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Accumulation of NKT cells in Progressive Nonalcoholic Fatty Liver Disease

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Abstract

Liver inflammation is greater in nonalcoholic steatohepatitis (NASH) than steatosis, suggesting that immune responses contribute to NAFLD progression. Livers normally contain many natural killer T (NKT) cells which produce factors that modulate inflammatory and fibrogenic responses. Such cells are relatively depleted in steatosis, but their status in more advanced NAFLD is uncertain. We hypothesized that NKT cells accumulate and promote fibrosis progression in NASH. We aimed to determine if livers become enriched with NKT cells during NASH-related fibrosis; identify responsible mechanisms; and assess if NKT cells stimulate fibrogenesis. NKT cells were analyzed in wild type mice and Ptc⁺/⁻ mice with an overly-active Hedgehog (Hh) pathway, before and after feeding methionine choline deficient (MCD) diets to induce NASH-related fibrosis; effects of NKT cell-derived factors on hepatic stellate cells (HSC) were examined and fibrogenesis was evaluated in CD1d-deficient mice which lack NKT cells; NKT cells were quantified in human cirrhotic and non-diseased livers. During NASH-related fibrogenesis in wild-type mice, Hh pathway activation occurred, leading to induction of factors that promoted NKT cell recruitment, retention and viability, plus liver enrichment with NKT cells. Ptc⁺/⁻ mice accumulated more NKT cells and developed worse liver fibrosis; CD1d-deficient mice which lack NKT cells were protected from fibrosis. NKT cell-conditioned medium stimulated HSC to become myofibroblastic. Liver explants were 2-fold enriched with NKT cells in patients with non-NASH cirrhosis, and 4-fold enriched in patients with NASH-cirrhosis. In conclusion, Hh pathway activation leads to hepatic enrichment with NKT cells that contribute to fibrosis progression in NASH.

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Keywords

cirrhosis; natural killer T cells; non-alcoholic fatty liver disease

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease. It encompasses a spectrum of histopathology, including hepatic steatosis (fatty liver) and nonalcoholic steatohepatitis (NASH)(1,2). Although hepatocyte injury and death are uniformly worse in NASH than in steatosis, the outcomes of NASH are variable.

Hepatic accumulation of inflammatory cells is generally greater in NASH than in steatosis, suggesting that activation of the immune system may contribute to progression of fatty liver damage. The liver harbors resident populations of cells that regulate innate immune responses(3). Natural killer T (NKT) cells, a subset of lymphocytes that express both cell surface receptors normally expressed on NK cells (such as NK1.1 or CD57 in mice and CD56 or CD57 in humans) and a T-cell receptor, are particularly abundant in healthy livers(4,5). For example, NKT cells with an invariant T cell receptor comprise up to 20% of T cells in murine livers. Such cells are also enriched in human livers which harbor a more diverse repertoire of NKT cells(5,6). In both species, NKT cells reside mainly in the hepatic sinusoids, where they provide intravascular immune surveillance(7,8). NKT cells specifically recognize glycolipid antigens and can produce both Th1 and Th2 cytokines when activated(4,9). This ability to skew the hepatic microenvironment cytokine milieu towards either proinflammatory/antifibrogenic (Th1) or anti-inflammatory/profibrogenic (Th2) responses is well-documented(10), and may explain the apparently contradictory outcomes that accompany hepatic enrichment with NKT cells in different liver diseases. For example, NKT cells accumulation appears to promote fibrosis in chronic viral hepatitis(11,12), Wilson's disease(13) and primary biliary cirrhosis(14,15), but may attenuate chronic carbon-tetrachloride-induced liver fibrosis in mice(16).

