with this concept, in both mice and humans, obesity, a major risk factor for hepatic steatosis, is strongly associated with conditions, such as autoimmunity and cancer, which typically increase as NKT cell function declines.^{14,58,59} Further research is needed to explore how obesity might reconfigure hepatic immune responses, given this novel evidence that links hepatic ER stress and fat accumulation with dysregulation of hepatic innate immunity.

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