Published in final edited form as:
Hepatology. 2012 August ; 56(2): 747–759. doi:10.1002/hep.25694.

INHIBITION OF NK CELLS PROTECTS THE LIVER AGAINST ACUTE INJURY IN THE ABSENCE OF GNMT

Laura Gomez-Santos[1], **Zigmund Luka**[2], **Conrad Wagner**[2,3], **Sara Fernandez-Alvarez**[1], **Shelly C Lu**[4], **Jose M Mato**[1], **Maria L Martinez-Chantar**[1], and **Naiara Beraza**[1,5]

[1]Department of Metabolomics, CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberedh), Technology Park of Bizkaia, 48160-Derio, Bizkaia, Spain

[2]Department of Biochemistry, Vanderbilt University, Nashville, TN

[3]Tennessee Valley Department of Medical Affairs Medical Center, Nashville, TN

[4]Division of Gastrointestinal and Liver Diseases, USC Research Center for Liver Diseases, Southern California Research Center for Alcoholic Liver and Pancreatic Diseases and Cirrhosis, Keck School of Medicine, University of Southern California, Los Angeles, CA

Abstract

Glycine N-methyltransferase (GNMT) catabolizes S-adenosylmethionine (SAMe), the main methyl donor of the body. Patients with cirrhosis show attenuated GNMT expression, which is absent in HCC samples. GNMT$^{-/-}$ mice develop spontaneous steatosis that progresses to steatohepatitis, cirrhosis and HCC. The liver is highly enriched with innate immune cells and plays a key role in the body's host defense and in the regulation of inflammation. Chronic inflammation is the major hallmark of NASH progression. The aim of our study was to uncover the molecular mechanisms leading to liver chronic inflammation in the absence of GNMT, focusing on the implication of NK/NKT cells. We found increased expression of Th1- over Th2-related cytokines, TRAIL-R2/DR5 and several ligands of NK cells in GNMT$^{-/-}$ livers. Interestingly, NK cells from GNMT$^{-/-}$ mice were spontaneously activated; expressed more TRAIL and had strong cytotoxic activity, suggesting their contribution to the pro-inflammatory environment in the liver. Accordingly, NK cells mediated hypersensitivity to ConA-mediated hepatitis in GNMT$^{-/-}$ mice. Moreover, GNMT$^{-/-}$ mice were hypersensitive to endotoxin-mediated liver injury. NK cell depletion and adoptive transfer of TRAIL$^{-/-}$ liver-NK cells protected the liver against LPS-liver damage.

Conclusions—Our data allow us to conclude that TRAIL-producing NK cells actively contribute to promote a pro-inflammatory environment at early stages of fatty liver disease suggesting that this cell compartment may contribute to the progression of NASH.

Keywords

GNMT; NASH; NK cells; TRAIL

GNMT is the most important methyltransferase of the liver and is responsible of the catabolism of SAMe, the main methyl donor of the body. GNMT is down-regulated in

[5]Corresponding Author: Naiara Beraza, PhD, Department of Metabolomics, CIC bioGUNE, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (Ciberedh), Technology Park of Bizkaia, 48160-Derio, Bizkaia, Spain. Tel. 0034/944044592, Fax. 0034944061301, nberaza@cicbiogune.es.

Conflict of Interest Statement: The authors declare that they have no competing financial interests.

patients at risk of developing cirrhosis and is absent in HCC samples(1). Accordingly, we described that mice lacking GNMT accumulate high levels of SAMe and develop spontaneous steatosis that progresses to steatohepatitis, cirrhosis and HCC(1–3).

The importance of the liver as an immune organ is well accepted. The liver receives the majority of its blood from the gut and represents an essential line of defense against products from the digestive tract. Several studies underlined the implication of gut-derived endotoxins in the pathogenesis of NASH in mice and humans as fatty liver disease sensitizes the liver to LPS-injury(4–6). In this line, several work highlighted NKT cells as especially relevant to preserve a balanced Th1/Th2 response in genetic- and diet induced-models of NASH(7, 8). More recently, the implication of TRAIL-producing NK cells as mediators of liver inflammation and NASH progression has been demonstrated(9).

Liver steatosis is a benign process that can be persistent with no further consequences for the patient. However, when liver inflammation is chronic, the progression of NASH might be irreversible. To better understand the molecular mechanisms triggering the progression from steatosis to chronic hepatitis will allow us to develop potential therapeutic strategies to counteract NASH progression.

Herein, we found spontaneous activation of NK cells in livers from GNMT$^{-/-}$mice that play a key role in mediating ConA and LPS-mediated liver injury as selective depletion of NK cells significantly attenuated liver damage. Moreover, adoptive transfer of liver-derived TRAIL$^{-/-}$NK cells significantly attenuated LPS/GalN-mediated acute liver injury in GNMT$^{-/-}$ animals.

Overall, our data supports the essential role of TRAIL-producing NK cells in mediating acute liver injury when GNMT is absent and points to this cell compartment as potential mediator of chronic inflammation in the onset of NASH progression.

MATERIALS AND METHODS

GNMT Knockout animals and models of liver injury

GNMT$^{-/-}$ mice were bred as previously described(10). TRAIL$^{-/-}$ mice were kindly provided by Amgen (Seatlle, WA, USA). Male 8–10 week old mice were used in our experiments. Animals were treated according to the guidelines of the National Academy of Sciences (National Institutes of Health publication 86–23, revised 1985).

Liver injury experimental models

Concanavalin A (15mg/kg) (Sigma) was injected i.v.. LPS (16mg/kg)/GalN (800mg/kg) (Sigma) was administered i.p.. Jo2 antibody (0.5 μg/g) (BDbioscience) was injected i.p.. TNF (6μg/Kg) (Preprotech) was administered i.v.. Nicotinamide (NAM) (50 μM; Sigma) was administered dissolved in the drinking water.

Determination of liver damage and apoptosis

Alanine aminotransferase (ALT) was determined in serum. Histological examination was performed in formalin-fixed liver sections stained with hematoxilin&eosin (H&E). Apoptotic cell death was determined on frozen livers by TUNEL assay using the In-Situ-Cell-Death Detection Kit (Roche-diagnosis). Caspase-3 activity was quantified in snap frozen livers(9).

RNA Isolation and Quantitative Real-Time PCR

RNA was isolated with TriZol Reagent (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green reagent (Quantas biosciences) in a MyiQ single color Real-time PCR detection system (Biorad). Gene expression was normalize with GAPDH and shown in times versus WT basal expression.

Western Blot Analysis

Protein extracts were resolved in SDS polyacrylamide gels, transferred to nitrocellulose membranes (Whatman) and probed with primary antibodies: p-JNK1/2 (Thr 183/Tyr185), p-c-jun (Ser73) and PARP (Cell signaling); Bcl2, Bcl-x_L and NOS2 (Santa Cruz Biotechnology). GAPDH antibody was used as (loading control Abcam). Secondary antibodies; anti-rabbit-IgG HRP-linked (Cell Signaling) and anti-mouse IgG HRP-linked (Santa Cruz Biotechnology).

MNC isolation and flow cytometric analysis

Spleen and liver MNCs (9) were stained with CD45-APC-Cy7, NK1.1-PE-Cy7, Annexin V FITC (BD), and CD3-APC (eBioscience). Flow cytometry analysis was performed on a FACS Canto II (BD). NK cells were purified from cell suspension by magnetic cell sorting (MACS; Miltenyi Biotec) using anti-DX5 conjugated magnetic beads (Miltenyi Biotec). Only cell preparations with a >90% purity were used for further experiments.

$NK1.1^+$ cell depletion and adoptive transfer experiments

NK cell-specific depletion was obtained with 250 μg/ mouse of Asialo-GM1 mAb (Wako Chemicals USA, Inc.) administered i.p. 48 and 2 hours before ConA or LPS/GalN treatment. Adoptive transfer experiments were performed by intrahepatic injection of $2x10^6$ splenic MNCs or $20x10^6$ liver NK cells from $TRAIL^{-/-}$ or $GNMT^{-/-}$ mice.

