Innate immune responses involving natural killer and natural killer T cells promote liver regeneration after partial hepatectomy in mice

Satoko Hosoya1, Kenichi Ikejima1*, Kazuyoshi Takeda2, Kumiko Arai1, Sachiko Ishikawa1, Hisafumi Yamagata1, Tomonori Aoyama1, Kazuyoshi Kon1, Shunhei Yamashina1, Sumio Watanabe1

1Department of Gastroenterology, and 2Department of Immunology, Juntendo University Graduate School of Medicine, Tokyo, Japan

Author contributions
Satoko Hosoya: Perform experiments, analyze data, and write manuscript.
Kenichi Ikejima: Design this study, analyze data, and write manuscript.
Kazuyoshi Takeda: Supervise immunological experiments, provide antibodies.
Kumiko Arai: Perform experiments.
Sachiko Ishikawa: Perform experiments.
Hisafumi Yamagata: Perform experiments.
Tomonori Aoyama: Perform experiments.
Kazuyoshi Kon: Provide technical assistance.
Shunhei Yamashina: Provide technical assistance.
Sumio Watanabe: Organize the experimental group, review manuscript.

Running head: NK and NKT cells in liver regeneration

*Corresponding author:
Kenichi Ikejima, MD, PhD, FACP, Associate Professor,
Department of Gastroenterology, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421 Japan
Phone: +81-3-3813-3111, Fax: +81-3-3813-8862, E-mail: ikejima@juntendo.ac.jp

Conflict of interest: All authors have no conflict of interest in terms of this study.
Abstract

To clarify the roles of innate immune cells in liver regeneration, here we investigated the alteration in regenerative responses after partial hepatectomy (PH) under selective depletion of natural killer (NK) and/or NKT cells. Male, wild-type (WT; C57Bl/6) and CD1d-knockout (KO) mice were injected with anti-NK1.1 or anti-asialo GM1 antibody, and then underwent the 70% PH. Regenerative responses after PH were evaluated and hepatic expression levels of cytokines and growth factors were measured by real-time RT-PCR and ELISA. Phosphorylation of STAT3 was detected by Western blotting. Depletion of both NK and NKT cells with an anti-NK1.1 antibody in WT mice caused drastic decreases in bromodeoxyuridine (BrdU) uptake, expression of PCNA and cyclin
D1 48 hour after PH. In mice given NK1.1 antibody, increases in hepatic TNFα,

IL-6/phospho-STAT3 and hepatocyte growth factor (HGF) levels following PH were

also blunted significantly, whereas IFNγ mRNA levels were not different. CD1d-KO

mice per se showed normal liver regeneration; however, pretreatment with an

anti-asialo GM1 antibody to CD1d-KO mice, resulting in depletion of both NK and

NKT cells, also blunted regenerative responses. Collectively, these observations

clearly indicated that depletion of both NK and NKT cells by two different ways results

in impaired liver regeneration. NK and NKT cells most likely up-regulate TNFα,

IL-6/STAT3, and HGF in a coordinate fashion, thus promoting normal regenerative

responses in the liver.
Keywords: innate immunity; TNFα; IL-6; STAT3; hepatocyte growth factor (HGF).
Introduction

Lines of evidence have suggested that alteration in innate immune system is involved in a variety of pathophysiological conditions in the liver (24). It is well known that the liver contains a variety of immune cells, with considerable proportion of natural killer (NK) and natural killer (NK) T cell fractions (6). NK cells are defined as large granular lymphocytes, which exert cytotoxic activity against tumors and viral-infected cells through the perforin and granzyme system (27). NK cells preferentially reside in the hepatic sinusoid, and these liver-specific NK cells are called as Pit cells (31). On the other hand, NKT cells are a heterogeneous subset of lymphocytes expressing both NK and T cell surface markers (8, 14). NKT cells recognize a glycolipid antigen presented
by CD1d, one of the major histo-compatibility complex molecules, on antigen presenting cells such as dendritic cells and macrophages (3, 8). Several studies suggested that NKT cells modulate hepatic inflammation and fibrogenesis (7, 12, 21, 23, 25); however, the precise role of these cells in liver patho-physiology is still controversial.