The possibility that NKT cells modulate disease outcomes in NAFLD is an attractive hypothesis because these cells are activated by lipid antigens and NAFLD is a disorder of fat homeostasis. However, the tendency for NKT cells to down-regulate expression of classical NK cell surface markers when activated or immature, and their heterogeneity in humans, have confounded efforts to investigate this hypothesis. Liver NKT cells appear to be relatively depleted in ob/ob mice, a model of obesity-related pro-inflammatory cytokine excess, insulin resistance, and mild NASH(17-19). Adoptive transfer of NKT cells to ob/ob mice resulted in a reduction of liver steatosis and improved glucose homeostasis(20). Reductions in hepatic NKT cell numbers have also been reported to occur when wild type mice are administered high-fat, high sucrose diets to induce obesity, insulin resistance, and hepatic steatosis(21),(22), further supporting the concept that relative depletion of hepatic NKT cells contributes to the metabolic and cytokine alterations that are involved in the pathogenesis of hepatic steatosis.

Neither ob/ob mice nor mice fed high-fat, high- sucrose diets develop much liver fibrosis, even after protracted exposure to steatogenic conditions(23), leaving the role of NKT cells in more advanced stages of NAFLD uncertain. A recent study of liver biopsy samples from 54 patients with varying severities of NASH-related liver injury suggested that livers become relatively enriched with NKT cells during more severe NASH(24). Around 13% of the liver mononuclear cells isolated from some of these patients were double-positive for CD56 and CD3 in patients with NAS >5, compared to about 9% from patients with NAS < 4 or chronic hepatitis C. Furthermore, more cells expressed V α 24, denoting invariant NKT

(iNKT) cells, in both groups of NASH patients compared with chronic hepatitis C. NKT cells isolated from subjects with NAS > 5 produced more IL4 than those with NAS < 4 or chronic hepatitis C, prompting the authors to speculate that NKT cells might contribute to fibrosis during NASH. However, the small number of subjects with advanced liver fibrosis and cross-sectional design of the study precluded definitive attribution of causality.

Feeding methionine choline-deficient (MCD) diets to rodents reproducibly elicits NASH with fibrosis, mimicking the severe form of NASH that occurs in some humans(23). The current study used this model to investigate the possibility that NKT cells may contribute to liver fibrogenesis in NASH. We aimed to determine if liver lymphocyte populations are relatively enriched with NKT cells in NASH-related fibrosis/cirrhosis; identify mechanisms that might account for this; and assess if NKT cells stimulate fibrogenesis.

Methods

A) Animal experiments

Mice—C57BL/6 wild type (WT) (Jackson Laboratories, Bar Harbor, ME), Patched-deficient (*Ptc*^{+/-}) mice (from R.J. Wechsler-Reya, Duke University, NC), and CD1d-deficient mice (from ZP Li, Johns Hopkins University, Baltimore, MD) were fed methionine-choline deficient (MCD) diet or control chow for 8 weeks. *Ptc*^{+/-} mice have only one copy of patched, a Hedgehog (Hh)-pathway repressor. Therefore, they are unable to silence Hh-signaling and exhibit excessive Hh-pathway activity(25). NKT cells are genetically absent in CD1d-deficient mice(26).

Analysis of Liver Architecture, Morphometry and Immunohistochemistry—

Formalin-fixed paraffin embedded (FFPE)-livers were analyzed(27). For detailed protocol and antibodies, see Supplemental Materials and Methods.

RNA extraction and mRNA quantification—Total liver RNA extraction and mRNA quantification by Real time QRT-PCR were performed as detailed in Supplemental Materials and Methods (27). Primers are in Supplemental Table 1.

Hydroxyproline Assay—Hydroxylproline content in whole liver specimens was quantified colorimetrically(27).

Mouse primary liver mononuclear cell (LMNC) isolation/characterization—

LMNC were isolated from WT mice (28,29) and characterized by fluorescent antibody cell sorting (FACS) as detailed in Supplemental Materials and Methods.

Effect of primary liver NKT cells on activation of primary HSC—LMNC were cultured in complete NKT media (RPMI 1640, supplemented with IL2 (10ng/ml; Biolegend) and 10% heat inactivated fetal bovine serum)(28), with or without the NKT cell ligand, α GalCer (100 ng/ml; (Axxora, Cat no 306027, CA), for 24 hours. This α GalCer dose elicits maximal iNKT activation(30). Conditioned medium were then added to primary murine hepatic stellate cells for 1 day and RNA was harvested for QRT-PCR. Experiments were performed in duplicate wells and repeated twice.