Statistical analysis

Data are expressed as mean ± standard deviation of the mean. Statistical signi**fi**cance was determined by two-way analysis of variance followed by a Student's t test. All data shown are representative of at less three independent experiments.

RESULTS

Hepatic $NK1.1^+$ cells are activated in the absence of GNMT

Down-regulation/deficiency of GNMT correlates with progression of steatohepatitis, cirrhosis and HCC development in humans and mice ($GNMT^{-/-}$)(1, 11). NALFD patients and $GNMT^{-/-}$ mice have increased free fatty acids and bile acids (BA) in serum (12). Previous work in NASH patients correlated high presence of FFA and BA with expression of TRAIL receptor-2 (DR5) that sensitizes hepatocytes to TRAIL-mediated apoptosis(13, 14). Similarly, we found strong DR5 expression in young (8–10 week old) $GNMT^{-/-}$ mice (Fig 1A), showing early signs of fatty liver disease (Suppl Fig 1A). We found higher IFNγ in $GNMT^{-/-}$ mice whereas TNF levels were lower, but not significantly, than in WTs (Fig 1A), suggesting lower presence or activation of Kupffer cells. Accordingly, we observed lower F4/80, IL-1β and IL-6 expression in $GNMT^{-/-}$ mice compared to WTs (Suppl. Fig. 2A). NK/NKT cell-related cytotoxic molecule perforin was significantly over-expressed in $GNMT^{-/-}$ mice whereas IL-4 and IL-10 were down-regulated when compared to WTs (Fig 1A).

Fatty liver disease is associated with alterations of the innate immune system(7–9). We observed depletion of $NK1.1^+$ cells in liver (Fig 1B) and spleen (Suppl. Fig. 3A) from

GNMT$^{-/-}$ mice, accompanied by higher expression of the early activation marker, CD69 and TRAIL (Fig 1B). Cytotoxic assays either co-culturing liver (Fig. 1C) or splenic (Suppl. Fig. 3A) MNCs with YAC-1 cells or MACS-isolated NK cells with primary hepatocytes from GNMT$^{-/-}$ mice (Fig. 1C) confirmed strong NK cell activation in GNMT$^{-/-}$. Moreover, analysis of isolated NK/NKT cells showed higher TRAIL, IFNγ, perforin, CCL5 and CCR5 but low IL-4 in GNMT$^{-/-}$-cells (Fig 1D). Interestingly, hepatocytes isolated from GNMT$^{-/-}$ animals showed increased expression of RAE-1 and MULT-1; both NK cells activating ligands expressed in stressed cells but not in normal hepatocytes(15–17). CCL5 and DR5 were also augmented in GNMT$^{-/-}$ hepatocytes (Fig 1D).

Overall, these data show spontaneous depletion, activation and cytotoxic activity of NK1.1^{+} cells and unbalanced cytokine expression shifted towards a proinflammatory Th1-response in GNMT$^{-/-}$ mice. This could potentially contribute to the chronic inflammation that precedes the progression of NASH in the absence of GNMT.

GNMT$^{-/-}$ mice are sensitive to T-cell mediated acute hepatitis

ConA induces acute hepatitis through activation of T and NKT cells(18). ConA promoted extensive liver injury in GNMT$^{-/-}$ mice as evidenced by significant increase in ALT levels 6 hours after administration (Fig 2A). Histological analysis revealed evident liver parenchyma degeneration in GNMT$^{-/-}$ mice (Fig 2B) further confirmed by TUNEL (Fig 2C) and quantification of caspase-3 activity (Fig 2D). ConA resulted in significant increase of IFN-γ but comparable TNF expression in GNMT$^{-/-}$ animals (Fig 2E). ELISA on serum samples confirmed these data (Suppl Fig 4A). Perforin and granzyme were augmented both at 3 and 6 hours after ConA (Fig 2E). In line with the strong proinflammatory response, we found low expression of IL-10 in ConA/GNMT$^{-/-}$mice (Fig 2E). Additionally, NOS2 up-regulation observed in ConA/GNMT$^{+/+}$ mice was significantly delayed in ConA/GNMT$^{-/-}$ (Fig 2E). We detected strong JNK-activation in GNMT$^{-/-}$ mice 3h after ConA whereas in WT animals this response was weaker and delayed (Fig 2H).

NK/NKT cell activation upon ConA was confirmed by FACS analysis of spleen-derived MNCs showing stronger depletion and apoptosis of NK1.1^{+} cells in GNMT$^{-/-}$ animals after 3 hours (Suppl Fig 5A).

GNMT deficiency does not sensitize the liver against Fas mediated cell death

During ConA-acute hepatitis, NKT cells undergo depletion, activation and apoptosis through activation of Fas-signaling pathway(18). In order to identify if this could be the mechanism mediating ConA-liver injury in GNMT$^{-/-}$ mice we administered the Fas-activating antibody Jo2.

As expected, 50% of GNMT$^{+/+}$ mice died within 4h after Jo2 (Fig 3A). On the contrary, all GNMT$^{-/-}$ survived up to 6h after Jo2. ALT levels were elevated, although not significantly different than WTs (Fig 3B). H&E staining on liver sections revealed severe but similar tissue damage within mouse strains 6h after Jo2 (Fig 3C). This observation was confirmed by TUNEL and Caspase-3 activity assays (Fig 3D). Accordingly, Jo2 promoted similar cleavage of PARP and down-regulation of Bcl-x_L (Fig 3E).

Improved survival of GNMT$^{-/-}$ mice over GNMT$^{+/+}$ after Fas engagement could be better explained by the lower expression of Fas in GNMT$^{-/-}$ hepatocytes and whole livers at basal conditions (Fig 3F).

In summary, lower presence of Fas may counteract higher sensitivity to apoptosis in GNTM$^{-/-}$ mice rendering an overall comparable impact of Fas engagement when compared

to WT littermates. Overall, our data suggest that Fas does not mediate the hypersensitivity of GNMT$^{-/-}$ livers to tissue damage.

NK cells mediate acute hepatitis after ConA in GNMT$^{-/-}$ mice

Several works highlighted the main role of NKT cells(18) and excluded NK cells(19) as mediators of ConA-hepatitis. Conversely, NK cells mediate ConA-liver injury in an animal model that spontaneously develops NASH(9). Since we found NK cell activation in GNMT$^{-/-}$ mice (Fig 1), we hypothesize that they may mediate ConA-hepatitis. To prove this, we pre-treated GNMT$^{-/-}$ mice with the anti-ASIALO-GM1 antibody, that selectively depletes NK but not NKT cells (20, 21) Accordingly, treatment with anti-ASIALO-GM-1 led to significantly lower ALT (Fig 4A), substantial improvement of liver parenchyma and fewer hepatocyte cell death in GNMT$^{-/-}$ mice (Fig 4B). Perforin, granzyme and IFNγ were significantly down-regulated in ConA/ASIALO/GNMT$^{-/-}$mice. Interestingly, despite ASIALO treatment TNF expression remained comparable to ConA/GNMT$^{+/+}$ animals (Fig 4C). ELISA on serum samples confirmed these data (Fig 4D). ASIALO/GNMT$^{-/-}$ mice showed stronger phosphorylation of JNK than GNMT$^{-/-}$ animals 6h after ConA (Fig 4E). c-jun and NOS2 are mediators of cell protection against ConA-hepatitis(22). In consonance, phosphorylation of c-jun correlated with elevated NOS2 in ConA/ASIALO/GNMT$^{-/-}$ mice (Fig 4E). Finally, stronger Bcl-x_L confirmed the protective effect of ASIALO in GNMT$^{-/-}$ mice (Fig 4E).

Overall, our data indicate that inhibition of NK cells protects the liver against ConA-acute hepatitis in the absence of GNMT.