Liver regeneration is one of the significant natures of this important organ. Normal liver is capable to regenerate when get injured by various pathogens and mechanical damages (20, 29). The mechanism underlying this process has been studied from various aspects; however, it still remains unclear. Recent lines of evidence indicated that innate immune responses play a key role in the trigger and promotion of regenerating
process (5). For example, pattern-recognition receptors such as toll-like receptors (TLRs) and down-stream signaling involved in production of cytokines from hepatic macrophages (Kupffer cells) are quite important in liver regeneration (11, 26). However, the role of other types of innate immune cells, such as NK and NKT cells, in liver regeneration has not been fully elucidated. Recent report indicated that NK cells negatively regulate liver regeneration through production of IFN\(\gamma\) (28). The role of NKT cells in liver regeneration is more obscure; poor regeneration in steatotic liver in ob/ob mice has been reported (16, 32), where hepatic NKT cells are depleted (17). Similarly, we have recently shown that KK-A\(^{\text{Y}}\) mice, which develop metabolic syndrome-like phenotype spontaneously, demonstrate poor regeneration following 70% partial
hepatectomy (PH) (2), where hepatic NKT cells are also depleted. Further, activation of NKT cells triggered by a specific ligand \(\alpha\)-galactosylceramide has been shown to accelerate liver regeneration after PH (22). These observations suggested that NKT cells promote regeneration process; however, mice lacking NKT cells caused by genetic knockout of CD1d have been shown to demonstrate almost normal liver regeneration after PH (28).

In the present study, we therefore investigated the role of hepatic NK and NKT cells in liver regeneration following PH using mice lacking NK and/or NKT cells generated by combination of knockout (KO) animals and selective depletion of these cells by specific antibodies.
Materials and Methods

Animal experiments

Male, wild-type (WT) C57Bl/6 mice 7 weeks after birth were obtained from CLEA Japan Inc. (Tokyo, Japan). A colony of CD1d-KO mice raised from C57Bl/6 strain (a generous gift from Department of Immunology, Juntendo University of Medicine, Tokyo, Japan) were maintained in the animal facility in our institution (9, 19). All animals received humane care in compliance with the experimental protocol approved by the Committee of Laboratory Animals according to institutional guidelines. Mice were housed in air-conditioned specific pathogen free animal quarters with lighting from 08:00 to 20:00 h, and given unrestricted access to a standard lab chow and water.
for 1 week prior to experiments. After overnight fasting, 70% PH was performed in the
mice according to the Higgins and Anderson method (10). Some mice were given a
single, intraperitoneal injection of a mouse monoclonal anti-NK1.1 antibody (PK136;
150 μg/body, provided from Department of Immunology, Juntendo University School of
Medicine) or an anti-asialo GM1 antibody (200 μg/body, provided from Department of
Immunology, Juntendo University School of Medicine) 24 hr prior to operation. For the
extended time course over 72 hr following PH, mice were given the second injection of
antibodies at 48 hr after PH. Mice were sacrificed by exsanguination from inferior vena
cava, and serum and liver samples were obtained. Some mice were pulse-labeled with a
single intraperitoneal injection of bromodeoxyuridine (BrdU; Sigma Chemical Co., St.
Louis, MO, USA; 50 mg/kg in PBS) 2 hr prior to sacrifice, and liver specimens were
fixed in buffered formalin for immunohistochemistry. Serum and liver samples were
kept frozen at -80°C until assayed.

**Immunohistochemistry**

For immunohistochemistry, formalin-fixed and paraffin-embedded tissue sections
were deparaffinized and incubated with 3% H$_2$O$_2$ for 10 min. To examine BrdU
incorporation to hepatocyte nuclei, tissue sections were incubated with 2N HCl for 30
min. After blocking with normal horse serum for 60 min, tissue sections were incubated
with a mouse monoclonal anti-BrdU antibody (DakoCytomation Norden A/S, Glostrup,
Denmark). After rinsing the primary antibody, the sections were incubated with
secondary biotinylated anti-mouse IgG antibody, and the specific binding was visualized with the avidin-biotin complex solution followed by incubation with a 3,3-diaminobenzidine tetrahydrochloride solution using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). BrdU-positive hepatocytes were counted in five 100× fields on each slide to determine the average number BrdU-labeling index (BrdU-positive hepatocytes/total hepatocytes). Expression of PCNA in hepatocytes was evaluated similarly by immunohistochemistry as previously described elsewhere (1). Specimens were observed and photographed using a microscope equipped with a digital imaging system (Leica DM 2000; Leica Microsystems GmbH, Germany).

