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

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Hosoya S, Ikejima K, Takeda K, Arai K, Ishikawa S, Yamagata H, Aoyama T, Kon K, Yamashina S, Watanabe S. Innate immune responses involving natural killer and natural killer T cells promote liver regeneration after partial hepatectomy (PH) (2), where hepatic NKT cells are also depleted in KK-Ay mice, which have been reported (16, 32), where hepatic NKT cells are depleted obscure; poor regeneration in steatotic liver in ob/ob mice has been reported (28). The role of NKT cells in liver regeneration is more controversial. 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 pathophysiology is still controversial.

Liver regeneration is one of the significant natures of this important organ. The normal liver is capable of regenerating when 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 the regenerating process (5). For example, pattern-recognition receptors, such as Toll-like receptors, and downstream 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. A recent report indicated that NK cells negatively regulate liver regeneration through production of IFN-γ (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 shown recently that KK-Ay mice, which develop a metabolic, syndrome-like phenotype spontaneously, demonstrate poor regeneration following 70% partial hepatectomy (PH) (2), where hepatic NKT cells are also depleted. Furthermore, activation of NKT cells triggered by a specific ligand α-galactosylceramide has been shown to accelerate liver regeneration after PH (22). These observations suggested that NKT cells promote the regeneration process; however, mice lacking NKT cells caused by genetic knockout (KO) 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 a combination of KO animals and selective depletion of these cells by specific antibodies.

MATERIALS AND METHODS

Animal experiments. Male, wild-type (WT) C57Bl/6 mice, 7 wk after birth, were obtained from CLEA Japan (Tokyo, Japan). A colony of CD1d-KO mice raised from the C57Bl/6 strain (a generous gift from the Department of Immunology, Juntendo University of Medicine, Tokyo, Japan) was maintained in the animal facility in our institution—Juntendo University Graduate School of Medicine (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-condi-
tioned, specific pathogen-free animal quarters with lighting from 0800 to 2000 and were given unrestricted access to standard lab chow and water for 1 wk 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 intra-peritoneal injection of a mouse anti-NK1.1 MAb (PK136; 150 µg/body; provided by the Department of Immunology, Juntendo University School of Medicine) or an anti-asialo ganglio-N-tetraosylceramide (GM1) antibody (200 µg/body; provided by the Department of Immunology, Juntendo University School of Medicine), 24 h prior to operation. For the extended time course over 72 h following PH, mice were given the second injection of antibodies at 48 h after PH. Mice were killed 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, St. Louis, MO; 50 mg/kg in PBS), 2 h prior to death, 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% H2O2 for 10 min. To examine BrdU incorporation to hepatocyte nuclei, tissue sections were incubated with 2 N HCl for 30 min. After blocking with normal horse serum for 60 min, tissue sections were incubated with a mouse anti-BrdU MAb (DakoCytomation Norden A/S, Glostrup, Denmark). After rinsing the primary antibody, the sections were incubated with secondary biotinylated antimouse IgG antibody, and specific binding was visualized with avidin-biotin complex solution, followed by incubation with a 3,3'-diaminobenzidine tetrahydrochloride solution using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). 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 proliferating cell nuclear antigen (PCNA) in hepatocytes was evaluated similarly by immunohistochemistry as described previously 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 protease inhibitor cocktail tablets; Roche Diagnostics, Mannheim, Germany), and a phosphatase inhibitor Na3VO4 (50 µM; Sigma Chemical), followed by centrifugation at 15,000 rpm for 10 min. Protein concentration was determined by the Bradford assay using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Twenty micrograms of protein was separated in 10% SDS-PAGE and electrophoretically transferred onto polyvinylamid fluoride membranes. After blocking with 5% non-fat dry milk in Tris-buffered saline, membranes were incubated overnight at 4°C with rabbit polyclonal anticyclin D1 or antiphospho-STAT3 (Tyr705; Cell Signaling Technology, Beverly, MA), followed by a secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (DakoCytomation Norden A/S). Subsequently, specific bands were visualized using the enhanced chemiluminescence detection kit (GE Healthcare, Buckinghamshire, UK). Images were captured using a lumino-image analyzer (LAS-3000; Fujifilm, Tokyo, Japan), and densitometry was performed using Multi Gauge software (Fujifilm).

ELISA. Hepatocyte growth factor (HGF) levels in the liver homogenate were determined using an ELISA kit (Institute of Immunology, Tokyo, Japan), according to the manufacturer’s instruction. Serum IL-6 levels were measured similarly by an ELISA kit (R&D Systems, Minneapolis, MN).

Cell culture. Hepatic stellate cell (HSC)-T6 cells, a rat HSC line, were cultured on polystyrene dishes using DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% FBS in a humidified air containing 5% CO2 at 37°C. Cells were then incubated with recombinant murine IFN-γ, TNF-α, IL-4, or IL-13 (10 ng/ml each; R&D Systems) for 3–6 h as appropriate.

RNA preparation and real-time RT-PCR. Total RNA was prepared from frozen tissue samples or culture cells using the 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. Furthermore, the integrity of RNA was verified by electrophoresis on formaldehyde-denaturing agarose gels.