GNMT deficiency sensitizes the liver to endotoxin-mediated injury

Gut-derived endotoxins contribute to chronification of inflammation in the onset of NASH, as fatty livers are hypersensitive to LPS(7, 23). We hypothesized that loss of GNMT may affect LPS-driven hepatic inflammation. LPS/GalN had no impact on WT animals whereas ALT was significantly increased in GNMT$^{-/-}$ mice after 8h (Fig 5A). H&E staining confirmed acute injury in GNMT$^{-/-}$ animals as liver parenchyma destruction, abundant red-blood cells infiltration and apoptotic bodies were observed (Fig 5B). Extensive hepatocyte apoptosis was underlined by TUNEL assay (Fig 5C), cleavage of PARP and low expression of Bcl-2 in livers from GNMT$^{-/-}$mice (Fig 5C). Moreover, JNK was strongly phosphorylated in LPS/GalN/GNMT$^{-/-}$ mice (Fig 5D). GNMT$^{-/-}$ mice had higher IFNγ levels than GNMT$^{+/+}$ littermates (Fig 5E) but despite strong impact of LPS on liver injury, no significant differences on TNF expression were observed (Fig 5E). NOS2 up-regulation in GNMT$^{-/-}$animals was significantly lower than that found in LPS/GalN/GNMT$^{+/+}$(Fig 5E). Finally, strong production of perforin was detected 8h after LPS/GalN whereas the significant up-regulation of IL-4 found in WTs was greatly attenuated in GNMT$^{-/-}$ (Fig 5E).

Overall these data demonstrate the hypersensitivity to endotoxin-mediated liver injury in the absence of GNMT at early stages of fatty liver disease.

Inhibition of NK cells protects the liver from LPS-injury in the absence of GNMT

Previous work showed that endotoxin (*Propionibacterium acnes)* reduced liver NKT cells promoting a polarization towards a Th1 response, a phenomenon also observed in genetically obese mice upon LPS administration(7, 8) and similar to what we found in GNMT$^{-/-}$ mice (Fig. 5). However, NK but not NKT cells mediate ConA-hepatitis in GNMT$^{-/-}$ animals (Fig 3). To better characterize the molecular mechanisms underlying the hypersensitivity of GNMT$^{-/-}$ mice to LPS-liver injury we selectively depleted NK cells. Liver damage was greatly attenuated after LPS/GalN in ASIALO/GNMT$^{-/-}$ mice as ALT levels were largely reduced (Fig 6A). H&E staining evidenced attenuation of red blood cell

infiltration, lower presence apoptotic bodies and less parenchyma disruption in ASIALO/GNMT$^{-/-}$ mice (Fig 6B). TUNEL assay confirmed the robust antiapoptotic effect of ASIALO (Fig 6B). Accordingly, the inflammatory response was greatly weakened; IFNγ expression was significantly lower in ASIALO/GNMT$^{-/-}$ mice compared to GNMT$^{-/-}$ (Fig 6C, D). Interestingly, decrease in TNF levels was not significant between treatment groups (Fig 6C). NOS2 was significantly induced in ASIALO/GNMT$^{-/-}$ animals upon LPS/GalN, reaching comparable levels to those found in WT animals (Fig 6C). In consonance with NK cell inactivation, perforin expression was lower in ASIALO/GNMT$^{-/-}$ livers after LPS/GalN (Fig 6C). Finally, IL-4 expression was enhanced but did not change significantly when compared to GNMT$^{-/-}$ suggesting that NK cell depletion does not alter NKT cells response to LPS/GalN (Fig 6C). Phosphorylation of both JNK (Fig 6E) and c-jun was detected in ASIALO/LPS/GalN/GNMT$^{-/-}$.

Taking together, our data suggest that NK cells mediate LPS/GalN-liver injury when GNMT is absent. Moreover, NK cell inhibition seems to shift cell signaling towards the c-jun/NOS2 pathway, with cell-protective characteristics.

Lack of TRAIL protects the GNMT-deficient liver against LPS/GalN-mediated acute injury

Liver NK cells constitutively express TRAIL and are the main producers of this cytokine in the body(20, 21). To uncover the implication of TRAIL as a mediator of liver injury we performed adoptive transfer experiments of TRAIL-deficient liver NK cells into ASIALO/GNMT$^{-/-}$ mice, which significantly protected against LPS/GalN-liver injury. Lower ALT levels (Fig 7A), restoration of the liver parenchyma status and attenuation of apoptosis in GNMT$^{-/-}$ mice receiving TRAIL$^{-/-}$NKs were patent (Fig 7B). Adoptive transfer of liver NK cells from GNMT$^{-/-}$ mice restored LPS-liver injury in ASIALO/GNMT$^{-/-}$ animals (Fig 7A,B). qRT-PCR analysis confirmed attenuation of the inflammatory response as IFNγ was significantly reduced in TRAIL$^{-/-}$ NKs/ASIALO/GNMT$^{-/-}$ mice (Fig 7C). Moreover, NOS2 expression was greatly increased in TRAIL$^{-/-}$NKs/ASIALO/GNMT$^{-/-}$ mice whereas perforin was significantly downregulated (Fig 7C). Finally, strong JNK phosphorylation observed in GNMT$^{-/-}$ livers after LPS/GalN was blunted in the presence of TRAIL$^{-/-}$NKs (Fig 7D). The damaging impact of LPS was restored by adoptive transfer of GNMT$^{-/-}$ liver NKs (Fig 7). Similar effects were observed when splenic MNCs were adoptively transferred into mice pretreated with ASIALO and further challenged with LPS (Suppl. Fig 7), confirming the activation of NK cells also in spleens in GNMT$^{-/-}$ mice (Suppl. Fig. 3).

These data showing systemic activation of NK cells in the absence of GNMT, suggest the direct impact of SAMe, which is greatly elevated in GNMT$^{-/-}$ mice, in the activation of NK cells.

SAMe depletion with NAM protects the liver against ConA- and LPS-liver injury

To further confirm this, we first depleted SAMe systemically in GNMT$^{-/-}$ mice and further challenged GNMT$^{-/-}$ mice with ConA and LPS. To do so, we fed GNMT$^{-/-}$ mice with nicotinamide (NAM), a substrate of the nicotinamide N-methyltransferase that leads to the normalization of SAMe content and prevents fatty liver and fibrosis formation in GNTM$^{-/-}$ mice(24). As shown in Fig. 8, ConA had only a minor impact on NAM/GNTM$^{-/-}$ mice showing ALT levels, liver damage and presence TUNEL positive cells comparable to those found in WT animals (Fig. 2). Moreover, JNK activation was blunted in GNTM$^{-/-}$ mice in the presence of NAM. Similarly, NAM/LPS GNTM$^{-/-}$ mice showed low ALT levels, normal histology and very low presence of TUNEL positive cells (Fig. 8D, E). Finally, JNK phosphorylation was almost undetectable in NAM pretreated mice (Fig. 8D).

Overall, these data points that SAMe depletion successfully protects the liver against acute liver injury, which we proved to be mediated by TRAIL/NK cells in the absence of GNMT, suggesting the implication of SAMe in promoting activation of the NK cell compartment.

GNMT deficiency does not sensitize the liver to TNF-injury

Interestingly, despite evident tissue protection upon NK cell inhibition, TNF levels remain elevated after ConA or LPS/GalN in ASIALO/GNMT$^{-/-}$ mice. We further investigated the impact of TNF on the liver in the absence of GNMT. GNMT$^{-/-}$ primary hepatocytes showed stronger JNK phosphorylation after TNF compared to WT cells (Suppl Fig 6A). JNK activation correlated with higher p-c-jun in GNMT$^{-/-}$hepatocytes (Suppl Fig 6A). Interestingly, TNF did not result in GNMT$^{-/-}$ hepatocyte apoptosis; PARP cleavage was dimly increased in TNF/GNMT$^{-/-}$ hepatocytes but densitometric quantification showed no significant differences with TNF/GNMT$^{+/+}$ hepatocytes (Suppl Fig 6B). The same was observed regarding the regulation of Bcl-x_L (Suppl Fig 6B). Finally, *in vivo* administration confirmed the overall modest effect of TNF on GNMT$^{-/-}$ mice as moderate increase in ALT levels (487.3 ± 121.1) (Suppl Fig 6C) was observed. H&E staining did not evidence tissue damage and both GNMT$^{+/+}$ and GNMT$^{-/-}$ livers lacked TUNEL-positive cells (Suppl Fig 6D). In accordance to what was found *in vitro*, TNF led to stronger phosphorylation of JNK and c-jun phosphorylation in GNMT$^{-/-}$ mice (Suppl Fig 6E).