Cell lines and static adhesion assay—Murine cholangiocyte 603B line (from Yoshiyuki Ueno, Tohoku University, Sendai, Japan, and G Gores, Mayo Clinic, Rochester, MN)(31), rat hepatic stellate cell (HSC) line 8B (from M Rojkind, George Washington University, Washington, DC)(32), and murine invariant hybridoma cell line DN32 (from Albert Bendelac, University of Chicago, Chicago, IL)(33) were cultured according to

established protocols(31-33). Static adhesion was performed as previous described(34) and detailed in Supplemental Materials and Methods.

B) Human subjects

Analysis of NKT cells subsets in human liver—Explanted diseased livers from individuals undergoing liver transplantation and non-diseased liver tissues removed during resection for colorectal hepatic metastases or from split-liver grafts, were used to isolate liver-derived lymphocytes as described(35). Primary hepatic lymphocytes were stained with PE-Cy7 conjugated anti-CD3 (eBioscience; clone UCHT1, Cat: 25-0038; Hatfield, UK) and FITC-conjugated anti-CD56 (BD; cat no 34058; Oxford, UK), and analyzed using Summit 4.3 software (Dako Cytomation).

Immunohistochemistry—Formalin-fixed, paraffin-embedded liver sections from de-identified controls and subjects with biopsy-proven NASH-related cirrhosis (n=6/group) from Departments of Pathology at Duke University and University Hospital Cassiano Antônio de Moraes were studied in accordance with NIH and Institutional guidelines for human subject research (see Supplemental Materials and Methods for immunohistochemistry protocol/antibodies).

Statistical analysis—Results are expressed as mean $\pm$ SEM. Statistical significance was determined using the Student's t-test. Significance was accepted at the 5% level, *p<0.05.

Results

MCD diets induce NASH and liver fibrosis in wild type mice

Compared to control mice that were fed normal chow (n = 25), MCD diet-treated mice (n = 25) developed significant macrovesicular steatosis, ballooning degeneration of hepatocytes, and liver necro-inflammation (Fig 1A), as well as fibrosis after 8 weeks. The latter was demonstrated by increased Sirius red staining (Fig 1B, C), and hepatic hydroxyproline quantification (Fig 1D). Collagen deposition was accompanied by the accumulation of α -SMA-immunoreactive cells (Fig 1E, F), and induction of pro-fibrogenic genes, including α -sma, tgf β collagen 1 α 1, mmp9, and timp1 (Suppl Fig 1 A-E).

Hedgehog (Hh)-pathway activation and Hh-related accumulation of hepatic NKT cells occur during MCD diet-induced NASH in wild type mice

These fibrotic livers also demonstrated increased activity of the Hh-pathway, a morphogenic signaling system that orchestrates wound healing responses(36). Sonic hedgehog ligand (Shh) mRNA expression tripled after MCD diet treatment and mRNA levels of the Hh-regulated transcription factor, glioblastoma (gli)2, increased 4 fold. This was accompanied by significant accumulation of Gli2-expressing cells, which tended to localize near portal tracts and along fibrous septa that contained immature ductular cells and fibroblastic cells (Fig 2A). mRNA levels of CXCL16, the Hh-inducible NKT cell chemokine, and VCAM1, a factor that promotes NKT cell adhesion, increased significantly by MCD diet treatment (Fig 2B-C).

Hh-dependent production of CXCL16 by immature ductular cells promotes NKT cell chemotaxis(37). To determine if Hh-pathway activation also promotes NKT cell adhesion, NKT cells were incubated with immature ductular cells in the presence of vehicle or Shh. Shh significantly increased adhesion of NKT cells to ductular cells; this was abrogated by adding 5E1 antibody to neutralize Shh activity (Fig 2D). In fibrotic livers, myofibroblastic stellate cells are a major source of Shh(36). Therefore, static adhesion assays were repeated after co-culturing immature ductular cells with a myofibroblastic cell line (8B) in a trans-

well system(38). Co-culture with stellate cells increased static adhesion of NKT cells to ductular cells; this was also markedly attenuated when Hh-neutralizing antibodies were added to the medium (Fig 2E). Therefore, Hh-pathway activation during NASH promotes the hepatic recruitment and retention of NKT cells.