**Western blot analysis**
Whole liver protein extracts were prepared by homogenizing frozen tissue in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors (Complete Mini, Roche Diagnostics Co., Mannheim, Germany), and a phosphatase inhibitor Na$_3$VO$_4$ (50 μM, Sigma Chemical Co., St. Louis, MO), followed by a centrifugation at 15,000 rpm for 10 min. Protein concentration was determined by Bradford assay using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms of protein was separated in 10% SDS-PAGE, and electrophoretically transferred onto polyvinylamide fluoride membranes. After blocking with 5% non-fat dry milk in Tris-buffered saline, membranes were incubated overnight at 4°C with rabbit polyclonal anti-cyclin D1 or
anti-phospho-STAT3 (Tyr705; Cell signaling Technology Inc., Beverly, MA, USA), followed by a secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (DakoCytomation Norden A/S, Glostrup, Denmark). Subsequently, specific bands were visualized using the ECL detection kit (GE healthcare, Buckinghamshire, UK). Images were captured using a lumino-image analyzer (LAS-3000; Fujifilm Corp., Tokyo, Japan), and densitometry was performed using Multi Gauge software (Fujifilm Corp.).

**ELISA**

Hepatocyte growth factor (HGF) levels in the liver homogenate were determined using an ELISA kit (HGF: Institute of Immunology Co.Ltd., Tokyo, Japan) according to the manufacturer’s instruction. Serum IL-6 levels were measured similarly by an ELISA
Cell culture

HSC-T6 cells, a rat hepatic stellate cell line, were cultured on polystyrene dishes using Dulbecco’s modified Eagle medium (D’MEM; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum in a humidified air containing 5% CO₂ at 37°C. Cells were then incubated with recombinant murine IFNγ, TNFα, IL-4, or IL-13 (10 ng/ml each, R&D Systems Inc.) for 3-6 hr, as appropriate.

RNA preparation and real-time RT-PCR

Total RNA was prepared from frozen tissue samples or culture cells using illustra...
RNAspin Mini RNA Isolation kit (GE healthcare). The concentration and purity of isolated RNA were determined by measuring optical density at 260 and 280 nm. Further, the integrity of RNA was verified by electrophoresis on formaldehyde-denaturing agarose gels.

For real-time RT-PCR, total RNA (1 µg) were reverse-transcribed using Moloney murine leukemia virus transcriptase (Super-Script II, Invitrogen Corp.) and an oligo dT [12-18] primer (Invitrogen Corp.) at 42°C for 1 hr. Obtained cDNA (1 µg) was amplified using SYBR Premix Ex Taq™ (Takara Bio, Tokyo, Japan) and specific primers for IFNγ, TNFα, HGF, IL-4, IL-6, suppressor of cytokine signal (SOCS)-3, and GAPDH, as appropriate (Table 1). After a 10 sec activation period at 95°C, 40 cycles
of 95°C for 5 sec and 60°C for 31 sec, followed by the final cycle of 95°C for 15 sec,

60°C for 1 min, and 95°C for 15 sec, were performed using ABI PRISM 7700 Sequence

Detection System (PE Applied Biosystems, Foster City, CA, USA), and the threshold cycle (C_T) values were obtained.

Statistical analysis

Data were expressed as means ± S.E.M. Statistical differences between means were
determined using two-way ANOVA or ANOVA on ranks followed by a post-hoc test
(Student-Newman-Keuls all pairwise comparison procedures) as appropriate.  A value
of p<0.05 was selected before the study to reflect significance.
Results

Liver regeneration is impaired in mice pretreated with anti-NK1.1 antibody

In order to evaluate the role of NK and NKT cells in liver regeneration, we first tried to evaluate the alteration in hepatic regeneration following 70% PH in mice pretreated with a mouse monoclonal anti-NK1.1 antibody (PK136). A single, intraperitoneal injection of this antibody caused depletion of both NK and NKT cells in the liver almost completely for at least 3 days, which was confirmed by fluorescence activated cell sorting (FACS) analysis (data not shown). Male C57Bl/6 mice (WT) 8 weeks after birth were pretreated with this antibody, and underwent 70% PH 24 hr later. BrdU uptake into hepatocyte nuclei was observed 48 hr after PH (Fig. 1 A). In the control C57Bl/6 mice,
the percentages of BrdU-positive hepatocytes reached nearly 20% as expected; however, pretreatment with an anti-NK1.1 antibody drastically blunted this increase (Fig. 1 B).