Overall, our data indicate that in GNMT$^{-/-}$ mice, the TNF signaling pathway activates both JNK and c-jun cascade, having a mild impact on liver homeostasis.

DISCUSSION

GNMT expression negatively correlates with progression of human liver disease and cirrhosis and it is markedly reduced in human HCC samples. GNMT deficient mice develop spontaneous steatosis that progresses to steatohepatitis, cirrhosis and HCC(1). Therefore, GNMT$^{-/-}$ mice are a valuable tool to investigate the molecular mechanisms underlying the progression of NASH.

The liver is considered an important organ of the innate immune system as it's highly enriched with lymphocytes, of which 30-50% are NK/NKT cells characterized by NK1.1^{+} expression. Interestingly, in young GNMT$^{-/-}$ animals we found significant depletion of NK1.1^{+} cells, which correlated with strong cytotoxic activity and cytokine release. We hypothesize that, at early stages of fatty liver disease, activated NK1.1^{+} cells may contribute to chronic inflammation in the liver, which precedes the progression of NASH.

Previous work highlighted the protective role of NKT cells in genetically or diet-induced fatty livers against inflammation, whereas the implication of NK cells during fatty liver disease has been neglected. The former studies correlated steatosis with reduced NKT cells number and function that lead to a shift towards a Th1 pro-inflammatory response(7, 8). Accordingly, in GNMT$^{-/-}$ mice we observed low expression of Th2-cytokines (IL-4, IL-10), likely derived from NKT cells, but strong presence of TRAIL, IFNγ and perforin; potentially due to NK cell activation. These data suggested a differential activation of NKT and NK cells in the absence of GNMT. To confirm this, we used an experimental model of fulminant hepatitis using ConA that promotes liver injury through T and NKT cells(18, 19). Herein, we show that GNMT deficiency hypersensitizes the liver to ConA-hepatitis. Notably, selective inactivation with ASIALO indicated that NK cells, but not NKT cells, are the main mediators of ConA-deleterious effects. Furthermore, Fas engagement did not hypersensitize livers from GNMT$^{-/-}$ mice to liver injury. This data suggests that the well-described mechanism by which NKT cells contribute to ConA-damage through FasL is not relevant in the absence of GNMT(18).

Liver NK cells get activated at early stages of infections, and express TRAIL and IFNγ in response to endotoxins(20, 25). The vast majority of the blood supply that the liver receives comes from the gut through the portal vein and it is enriched with endotoxins and toxic products derived from food intake. Small intestine bacterial overgrowth and disruption of the intestinal barrier (leaky gut) are features of steatohepatitis and potentially contribute to endotoxemia in patients with NASH(5, 26). Moreover, fat accumulation sensitizes the liver to endotoxin-mediated tissue damage(4, 7, 8). In this line, herein we show a shift towards a Th1-(IFNγ)-response in LPS/GalN-treated GNMT$^{-/-}$ mice that are strongly susceptible to endotoxin-injury. Selective depletion confirmed that NK cells but not NKT mainly mediate LPS-damage in the absence of GNMT.

Previous work suggested that the higher susceptibility to LPS-damage in obese individuals is mediated by liver-cell hypersensitivity to TNF(4). Interestingly, attenuation of ConA and LPS-liver injury in ASIALO/GNMT$^{-/-}$was accompanied by sustained TNF expression and c-jun. In the liver, c-jun strongly protects hepatocytes(22) and thus we hypothesize that TNF may have a dual role; promoting cell damage in GNMT$^{-/-}$ mice through JNK activation but counteracting it by activation of c-jun/NOS2. Our data showing that TNF administration promoted JNK, c-jun activation but had a minor impact on GNMT$^{-/-}$ hepatocyte death supports this hypothesis.

Based on our data, it is tempting to speculate that in the context of GNMT deficiency the ability of TNF to activate a protective signaling could be overshadowed by the activation of pro-apoptotic pathway; potentially mediated by TRAIL. Moreover, lower presence of Kupffer cell activation markers could explain the lower TNF expression in untreated GNMT$^{-/-}$ mice and argue in favor of the implication of other cell compartments in mediating the pro-inflammatory status found in these KO animals.

Liver NK cells express TNF, IFNγ, cytotoxic molecules like perforin and granzyme and mainly produce TRAIL(20, 21), which promote cell death through activation of JNK upon binding to DR5 in transformed cells but not in healthy hepatocytes(27, 28). Interestingly, GNMT$^{-/-}$ mice show strong presence of DR5 in hepatocytes along with activation of NK cells. Our data suggests that two potential mechanisms may underlay the implication of NK cell in mediating chronic liver inflammation in GNMT$^{-/-}$ mice; an indirect one, implicating BAs and a direct one, involving the impact of SAMe on NK cell biology. Firstly, bile acids (BAs) and fatty acids promote DR5 expression, which is increased in NASH patients contributing to apoptosis, which is directly linked to inflammation(14). Thus, accumulating BAs and increased DR5 levels found in GNMT$^{-/-}$ mice would sensitize hepatocytes to apoptosis, contributing to chronic inflammation in the onset of NASH development. Activation of TRAIL-producing NK cells found and the strong liver protection elicited by selective depletion and adoptive transfer of liver-derived TRAIL$^{-/-}$NK cells, further supports the deleterious effect of TRAIL-producing NK cells in the absence of GNMT. Recent work describing the implication of TRAIL/NK cells as contributors of chronic inflammation at early stages of NASH supports this mechanism(9).

Finally, our data showing that systemic SAMe depletion by NAM elicits a similar degree of protection than NK cell inactivation against ConA and LPS-injury in GNMT$^{-/-}$ mice suggests a direct impact of SAMe in NK cell biology. This is further supported by the depletion and activation of splenic NK cells observed in GNMT$^{-/-}$ mice, that would argue in favor of a systemic effect of SAMe over NK cell activation. Moreover, our work highlights the importance to mantain the fine balance of SAMe in the body as lack of SAMe promotes liver injury and HCC development(29), whereas an excess leads to chronic inflammation, fibrosis and HCC development(1).

Overall, our present study provides new insights on the implication of TRAIL-producing NK cells in mediating liver injury at early stages of fatty liver disease when GNMT is absent. This knowledge may facilitate development of potential therapeutic strategies based on NK cell inactivation to counteract hepatocyte injury, which contributes to the chronification of the inflammatory response, a scenario that is critical for the progression of NASH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We want to thank Virginia Gutierrez, Begoña Rodriguez Iruretagoyena and Karoline Fechter for technical assistance. This work is supported by grants from Instituto de Salud Carlos III (to NB; FIS, PS09/02010; Ministry of Health, Spain), NIH AT-1576 (to S.C.L., M.L.M-C. and J.M.M.), SAF 2008-04800 (to J.M.M), ETORTEK-2010 (to M.L.M.-C), Sanidad Gobierno Vasco 2008 (to M.L.M.-C), Educación Gobierno Vasco 2011 (to M.L.M.-C), PI11/01588 (to M.L.M.-C). Ciberehd is funded by the Instituto de Salud Carlos III. NB is funded by the Program Ramón y Cajal (Ministry of Science and Innovation, Spain).