Fibrotic livers with NASH also expressed higher levels of CD1d (Fig 3A) and IL15 (Fig 3B), both of which can promote NKT cell survival. Consistent with evidence that MCD diet-induced NASH results in a nurturing microenvironment for NKT cells, the livers from MCD diet-treated mice were significantly enriched in CD1d tetramer-reactive NKT cells (Fig 3 C-D). Immunostaining for CD57, another NKT cell marker, confirmed that NASH liver parenchyma harbored about 2X more CD57(+) cells than control livers (Fig 3E-F).

Genetic over-activation of the Hedgehog-pathway exacerbates hepatic NKT cell accumulation in MCD diet-fed mice

Ptc^{+/-} mice (which exhibit sustained Hh-signaling after pathway activation(25)) develop more liver fibrosis than wild type mice when fed MCD diets(39). Therefore, we fed Ptc^{+/-} mice MCD or control diets for 8 weeks, isolated liver mononuclear cells (LMNC), and performed FACS to determine if over-activating the Hh-pathway influenced NKT cell accumulation. NKT cells comprised ~ 10% of the LMNC in chow-fed Ptc^{+/-} mice (Fig 4A), and 8 weeks of MCD diet feeding resulted in ~ 4 fold enrichment of NKT cells (Fig 4B-C). Thus, although LMNC from Ptc^{+/-} and wild type C57Bl6 mice contained similar proportions of NKT cells before injury-related activation of hepatic Hh signaling, the degree of both Hh pathway activation and NKT cell enrichment was greater in Ptc^{+/-} mice during NASH. The findings may be relevant because Ptc^{+/-} mice are also known to develop more severe liver fibrosis than WT mice during MCD diet-induced NASH.

To more directly evaluate the significance of hepatic NKT accumulation for NASH-related fibrogenesis, MCD diet feeding was repeated in CD1d-deficient mice which lack NKT cells(26) and littermate controls (n = 3/group). Livers were harvested after 8 weeks for assessment of collagen gene expression and hepatic hydroxyproline content. Both assays demonstrated significant attenuation of fibrogenesis in the NKT cell-deficient mice (Fig 4 D-E).

Activated mouse primary liver NKT cells promote myofibroblastic activation of mouse primary liver stellate cells

Primary hepatic NKT cells produce Shh ligand, and Hh ligands stimulate them to produce fibrogenic factors, including IL4 and IL13 (29), suggesting that soluble factors from NKT cells promote liver fibrosis. To investigate this more directly, primary LMNC were isolated from healthy mice and incubated with α -Galcer(40), which specifically activates NKT cells; cell-conditioned medium was then added to primary cultures of mouse stellate cells. One day later, cultures were harvested to obtain RNA for PCR analysis. Parallel studies were done with conditioned medium from LMNC that were treated with vehicle. Conditioned medium from LMNC that contained activated NKT cells induced expression of the Hh-target gene, *gli1*, increased expression of the stellate cell activation marker, foxhead box (*Foxf1*), and up-regulated expression of several fibrosis-related genes (Fig 5A-F), supporting the concept that NKT cell-derived factors promote liver fibrosis.