Similarly, increases in PCNA-positive hepatocytes 48 hr after PH were blunted largely by pretreatment with an anti-NK1.1 antibody (Fig. 1 C), the values reaching only 40% of control values (Fig. 1 D). To determine whether delayed regeneration occurs in the late phase, we observed extended time course with the second injection of anti-NK1.1 antibody at 48 hr after PH. At day 5 after PH, all animals were surviving, and the liver/body weight ratio in NK1.1 antibody-treated mice reached 94% of those without NK1.1 antibody treatment, clearly indicating that depletion of NK and NKT cells retards, but not irreversibly impairs, regenerating process.
Further, we detected the hepatic expression of cyclin D1 following PH by Western blotting (Fig. 2 A). The hepatic expression levels of cyclin D1 were peaked at 48 hr after PH in control mice as expected; however, the levels in mice pretreated with an anti-NK1.1 antibody only reached 1/5 of controls at the same time point (Fig. 2 B). While the expression levels of cyclin D1 in WT mice were decreased after the peak at 48 hr, the levels in anti-NK1.1 antibody-treated mice were gradually increased in 72 hr after PH, indicating that regenerative process is indeed retarded by pretreatment with an anti-NK1.1 antibody. Taken together, these findings clearly indicated that hepatic regeneration is impaired in mice lacking both NK and NKT cells caused by an anti-NK1.1 antibody.
Pretreatment with anti-NK1.1 antibody blunts expression of cytokines and growth factors triggering liver regeneration

We then evaluated the induction of cytokines affecting regenerative responses following PH. Since IFNγ produced from NK cells has been demonstrated to down-regulate liver regeneration process (28), we first evaluated the hepatic expression of IFNγ mRNA in mice pretreated with an anti-NK1.1 antibody (Fig. 3 A). In control mice, the hepatic expression levels of IFNγ mRNA were elevated in 6 hr after PH, followed by a gradual decrease. The pretreatment with anti-NK1.1 antibody, however, did not alter the levels throughout the time course up to 24 hr. In contrast, induction of IL-4, a cytokine produced mainly from NKT cells, following PH was almost completely
abolished in mice given anti-NK1.1 antibody (Fig. 3 B). Next, we measured TNFα mRNA levels following PH (Fig. 3 C). In control mice, TNFα mRNA levels were markedly increased 1 hr after PH, with rapid decreases thereafter, as expected. In sharp contrast, this swift increase in TNFα mRNA following PH was blunted significantly in mice pretreated with anti-NK1.1 antibody.

Since IL-6 and the Janus kinase (JAK)-STAT pathway also play a pivotal role in liver regeneration (29), we further evaluated the changes in these molecules. PH-induced increases in hepatic IL-6 mRNA and serum IL-6 levels were peaked at 1 hr and 6 hr after PH, respectively, followed by a gradual decrease in 24 hr. Pretreatment with an anti-NK1.1 antibody significantly blunted IL-6 both in mRNA and serum
protein levels (Fig. 3 D, E). Further, phosphorylation levels of STAT3 were peaked at 3 hr after PH in WT controls; however, the levels were markedly blunted in mice pretreated with an anti-NK1.1 antibody (Fig. 4 A, B). Moreover, hepatic mRNA levels of SOCS-3, the downstream inhibitory molecules of the JAK-STAT pathway, were also blunted significantly (Fig. 4 C). Taken together, these findings clearly indicated IL-6 and the JAK-STAT signaling following PH were thoroughly down-regulated by pretreatment with an anti-NK1.1 antibody.

In addition to cytokine responses, we further evaluated the changes in HGF, which plays a key role in normal regenerating process in the liver. Interestingly, elevations in HGF mRNA and protein levels in the liver following PH were also blunted significantly
Since one of the major sources of HGF is hepatic stellate cells (HSCs), we evaluated whether cytokines derived from NK and NKT cells elicit HGF production in vitro using HSC-T6 cells. Indeed, steady state mRNA levels of HGF were significantly elevated in HSC-T6 cells following incubation with IFNγ, TNFα, IL-4, and IL-13 (Fig. 5 C). Collectively, these findings indicated that pretreatment with an anti-NK1.1 antibody blunts cytokines/growth factors triggering regeneration and hepatocyte proliferation.