References

1. Martinez-Chantar ML, Vazquez-Chantada M, Ariz U, Martinez N, Varela M, Luka Z, Capdevila A, et al. Loss of the glycine N-methyltransferase gene leads to steatosis and hepatocellular carcinoma in mice. Hepatology. 2008; 47:1191–1199. [PubMed: 18318442]
2. Mudd SH, Cerone R, Schiaffino MC, Fantasia AR, Minniti G, Caruso U, Lorini R, et al. Glycine N-methyltransferase deficiency: a novel inborn error causing persistent isolated hypermethioninaemia. Journal of inherited metabolic disease. 2001; 24:448–464. [PubMed: 11596649]
3. Augoustides-Savvopoulou P, Luka Z, Karyda S, Stabler SP, Allen RH, Patsiaoura K, Wagner C, et al. Glycine N -methyltransferase deficiency: a new patient with a novel mutation. Journal of inherited metabolic disease. 2003; 26:745–759. [PubMed: 14739680]
4. Yang SQ, Lin HZ, Lane MD, Clemens M, Diehl AM. Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. Proceedings of the National Academy of Sciences of the United States of America. 1997; 94:2557–2562. [PubMed: 9122234]
5. Wigg AJ, Roberts-Thomson IC, Dymock RB, McCarthy PJ, Grose RH, Cummins AG. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis. Gut. 2001; 48:206–211. [PubMed: 11156641]
6. Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, Fearnside J, et al. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. Proceedings of the National Academy of Sciences of the United States of America. 2006; 103:12511–12516. [PubMed: 16895997]
7. Li Z, Soloski MJ, Diehl AM. Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. Hepatology. 2005; 42:880–885. [PubMed: 16175608]
8. Guebre-Xabier M, Yang S, Lin HZ, Schwenk R, Krzych U, Diehl AM. Altered hepatic lymphocyte subpopulations in obesity-related murine fatty livers: potential mechanism for sensitization to liver damage. Hepatology. 2000; 31:633–640. [PubMed: 10706553]
9. Beraza N, Malato Y, Sander LE, Al-Masaoudi M, Freimuth J, Riethmacher D, Gores GJ, et al. Hepatocyte-specific NEMO deletion promotes NK/NKT cell- and TRAIL-dependent liver damage. The Journal of experimental medicine. 2009; 206:1727–1737. [PubMed: 19635861]
10. Luka Z, Capdevila A, Mato JM, Wagner C. A glycine N-methyltransferase knockout mouse model for humans with deficiency of this enzyme. Transgenic research. 2006; 15:393–397. [PubMed: 16779654]
11. Avila MA, Berasain C, Torres L, Martin-Duce A, Corrales FJ, Yang H, Prieto J, et al. Reduced mRNA abundance of the main enzymes involved in methionine metabolism in human liver

cirrhosis and hepatocellular carcinoma. Journal of hepatology. 2000; 33:907–914. [PubMed: 11131452]

12. Barr J, Vazquez-Chantada M, Alonso C, Perez-Cormenzana M, Mayo R, Galan A, Caballeria J, et al. Liquid chromatography-mass spectrometry-based parallel metabolic profiling of human and mouse model serum reveals putative biomarkers associated with the progression of nonalcoholic fatty liver disease. Journal of proteome research. 2010; 9:4501–4512. [PubMed: 20684516]
13. Malhi H, Barreyro FJ, Isomoto H, Bronk SF, Gores GJ. Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. Gut. 2007; 56:1124–1131. [PubMed: 17470478]
14. Higuchi H, Bronk SF, Takikawa Y, Werneburg N, Takimoto R, El-Deiry W, Gores GJ. The bile acid glycochenodeoxycholate induces trail-receptor 2/DR5 expression and apoptosis. The Journal of biological chemistry. 2001; 276:38610–38618. [PubMed: 11507096]
15. Nice TJ, Coscoy L, Raulet DH. Posttranslational regulation of the NKG2D ligand Mult1 in response to cell stress. The Journal of experimental medicine. 2009; 206:287–298. [PubMed: 19171762]
16. Diefenbach A, Jensen ER, Jamieson AM, Raulet DH. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. Nature. 2001; 413:165–171. [PubMed: 11557981]
17. Gao B. Natural killer group 2 member D, its ligands, and liver disease: good or bad? Hepatology. 2010; 51:8–11. [PubMed: 20034045]
18. Takeda K, Hayakawa Y, Van Kaer L, Matsuda H, Yagita H, Okumura K. Critical contribution of liver natural killer T cells to a murine model of hepatitis. Proceedings of the National Academy of Sciences of the United States of America. 2000; 97:5498–5503. [PubMed: 10792025]
19. Toyabe S, Seki S, Iiai T, Takeda K, Shirai K, Watanabe H, Hiraide H, et al. Requirement of IL-4 and liver NK1+ T cells for concanavalin A-induced hepatic injury in mice. Journal of immunology. 1997; 159:1537–1542.
20. Smyth MJ, Cretney E, Takeda K, Wiltrout RH, Sedger LM, Kayagaki N, Yagita H, et al. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. The Journal of experimental medicine. 2001; 193:661–670. [PubMed: 11257133]
21. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, Iwakura Y, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nature medicine. 2001; 7:94–100.
22. Hasselblatt P, Rath M, Komnenovic V, Zatloukal K, Wagner EF. Hepatocyte survival in acute hepatitis is due to c-Jun/AP-1-dependent expression of inducible nitric oxide synthase. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:17105–17110. [PubMed: 17940019]
23. Matsui K, Yoshimoto T, Tsutsui H, Hyodo Y, Hayashi N, Hiroishi K, Kawada N, et al. Propionibacterium acnes treatment diminishes CD4+ NK1. 1+ T cells but induces type I T cells in the liver by induction of IL-12 and IL-18production from Kupffer cells. Journal of immunology. 1997; 159:97–106.
24. Varela-Rey M, Martinez-Lopez N, Fernandez-Ramos D, Embade N, Calvisi DF, Woodhoo A, Rodriguez J, et al. Fatty liver and fibrosis in glycine N-methyltransferase knockout mice is prevented by nicotinamide. Hepatology. 2010; 52:105–114. [PubMed: 20578266]
25. Matsumoto A, Kinoshita M, Ono S, Tsujimoto H, Majima T, Habu Y, Shinomiya N, et al. Cooperative IFN-gamma production of mouse liver B cells and natural killer cells stimulated with lipopolysaccharide. Journal of hepatology. 2006; 45:290–298. [PubMed: 16644058]
26. Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palu G, Martines D. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. American journal of physiology Gastrointestinal and liver physiology. 2007; 292:G518–525. [PubMed: 17023554]
27. Herr I, Wilhelm D, Meyer E, Jeremias I, Angel P, Debatin KM. JNK/SAPK activity contributes to TRAIL-induced apoptosis. Cell death and differentiation. 1999; 6:130–135. [PubMed: 10200559]
28. Muhlenbeck F, Haas E, Schwenzer R, Schubert G, Grell M, Smith C, Scheurich P, et al. TRAIL/Apo2L activates c-Jun NH2-terminal kinase (JNK) via caspase-dependent and caspase-

independent pathways. The Journal of biological chemistry. 1998; 273:33091–33098. [PubMed: 9830064]

29. Lu SC, Alvarez L, Huang ZZ, Chen L, An W, Corrales FJ, Avila MA, et al. Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. Proceedings of the National Academy of Sciences of the United States of America. 2001; 98:5560–5565. [PubMed: 11320206]

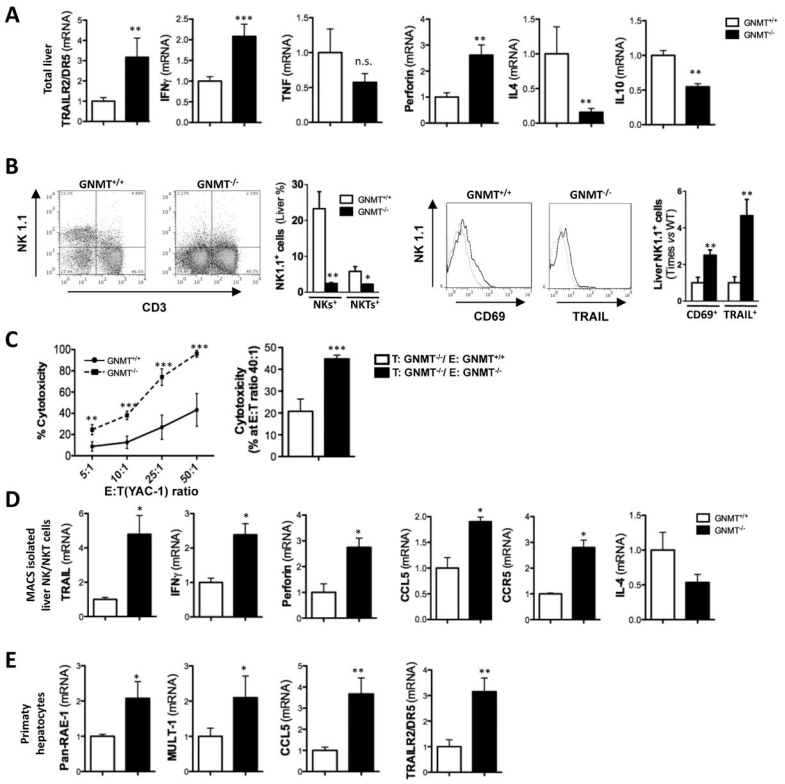

Figure 1. GNMT deficiency promotes activation and increased cytotoxicity of NK/NKT cells