Hepatic accumulation of NKT cells accompanies Hh pathway activation during NASH-related liver fibrosis in humans

NKT cells comprise a large proportion of LMNC in mice but are much less abundant in humans(5,6). Also, the mouse model of MCD diet-induced NASH differs in several regards from typical NASH in humans(23),(41). Thus, it was important to determine whether

alterations in hepatic NKT cells seen in the murine model of MCD diet-induced NASH also occurred in patients with NASH-related liver fibrosis. As we observed in mice with MCD diet-induced NASH and liver fibrosis, liver biopsies from patients with NASH and stage 3-4 liver fibrosis (Supplemental Fig 2) demonstrated accumulation of α -SMA-expressing cells (Fig 6A), and cells that expressed Shh ligand (Suppl Fig 2B), the Hh-regulated transcription factor, Gli2 (Suppl Fig 2C), and the Hh-inducible NKT cell chemokine, CXCL16 (Fig 6B), particularly within fibrous septa. Cells expressing markers of NKT cells (i.e., CD56/CD3 or CD57/CD3) also tended to accumulate in these areas (Fig 6C, Supplemental Fig 2). FACS analysis of LMNC from the explanted livers of 2 other patients that were undergoing liver transplantation for NASH-related cirrhosis confirmed that LMNC populations remained significantly enriched with NKT cells. NKT cells comprised 20-24% of LMNC in NASH-related cirrhosis (Fig 7 A, B), 10% in hepatitis C-related cirrhosis (Fig 7C) and 6% in cirrhosis due to autoimmune hepatitis (Fig 7D). Consistent with published data(42), we found that NKT cells comprised 3-5% of the LMNC in 2 non-diseased livers (Fig 7 E, F). Thus, intrahepatic mononuclear cells become enriched with NKT cells in cirrhosis.

Discussion

The present study demonstrates that LMNC populations are enriched with NKT cells in rodent and human livers with NASH-related fibrosis. Thus, fibrosing NASH and NASH-induced cirrhosis differ from hepatic steatosis, which is characterized by relative depletion of liver NKT cell populations (17-20,43). Our findings are consistent with a recent publication from another group which also demonstrated that hepatic NKT cells increase in parallel with the NAS score(24). In addition, we identified a novel mechanism that may contribute to hepatic NKT cell accumulation, namely liver injury-related activation of the Hh pathway.

In mice(39,44) and humans(39), hepatic Hh-pathway activation strongly correlates with fibrogenic repair of liver injury. This leads to accumulation of cells that produce and/or respond to Hh ligands. Hh-mediated cross-talk between such cells induces production of various chemokines, including CXCL16, an NKT cell chemoattractant(37). Here, we show that adhesion of NKT cells to liver cells is also Hh-regulated, and that, hepatic expression of IL-15 and Cd1d, two factors that enhance NKT cell viability, are significantly up-regulated in mice with increased Hh pathway activity and NASH-fibrosis. Increased hepatic CD1d has also been noted in patients with severe NASH.(24) Thus, factors that promote NKT cell recruitment, retention, and viability are induced in human and rodent livers with NASH-related fibrosis. Hh-pathway activation plays an important role in this process because a genetic manipulation that increases Hh-pathway activity in murine livers exacerbates NASH-related enrichment of liver NKT cells. Hh-signaling may also mediate hepatic NKT cell accumulation in human NASH given the striking correlation between hepatic Hh-pathway activity and the level of enrichment of liver mononuclear cells with NKT cells in patients with NAFLD-related cirrhosis.

Our work also suggests that NKT cells actively promote fibrogenesis in NASH because CD1d-deficient mice which lack NKT cells are protected from NASH-related fibrosis and treatment of mouse primary hepatic stellate cells with conditioned medium from LMNC that contained α GalCer-activated NKT cells stimulated stellate cells to become myofibroblastic. Previously, we showed that primary liver NKT cells from mice produce Shh, and that Shh stimulates NKT cells to produce the pro-fibrogenic cytokines, IL-4 and IL-13(29). Shh also directly stimulates myofibroblastic activation of hepatic stellate cells, and promotes the proliferation and survival of liver myofibroblasts(36). *Ptc*^{+/-} mice develop worse fibrosis after either bile duct ligation(27) or MCD diets(39). Others have reported that IL-13 production increases in mice with NASH-fibrosis, and shown that treatments that neutralize

IL-13 reduce fibrogenesis(45). Likewise, inhibiting IL-4 activity is known to diminish hepatic fibrosis in mice(46).