Pretreatment with anti-asialo GM1 antibody to CD1d-KO mice impairs liver regeneration

To confirm whether depletion of both NK and NKT cells causes impaired
regeneration, we utilized CD1d-KO mice, which lack CD1d-restricted NKT cells systemically, in combination with anti-asialo GM1 antibody. Anti-asialo GM1 antibody is well-known to deplete NK cells specifically (13), and depletion of NK cells using this antibody has been shown to enhance regenerating process following PH (28). Here we applied this antibody to both WT and CD1d-KO mice 24 hr prior to PH, and observed the regenerating process (Fig. 6). WT mice given an anti-asialo GM1 antibody showed almost normal uptake of BrdU and PCNA expression in hepatocytes 48 hr after PH. Similarly, CD1d-KO mice, which lack NKT cells, also demonstrated normal BrdU uptake and PCNA expression. In sharp contrast, CD1d-KO mice pretreated with an anti-asialo GM1 antibody showed significant decreases in both BrdU uptake and PCNA
expression 48 hr after PH (Fig. 6 A, B). Further, hepatic expression of cyclin D1 was largely blunted in CD1d-KO given an anti-asialo GM1 antibody (Fig. 6 C, D), the pattern being quite similar to WT mice pretreated with an anti-NK1.1 antibody (Fig. 2 A). Collectively, depletion of NK and NKT cells by two different approaches resulted in impaired liver regeneration after PH, supporting the hypothesis that NK and NKT cells cooperatively promote normal regenerative responses in the liver.


269 Discussion

270 Here, we demonstrated that depletion of both NK and NKT cells by anti-NK1.1 antibody impairs liver regeneration following PH (Fig. 1, 2). The mechanisms underlying this phenomenon appear to be the down-regulation of regeneration-triggering cytokine responses involving TNFα, IL-6 and the JAK-STAT signaling pathway, and induction of HGF following PH (Fig. 3-5). Moreover, pretreatment with anti-asialo GM1 antibody to CD1d-KO mice, which results in the depletion of both NK and NKT cells, also exhibited poor regeneration after PH (Fig. 6).

277 These observations obviously excluded a possibility of antibody-specific artifact, confirming the fact that liver regeneration is indeed impaired through depletion of these
two innate immune cells. Given the previous findings that depletion of NK cells
enhances liver regeneration (28); our findings are quite striking because NK
cell-depletion in the absence of NKT cells paradoxically inhibits regenerative responses
after PH.

The down-regulation in TNFα and IL-6 in anti-NK1.1 antibody-pretreated mice
after PH (Fig. 3 C, D, E) suggested that NK and NKT cells participate in the production
of these cytokines in corporation with Kupffer cells and other cytokine-producing cells.

Further, pretreatment with anti-NK1.1 antibody almost completely abolished induction
of IL-4 following PH (Fig. 3 B). Since IL-4 has been demonstrated to control IL-6
production following PH in cooperation with the complement system (4), abrogation of
IL-4 explains in part the mechanism of retarded hepatic regeneration caused by anti-NK1.1 antibody. Another possibility is that depletion of NK and NKT cells alters immune microenvironment, thus preventing activation of Kupffer cells in the early stage of regeneration. Anyway, blunting expression of TNFα, which triggers regenerative responses following PH, seems to be quite important in the mechanisms of impaired regeneration in the absence of hepatic NK and NKT cells. Indeed, we observed the blunting induction of HGF following PH in anti-NK1.1 antibody-treated mice (Fig. 5 A, B), where induction of HGF by NKT cell-derived cytokines in HSCs most likely plays a role (Fig. 5 C).

Here in this study, mice pretreated with an anti-NK1.1 antibody showed blunted
induction of IL-6 (Fig. 3 D, E) and subsequent activation of the JAK-STAT pathway (Fig. 4), clearly indicating that down-regulation of this signaling pathway causes poor regenerative responses following PH. These findings are coincident with the lines of evidence that these factors promote liver regeneration through investigations of KO animals (29). In terms of IL-6 and the JAK-STAT pathways, however, there are some controversial observations; prolonged, enhanced activation of STAT3 reciprocally inhibits regenerative responses (15, 18, 30). Indeed, recently we reported that KK-A\(^y\) mice, which spontaneously develop steatohepatitis with metabolic syndrome-like phenotypes, showed poor liver regeneration following PH, where augmented activation of STAT3 with delayed peak was observed (2). This phenomenon is most likely due to
tremendous over-expression of IL-6 and leptin, which share the same JAK-STAT signaling. Interestingly, KK-A^y mice also demonstrated depletion of hepatic NKT cells; however, the mechanism underlying the impaired regeneration seems to be different from the observations in this study, especially in respect with IL-6 and the JAK-STAT pathway. Nonetheless, NK and NKT cells most likely play a pivotal role in regulation of IL-6 and the JAK-STAT pathway, thereby modulating regenerating responses in the liver.