(A) qRT-PCR of livers from GNMT$^{-/-}$ and GNMT$^{+/+}$ mice. (B) FACS analysis of livers. The graph represents the percentage of NK1.1^{+} cells vs. CD45^{+} cells. Histogram/graph showing NK1.1^{+}/CD69^{+} and NK1.1^{+}/TRAIL^{+} in livers. (C) Cytotoxicity assay co-culturing liver-derived MNCs with YAC-1 and liver-MACS isolated NK cells with GNMT$^{-/-}$ hepatocytes at the E:T ratios shown in the figure (D) qRT-PCR of MACS-isolated NK/NKT cells. (E) qRT-PCR of hepatocytes. n=4. *P<0.05; **P<0.01; ***P<0.001 (GNMT$^{-/-}$ vs. GNMT$^{+/+}$). Error bars represent SD.

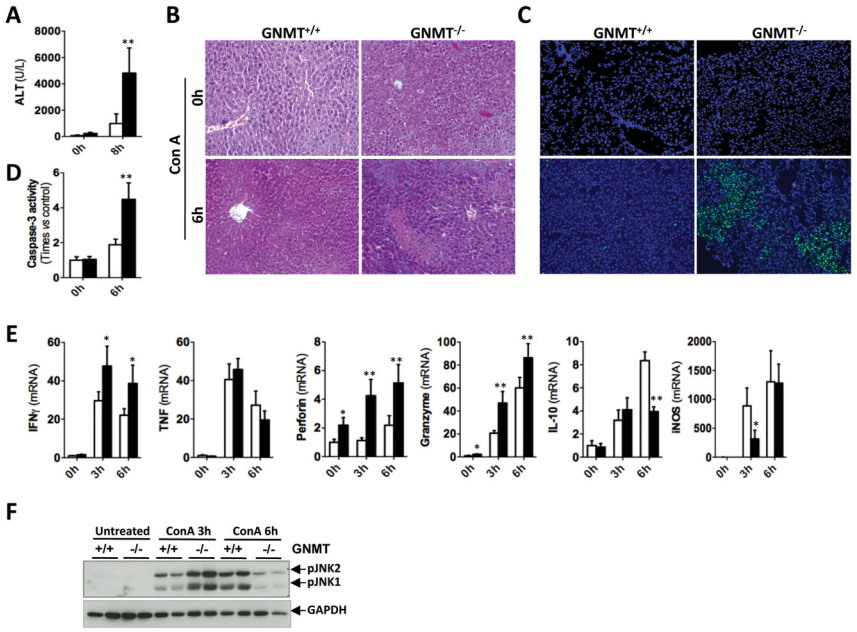

Figure 2. GNMT deficiency hyper-sensitizes the liver to Concanavalin A-acute hepatitis

(A) ConA increased ALT serum levels. (B) H&E staining (C) TUNEL assay in liver sections (200x) (D) Caspase-3 activity on liver extracts. (E) qRT-PCR (F) Western blot analysis. n=4. *P<0.05; **P<0.01 ($GNMT^{-/-}$ vs. $GNMT^{+/+}$). Error bars represent SD.

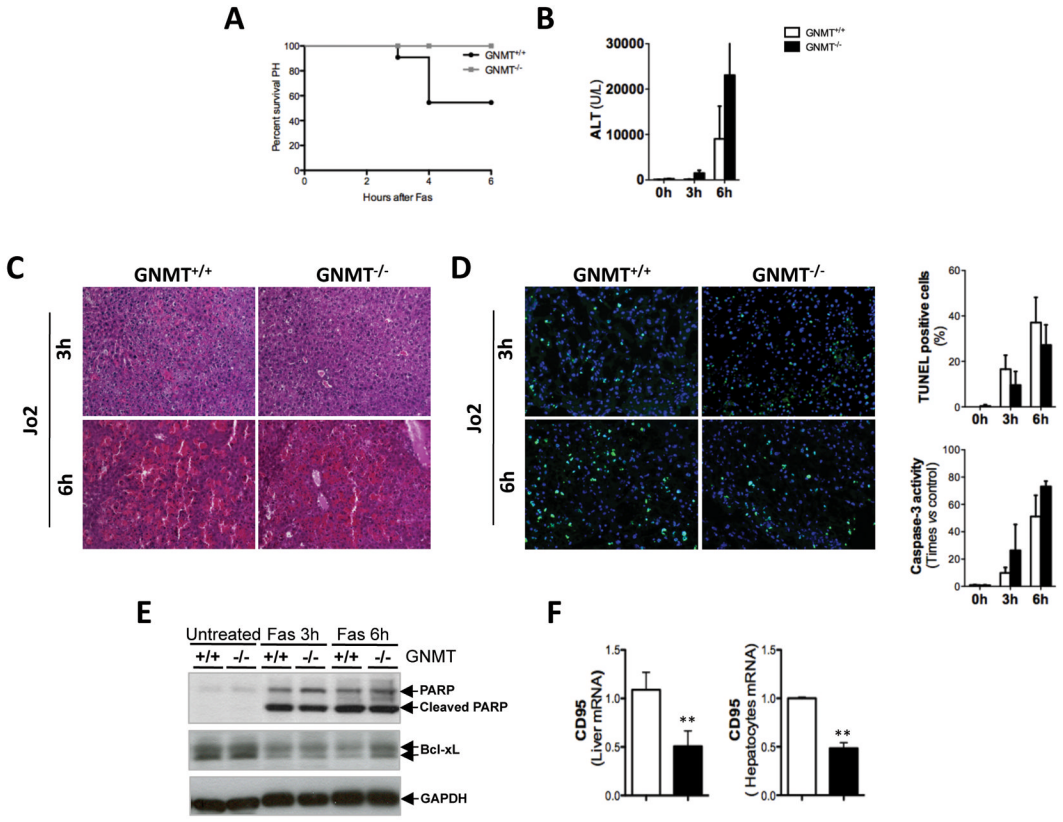

Figure 3. GNMT deficiency does not hyper-sensitize the liver to Fas-mediated liver injury

(A) Jo2 injection was lethal for 50% WT animals after 4h whereas no GNMT$^{-/-}$ mice died. (B) ALT levels on serum (C) H&E staining (200x) (D) TUNEL assay (200x), quantification of TUNEL positive (% vs DAPI^{+} cells) and Caspase3 activity. (E) Western blot analysis (F) qRT-PCR (times vs WT control). n=4. *P<0.05; **P<0.01 (GNMT$^{-/-}$ vs. GNMT$^{+/+}$). Error bars represent SD.