For real-time RT-PCR, total RNA (1 µg) was reverse transcribed using Moloney murine leukemia virus transcriptase (SuperScript II, Invitrogen) and an oligo(dT)12–18 primer (Invitrogen) at 42°C for 1 h. Obtained cDNA (1 µl) was amplified using SYBR Premix Ex Taq (Takara Bio, Tokyo, Japan) and specific primers for IFN-γ, TNF-α, HGF, IL-4, IL-6, suppressor of cytokine signal (SOCS)-3, and GAPDH, as appropriate (Table 1). After a 10-s activation period at 95°C, 40 cycles of 95°C for 5 s and 60°C for 31 s, followed by the final cycle of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s, were performed using the ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Foster City, CA), and the threshold cycle values were obtained.

Statistical analysis. Data were expressed as means ± SE. 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.

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>
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<tbody>
<tr>
<td>IFN-γ (NM_008337.3)</td>
<td>forward: 5′-CCGACACGATCATGAAAGGCTA-3′</td>
<td>199 bp</td>
</tr>
<tr>
<td></td>
<td>reverse: 5′-GGTCTGCTAGGCGCTGATTGTC-3′</td>
<td></td>
</tr>
<tr>
<td>TNF-α (NM_013693.2)</td>
<td>forward: 5′-AAATCCTGTACCAGCGCTGCTA-3′</td>
<td>122 bp</td>
</tr>
<tr>
<td></td>
<td>reverse: 5′-GGGCTCACTGTGGTCTTCTCTTGG-3′</td>
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</tr>
<tr>
<td>HGF (NM_010427.4)</td>
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<td>179 bp</td>
</tr>
<tr>
<td></td>
<td>reverse: 5′-GATGGGCACATCCACAGGGAAGG-3′</td>
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<tr>
<td>IL-4 (NM_021283.2)</td>
<td>forward: 5′-ACGGGAGATGGATGCCAAACG-3′</td>
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<td></td>
<td>reverse: 5′-AGCAGCCCTGGAGTGGCAAC-3′</td>
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<tr>
<td>IL-6 (NM_031168.1)</td>
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</tr>
<tr>
<td></td>
<td>reverse: 5′-GCAAGTTGCACTGACACGTCATGAC-3′</td>
<td></td>
</tr>
<tr>
<td>SOCS-3 (NM_007707)</td>
<td>forward: 5′-CAATACCTTTTGCAAAGGAGCTCGTC-3′</td>
<td>146 bp</td>
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<td></td>
<td>reverse: 5′-TCAAAAGGCAAACAGTTCCAGG-3′</td>
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<td>GAPDH (NM_008084.2)</td>
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Liver regeneration is impaired in mice pretreated with anti-NK1.1 antibody. 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 anti-NK1.1 MAb (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 analysis (data not shown). Male C57Bl/6 mice (WT), 8 wk after birth, were pretreated with this antibody and underwent 70% PH 24 h later. BrdU uptake into hepatocyte nuclei was observed 48 h after PH (Fig. 1A). 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. 1B). Similarly, increases in PCNA-positive hepatocytes, 48 h after PH, were blunted largely by pretreatment with an anti-NK1.1 antibody (Fig. 1C). The hepatic expression levels of cyclin D1 in WT mice were decreased after the peak at 48 h, the levels in anti-NK1.1 antibody-treated mice were increased gradually in 72 h after PH, indicating that the regenerative process is indeed retarded by pretreatment with an anti-NK1.1 antibody. Taken together, these findings clearly

controls at the same time point (Fig. 2B). Whereas the expression levels of cyclin D1 in WT mice were decreased after the peak at 48 h, the levels in anti-NK1.1 antibody-treated mice were increased gradually in 72 h after PH, indicating that the 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 an 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 IFN-γ produced from NK cells has been demonstrated to downregulate the liver regeneration process (28), we first evaluated the hepatic expression of IFN-γ mRNA in mice pretreated with an anti-NK1.1 antibody (Fig. 3A). In control mice, the hepatic expression levels of IFN-γ mRNA were elevated in 6 h after PH, followed by a gradual decrease. The pretreatment with an anti-NK1.1 antibody, however, did not alter the levels throughout the time course up to 24 h. In contrast, induction of IL-4, a cytokine produced mainly from NKT cells, following PH, was almost completely abolished in mice given an anti-NK1.1 antibody (Fig. 3B). Next, we measured TNF-α mRNA levels following PH (Fig. 3C). In control mice, TNF-α mRNA levels were increased markedly, 1 h after PH, with rapid decreases thereafter as expected. In sharp contrast, this swift increase in TNF-α mRNA following PH was blunted significantly in mice pretreated with an anti-NK1.1 antibody.