In conclusion, our findings in the present study clearly indicated that depletion of both NKT and NK cells by two different ways results in impaired liver regeneration. The role of NK cells in hepatic regeneration appears to be paradoxical in the presence or
absence of NKT cells, and this phenomenon cannot be explained simply by the secretion of IFN\(\gamma\). Rather, these two innate immune cells most likely up-regulate TNF\(\alpha\), IL-6 and the JAK-STAT pathway, and HGF in a coordinate fashion, thus promoting normal regenerative responses in the liver (Fig. 7).

**Grants**

This work was supported in part by Grant-in-Aid (No. 21590859 to KI, No. 21390234 to SW) and High Technology Research Center Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Health and Labour Science Research Grant from the Ministry of Health, Labour and Welfare of Japan (to SH, SW), and
grants from Liver Forum in Kyoto (co-sponsored by Viral Hepatitis Research Foundation in Japan and Dainippon Sumitomo Pharma Co. Ltd., to KI), and Research Conference on Alcohol and Health (sponsored by Suntory Co. Ltd., to KI).
References


7. Ginsburg I, Koren E, Horani A, Mahamid M, Doron S, Muhanna N, Amer J, and Safadi R. Amelioration of hepatic fibrosis via Padma Hepaten is associated...


28. Sun R, and Gao B. Negative regulation of liver regeneration by innate immunity


Figure legends

Fig. 1. Anti-NK1.1 antibody inhibits liver regeneration after PH

WT mice were given a single injection of anti-NK1.1 antibody (150 μg/body) 24 hr before PH. Representative photomicrographs of immunohistochemistry for BrdU (A) and PCNA (C) before and 48 hr after PH are shown (original magnification: ×100). The average labeling indices for BrdU (B) and PCNA (D) for WT controls (closed circle) and anti-NK1.1 antibody-treated WT (closed square) after PH are plotted (n=5, **; p<0.01, ***; p<0.001 vs. WT before PH, †††; p<0.001 vs. antibody-treated WT before PH, ###; p<0.001 vs. WT at the same time point).
Fig. 2. Anti-NK1.1 antibody blunts induction of cyclin D1 after PH

Representative photographs of Western blotting for hepatic cyclin D1 (A), and densitometrical data (B) for WT controls (closed circle) and anti-NK1.1 antibody-treated WT (closed square) are shown (n=5, ***; p<0.001 vs. WT before PH, †††; p<0.001 vs. antibody-treated WT before PH, #; p<0.05, ###; p<0.001 vs. WT at the same time point).

Fig. 3 Cytokine expression following PH in mice pretreated with an anti-NK1.1 antibody

Steady state mRNA levels for IFNγ, IL-4, TNFα, and IL-6 in the liver were measured
by real-time RT-PCR, and serum IL-6 levels were measured by ELISA. Average values
of IFNγ (A), IL-4 (B), TNFα (C), and IL-6 (D) mRNA levels and serum IL-6 levels (E)
in WT (closed circle) and anti-NK1.1 antibody-treated WT (closed square) are plotted
(n=5, *, p<0.05, **, p<0.01, ***, p<0.001 vs. WT before PH, #, p<0.05, ##, p<0.01 vs.
WT at the same time point).

Fig. 4  Phosphorylation of STAT3 and expression of SOCS-3 following PH in mice
pretreated with an anti-NK1.1 antibody.

Phosphorylation of STAT3 was detected by Western blotting. Steady state mRNA levels
for SOCS-3 were determined by real-time RT-PCR. Representative photographs of
specific bands for phospho-STAT3 in WT and WT pretreated with an anti-NK1.1 antibody are shown (A). Densitometrical data for phospho-STAT3/STAT3 (B), and average expression levels of SOCS-3 mRNA (C) are plotted (n=5, *; p<0.05, **; p<0.01, ***; p<0.001 vs. WT before PH, #; p<0.05, ##; p<0.01, ###; p<0.001 vs. WT at the same time point).