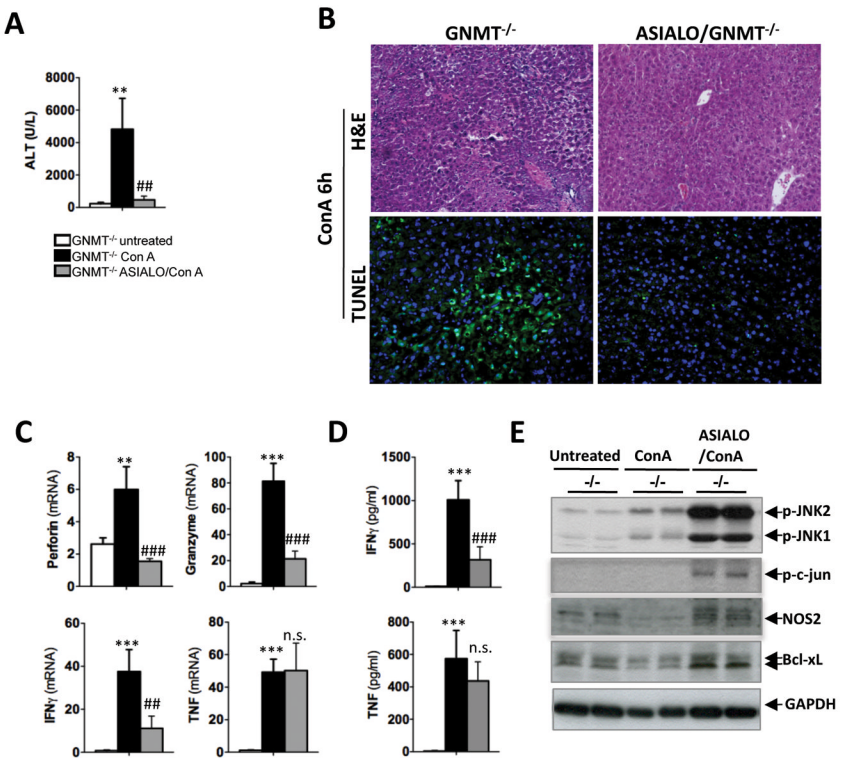

Figure 4. Specific NK cell depletion ameliorates acute liver injury after ConA in GNMT$^{-/-}$ mice

(A) ASIALO pre-treatment decreased ALT levels, (B) improved liver parenchyma and lower apoptosis (H&E and TUNEL; 200x). (C) qRT-PCR (times vs WT control) (D) ELISA on serum samples. (E) Western blot analysis of livers n=4. *P<0.05; **P<0.01 (GNMT$^{-/-}$ vs. GNMT$^{+/+}$). Error bars represent SD.

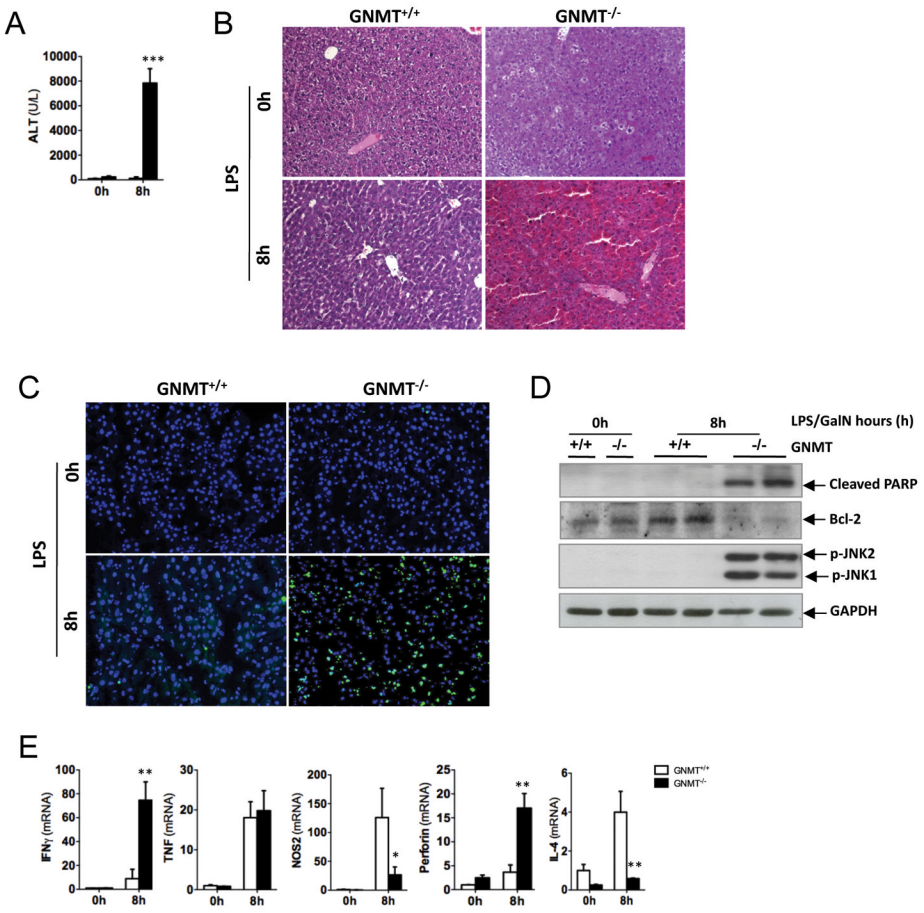

Figure 5. GNMT deficiency over-sensitizes the liver to LPS/GalN-mediated liver injury

(A) Increased serum ALT levels, (B) H&E and (C) TUNEL assay (200x) confirmed that LPS/GalN (16μg/kg/800mg/kg) promotes liver injury in GNMT$^{-/-}$mice. (D) Western blot analysis (E) qRT-PCR. n=4. *P<0.05; **P<0.01 (GNMT$^{-/-}$ vs. GNMT$^{+/+}$). Error bars represent SD.

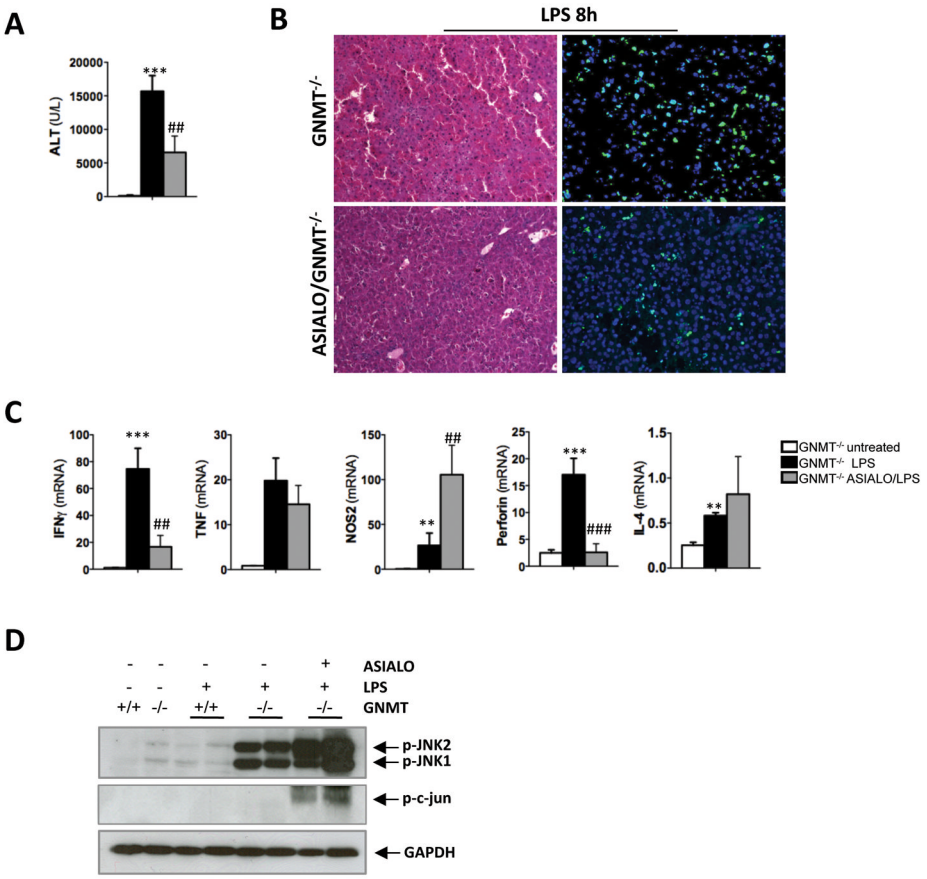

Figure 6. Selective depletion of NK cells attenuates strong liver injury after LPS/GalN

(A) Lower ALT serum levels, (B) H&E and TUNEL assay on liver sections confirmed amelioration of liver damage in ASIALO/GNMT$^{-/-}$ mice (200x). (C) qRT-PCR detecting IFNg, TNF, NOS2, Perforin and IL-4 (times vs WT control). (D) Western blot analysis showed strong p-JNK1/2 and p-c-jun in ASIALO/GNMT$^{-/-}$ mice 8h after LPS/GalN. GAPDH was used as loading control. n= 4. *P<0.05; **P<0.01 (GNMT$^{-/-}$ vs. GNMT$^{+/+}$). All data are representative of three independent experiments. Error bars represent SD.