Our human studies demonstrate that hepatic enrichment with NKT cells is a feature of cirrhosis. Definitive NKT cell enrichment was observed in patients with NASH-related cirrhosis, in whom we detected a 4-5 fold relative increase in liver NKT cells. The present study confirms the previous reports that increased hepatic expression of CD1d occurs in advanced NAFLD, raising the possibility that antigen presentation to NKT cells may be enhanced. This is intriguing because CD1d presents lipid antigens to NKT cells and lipid homeostasis is abnormal in NAFLD. However, further research is needed to determine if and why there might be disease-related differences in hepatic accumulation of NKT cells. Additional studies to address the possibility that NKT cells may interact with other types of innate immune cells to modulate fibrosis progression are also justified because CD56(+)/CD3(-) cells (i.e., NK cells) were relatively depleted in the human cirrhotic livers that we examined, and liver NK cells are thought to serve anti-fibrogenic functions(6). Also, it has been suggested that liver macrophages, which are a rich source of immunomodulatory cytokines, may be altered in NASH(18), and this could further influence fibrotic activity.

In summary, this analysis of mice and humans with NASH-related liver fibrosis demonstrates significant enrichment of liver mononuclear cell populations with NKT cells, identifies a role for Hh-pathway activation in this process, and proves that activation of liver NKT cells generates soluble factors that promote fibrogenesis via a mechanism that involves myofibroblastic activation of hepatic stellate cells. Because these results identify novel immune-mediated mechanisms that contribute to fibrosis progression in NAFLD, the findings have potential clinical implications for one of the most common types of chronic liver injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

αGalCer	alpha-Galactosylceramide
α-sma	alpha-smooth muscle actin
Ctgf	connective tissue growth factor

Facs	fluorescent activated cell sorting
Foxf1	Forkhead box F1
FFPE	formalin-fixed paraffin embedded
Gli	glioblastoma
Hh	Hedgehog
iNKT	invariant natural killer T
mmp9	matrix metalloproteinase 9
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NK	natural killer
NKT	natural killer T
MCD	methionine choline deficient
Ptc^{+/-}	patched
Shh	sonic hedgehog
Tgf β	transforming growth factor beta
Timp1	tissue inhibitor of metalloproteinase 1
Vcam1	vascular cell adhesion molecule 1

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Figure 1. Mice develop NASH-fibrosis when fed a methionine-choline deficient (MCD) diet
Wild-type mice were fed control chow (n=25) or MCD diet (n=25) for 8 weeks, and then sacrificed. (A) Representative hematoxylin & eosin (Final magnification 400X, insert in H&E-stained section from MCD mouse liver shows a ballooned hepatocyte) (B) Sirius red staining after MCD diet (Final magnification 200X). (C) Sirius red quantification by morphometric analysis, and (D) hepatic hydroxyproline content at the end of the treatment period. Results are expressed as fold change relative to control-chow fed mice. (E) αSMA immunoreactivity (final magnification 400X) and (F) αSMA morphometry. Sections from 5 animals were used at each time point and 10 randomly selected, 400X fields chosen for analysis by the Metaview software. Results are expressed as fold change relative to chow-fed control mice and graphed as mean ± SEM. *P<0.05 vs. control mice.

Figure 2. Activation of the Hedgehog-pathway promotes NKT recruitment and static adhesion in murine NASH

Liver tissue from mice described in Figure legend 1 was harvested for QRT-PCR and immunohistochemistry. (A) representative Gli2-staining in MCD diet-treated mice (Final magnification 400X) and mRNA expression of gli2, (B) cxcl16, and (C) vcam-1. Results are expressed as fold change relative to chow-fed control mice, and graphed as mean $\pm$ SEM. (D) DN32 static adhesion to immature ductular cells (603B) treated with Sonic hedgehog and / or 5E1, the hedgehog-neutralizing antibody. (E) DN32 static adhesion to 603B cells co-cultured with the myofibroblastic cell line (8B) for 24 hours. Results are expressed as fold change relative to the number of adherent DN32 cells to vehicle-treated ductular cells or ductular cell mono-culture. * $P < 0.05$ vs. control mice. Experiments were performed in duplicate and repeated twice.