Fig. 5  Expression of HGF following PH in mice pretreated with an anti-NK1.1 antibody

Steady state mRNA and protein levels for HGF in the liver were measured by real-time RT-PCR and ELISA, respectively. Average values of HGF mRNA levels (A) and HGF
protein levels (B) in WT (closed circle) and anti-NK1.1 antibody-treated WT (closed square) are plotted (n=5, *; p<0.05, **; p<0.01, ***; p<0.001 vs. WT before PH, #; p<0.05, ##; p<0.01 vs. WT at the same time point). HSC-T6 cells were incubated with IFN\(\gamma\), TNF\(\alpha\), IL-4, or IL-13 (10 ng/ml each) for 3-6 hr, and mRNA levels for HGF were quantified using real-time RT-PCR. Average mRNA levels from 5 separate dishes are plotted (C; *; p<0.05, **; p<0.01, vs. controls).

**Fig. 6** Anti-asialo GM1 antibody impairs liver regeneration following PH in CD1d-KO mice

WT and CD1d-KO mice were given a single injection of anti-asialo GM1 antibody (ASGM1Ab; 200 \(\mu\)g/body) 24 hr before PH. The average percentages of BrdU-positive
hepatocytes (A) and PCNA-positive hepatocytes (B) 48 hr after PH are plotted (A, n=5, *; p<0.05 vs. WT without ASGM1Ab, #; p<0.05 vs. CD1d-KO without ASGM1Ab).

Hepatic expression of cyclin D1 was detected by Western blotting (C). Densitometrical data (D) for CD1d-KO (closed circle) and CD1d-KO pretreated with ASGM1Ab (closed square) are shown (n=5, *; p<0.05, **; p<0.01 vs. CD1d-KO before PH, †; p<0.05 vs. ASGM1Ab-treated CD1d-KO before PH, #; p<0.05 vs. WT at the same time point).

Fig. 7 Working hypothesis: NK and NKT cells contribute to the normal regenerative responses in the liver
### Table 1. Primer sets for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene (GeneBank accession)</th>
<th>Primer sequences</th>
<th>Product size</th>
</tr>
</thead>
</table>
| **IFNγ** (NM_008337.3)   | forward: 5'-CGGCACAGTCATTGAAAGCCTA-3'  
reverse: 5'-GTTGCTGATGGCCTGATTGTC-3' | 199 bp |
| **TNFα** (NM_013693.2)   | forward: 5'-AAGCCTGTAGCCACGTCGTA-3'  
reverse: 5'-GGCACCACCTAGTTGGTCTTGTG-3' | 122 bp |
| **HGF** (NM_010427.4)    | forward: 5'-AGAAATGCGTCAGCACCCTCAAG-3'  
reverse: 5'-AGATGGCACTCCAGACCAG-3' | 179 bp |
| **IL-4** (NM_021283.2)   | forward: 5'-ACGGAGATGGATGTGCAAAC-3'  
reverse: 5'-AGCACCTTGGAAGCCCTACAGA-3' | 83 bp |
| **IL-6** (NM_031168.1)   | forward: 5'-CCAATTCACAAAGTGAGGCTA-3'  
reverse: 5'-GCAAGTCATCGTTCAGCTAC-3' | 112 bp |
| **SOCS-3** (NM_007707)   | forward: 5'-CAATACCTTTTGACAAGCGGACTCT-3'  
reverse: 5'-TCAAAGCGCAAACAAGTTCCAG-3' | 146 bp |
| **GAPDH** (NM_008084.2)  | forward: 5'-TTGCTGCATTGGAAGTCGAGGAG-3'  
reverse: 5'-TTGCTACGTGGAATTCGAGGAG-3' | 150 bp |
A

<table>
<thead>
<tr>
<th>Hour after PH</th>
<th>WT</th>
<th>WT + NK1.1Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**cyclin D1**

**GAPDH**

B

**Cyclin D1 (% induction)**

**Hour after PH**

- WT: Black circles
- WT + NK1.1Ab: Black squares

Significance levels:

- ***: p < 0.001
- ###: p < 0.001
- #: p < 0.05