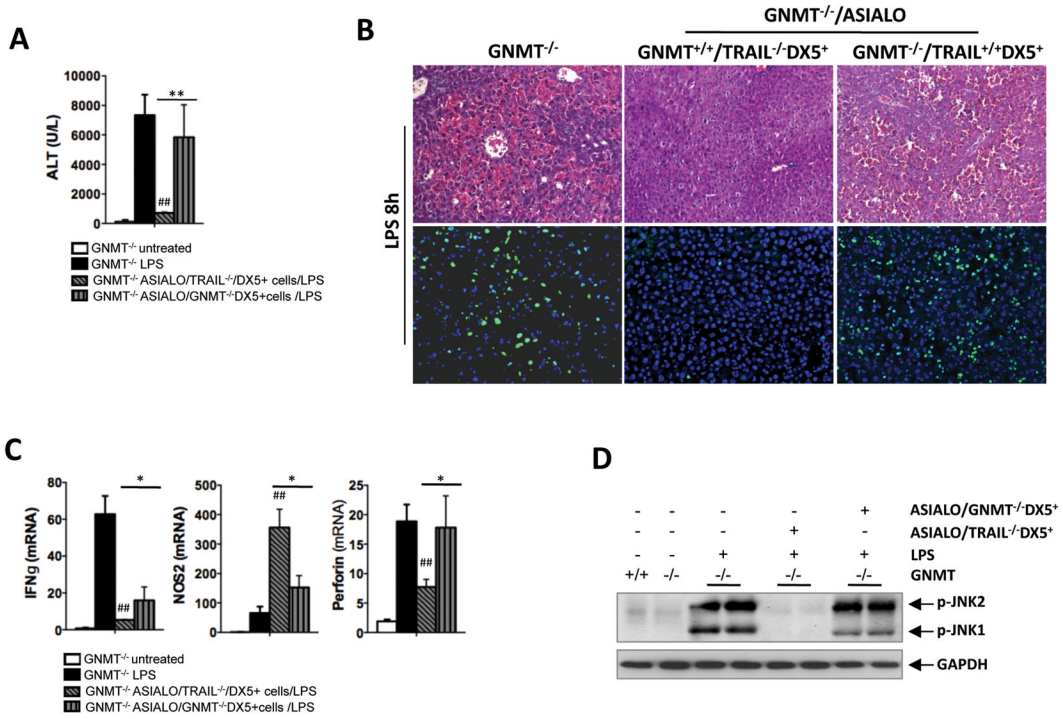

Figure 7. Adoptive transfer of liver-TRAIL deficient NK cells protects the liver against LPS/GalN damage in GNMT$^{-/-}$ mice

ASIALO/GNMT$^{-/-}$ mice received TRAIL$^{-/-}$ or GNMT$^{-/-}$ NK cells isolated from liver. (A) Serum ALT, (B) H&E staining and TUNEL. (C) qRT-PCR (D) Western blot analysis n= 4. *P<0.05; **P<0.01 (GNMT$^{-/-}$ASIALO/GNMT$^{-/-}$NKs vs. GNMT$^{-/-}$ASIALO/TRAIL$^{-/-}$NKs); ##p<0.01 (GNMT$^{-/-}$ vs. GNMT$^{-/-}$ ASIALO/TRAIL$^{-/-}$ NKs). Error bars represent SD.

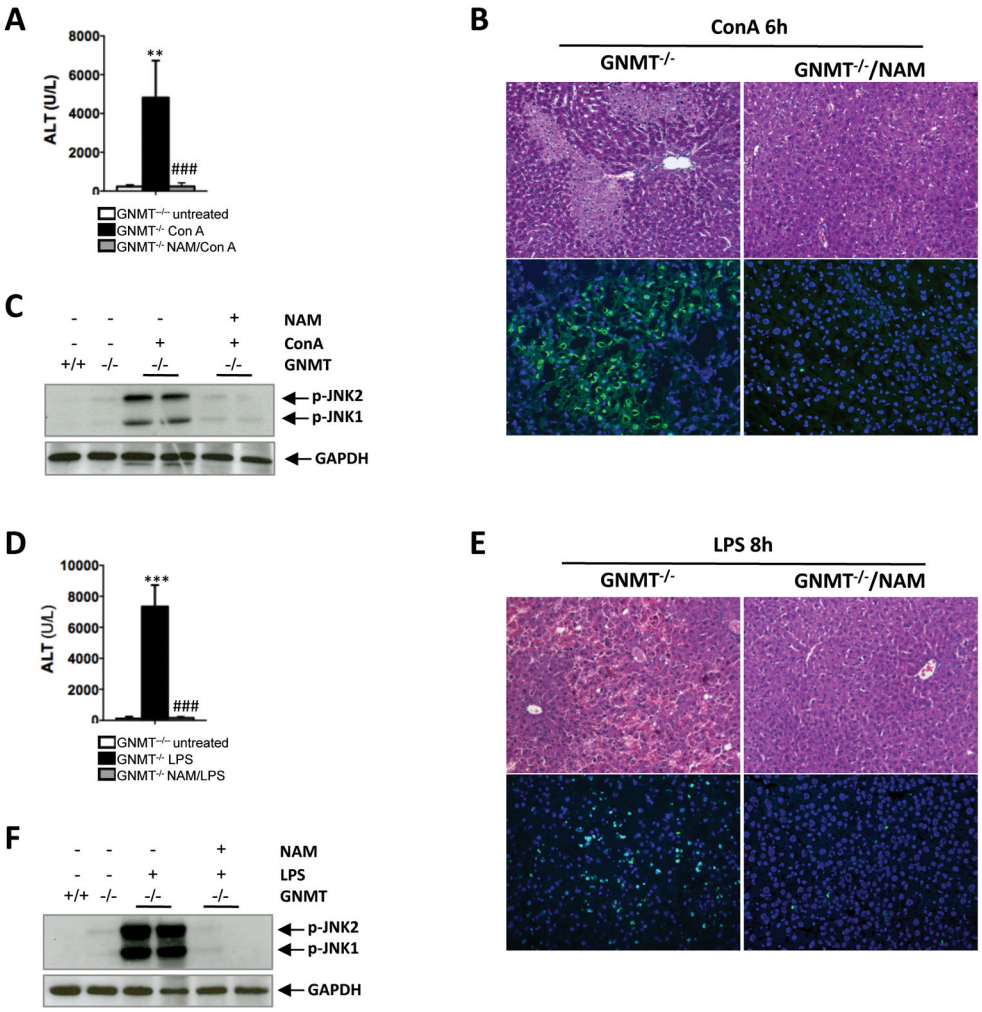

Figure 8. SAMe depletion with NAM protects the liver against LPS/GalN damage in GNMT$^{-/-}$ mice

GNMT$^{-/-}$ mice fed with NAM were protected against ConA- and LPS-damage. (A, D) ALT, (B, E) H&E and TUNEL and (C, F) p-JNK western blot. **P<0.01 (GNMT$^{-/-}$ vs. GNMT$^{-/-}$/LPS or ConA); ###p<0.001 (GNMT$^{-/-}$/LPS or ConA vs. GNMT$^{-/-}$/NAM/LPS or ConA). Error bars represent SD.