Figure 3. NASH fibrosis is associated with enrichment of liver NKT cells

At the end of 8 weeks, chow-fed control and MCD-fed mice (n=15/group) were sacrificed and QRT-PCR analysis of total liver RNA was done to assess the expression of NKT viability factors. (A) *cd1d*, and (B) *il15* mRNA. Results are expressed as fold change relative to the chow-fed control mice and graphed as mean $\pm$ SEM. *P<0.05 vs. control chow-fed mice. (C-D) Additional mice (n=10/group) were treated with control chow or MCD diet for 8 weeks; whole livers were used for FACS analysis. CD4 / CD1d-tetramer double-staining in chow-fed control (C), or MCD-treated mice (D). Livers from all mice were used for FACS analysis. (E-F) CD57 immunoreactive T cells in the hepatic parenchyma of NASH livers (E) compared with normal livers (F) (Final magnification 400X). For each group, sections from 4 mice were analyzed.

Figure 4. Increased accumulation of intrahepatic NKT cells during diet-induced NASH in *Ptc*^{+/-} mice with an overly-active Hedgehog-pathway and protection from MCD diet-induced fibrosis in *CD1d*-deficient mice that lack NKT cells

Ptc^{+/-} mice were fed regular chow (n=2) or MCD diets (n=2) for 8 weeks. At the end of the treatment period, all mice were sacrificed, and intrahepatic mononuclear cells were isolated for FACS analysis. (A) CD4 / CD1d-tetramer double positive cells in chow-fed *Ptc*^{+/-} mice and (B-C) MCD diet-fed *Ptc*^{+/-} mice. *CD1d*-deficient mice and littermate wild type (WT) controls were fed either control chow or MCD diets for 8 weeks (n=3 mice/group/dietary treatment). Fibrosis was evaluated by assessing (E) hepatic hydroxyproline content and performing QRT PCR analysis of (F) hepatic collagen gene expression. Results are expressed as Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.005$

Figure 5. α Galactosylceramide-treated mouse primary liver NKT cells induce primary liver hepatic stellate cell activation

Isolated mouse primary liver mononuclear cells (MNC) (5×10^4) were cultured in complete NKT media, and treated with vehicle or α Galactosylceramide for 24 hours. Cell conditioned-medium were then added to primary cultures of mouse hepatic stellate cells for a further 24 hours; stellate cells were then harvested, and changes in gene expression were assessed by QRT-PCR analysis. (A) gli1, (B) foxf1, (C) collagen 1 α 1, (D) α sma, (E) tgfb, and (F) ctgf. Results are expressed as fold change relative to stellate cells treated with 'vehicle-MNC' conditioned-medium. Mean $\pm$ SEM of duplicate experiments are graphed.

*P<0.05 vs. vehicle-MNC treated stellate cells.

Figure 6. Accumulation of liver NKT cells accompanies Hedgehog-pathway activation in human NASH

(A-C) Liver sections from patients with NASH and either little fibrosis (F0-1) or advanced fibrosis (F3-4) were stained for the marker of activated myofibroblast, α SMA (A), the Hh-regulated NKT chemokine, CXCL16 (B) and NKT cells, i.e., CD3 (blue) / CD57 (brown) double-immunoreactive cells. Representative photomicrographs are shown. Final magnification 400X (A,B) and 600X (C).

Figure 7. Enrichment of liver mononuclear cells with NKT cells in human cirrhosis

Liver infiltrating leucocytes were isolated from explanted livers of individuals undergoing liver transplantation (n=4), non-diseased liver tissues removed during resection for colorectal hepatic metastases (n=2), as well as excess liver tissues obtained during split-liver grafts (n=2), and analyzed by FACS. (A-B) NASH cirrhosis, (C) hepatitis C cirrhosis, (D) autoimmune hepatitis, and (E-F) non-diseased control livers. NKT cells were identified as CD3 / CD56 double-positive cells.