Since IL-6 and the 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 h and 6 h after PH, respectively, followed by a gradual decrease in 24 h. Pretreatment with an anti-NK1.1 antibody significantly blunted IL-6, both in mRNA and serum protein levels (Fig. 3D and E). Furthermore, phosphorylation levels of STAT3 were peaked at 3 h after PH in WT controls; however, the levels were blunted markedly, 1 h after PH, with rapid decreases thereafter as expected. In sharp contrast, this swift increase in TNF-α mRNA following PH was blunted significantly in mice pretreated with an anti-NK1.1 antibody.

In addition to cytokine responses, we further evaluated the changes in HGF, which plays a key role in the normal regenerating process in the liver. Interestingly, elevations in HGF mRNA and protein levels in the liver following PH were also blunted significantly (Fig. 5A and B). Since one of the major sources of HGF is 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 IFN-γ, TNF-α, IL-4, and IL-13 (Fig. 5C). Collectively, these findings indicated that pretreatment with an anti-NK1.1 antibody downregulated IL-6 and JAK-STAT signaling following PH.

![Fig. 3.](http://ajpgi.physiology.org/) Cytokine expression following PH in mice pretreated with an anti-NK1.1 antibody. Steady-state mRNA levels for IFN-γ, IL-4, TNF-α, 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 IFN-γ (A), IL-4 (B), TNF-α (C), and IL-6 (D) mRNA levels and serum IL-6 levels (E) in WT (■) and anti-NK1.1 antibody-treated WT (○) 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. 4.](http://ajpgi.physiology.org/) Phosphorylation of STAT3 (p-STAT3) and expression of suppressor of cytokine signal (SOCS)-3 following PH in mice pretreated with an anti-NK1.1 antibody. p-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 p-STAT3 in WT and WT pretreated with an anti-NK1.1 antibody are shown (A). Densitometrical data for p-STAT3/STAT3 (B) and average expression levels of SOCS-3 mRNA (C) are plotted (n = 5; **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).
NK1.1 antibody blunts cytokines/growth factors triggering regeneration and hepatocyte proliferation.

Pretreatment with the antiasialo GM1 antibody to CD1d-KO mice impairs liver regeneration. To confirm whether depletion of both NK and NKT cells causes impaired regeneration, we used CD1d-KO mice, which lack CD1d-restricted NKT cells systemically, in combination with antiasialo GM1 antibody. Antiasialo GM1 antibody is well known to deplete NK cells specifically (13), and depletion of NK cells using this antibody has been shown to enhance the regenerating process following PH (28). Here, we applied this antibody to both WT and CD1d-KO mice, 24 h prior to PH, and observed the regenerating process (Fig. 6). WT mice given an antiasialo GM1 antibody showed almost normal uptake of BrdU and PCNA expression in hepatocytes, 48 h 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 antiasialo GM1 antibody showed significant decreases in both BrdU uptake and PCNA expression, 48 h after PH (Fig. 6, A and B). Furthermore, hepatic expression of cyclin D1 was largely blunted in CD1d-KO given an antiasialo GM1 antibody (Fig. 6, C and D), the pattern being quite similar to WT mice pretreated with an anti-NK1.1 antibody (Fig. 2A). 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.

DISCUSSION

Here, we demonstrated that depletion of both NK and NKT cells by an anti-NK1.1 antibody impairs liver regeneration following PH (Figs. 1 and 2). The mechanisms underlying this phenomenon appear to be the downregulation of regeneration-triggering cytokine responses involving TNF-α, IL-6, and the JAK-STAT signaling pathway and induction of HGF following PH (Figs. 3–5). Moreover, pretreatment with the antiasialo 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). These observations obviously excluded a possibility of an 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 downregulation in TNF-α and IL-6 in anti-NK1.1 antibody-pretreated mice after PH (Fig. 3, C–E) suggested that NK and NKT cells participate in the production of these cytokines in cooperation with Kupffer cells and other cytokine-producing cells. Furthermore, pretreatment with the anti-NK1.1 antibody almost completely abolished induction of IL-4 following PH (Fig. 3B). 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 cells alters the immune microenvironment, thus preventing activation of Kupffer cells in the early stage of regeneration. Anyway, blunting expression of TNF-α, 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 and B), where induction of HGF by NKT cell-derived cytokines in HSCs most likely plays a role (Fig. 5C).

Here, in this study, mice pretreated with an anti-NK1.1 antibody showed blunted induction of IL-6 (Fig. 3, D and E) and subsequent activation of the JAK-STAT pathway (Fig. 4), clearly indicating that downregulation 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′ mice, which spontaneously develop steatohepatitis with metabolic syndrome-like phenotypes, showed poor liver regeneration following PH, where augmented activation of STAT3 with a delayed peak was observed (2). This phenomenon is most likely due to tremendous overexpression of IL-6 and leptin, which share the same JAK-STAT signaling. Interestingly, KK-A′ 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 with respect to 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-γ. Rather, these two innate immune cells most likely upregulate TNF-α, IL-6, and the JAK-STAT pathway and HGF in a coordinate fashion, thus promoting normal regenerative responses in the liver (Fig. 7).

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**DISCLOSURES**

All authors have no conflict of interest in terms of this study.
AUTHOR CONTRIBUTIONS


REFERENCES


