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 in mice. Am J Physiol Gastrointest Liver Physiol 304: G293–G299, 2013. First published October 18, 2012; doi:10.1152/ajpgi.00083.2012.—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 ganglio-N-tetraosylceramide (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 bro-modeoxyuridine uptake, expression of proliferating cell nuclear antigen, and cyclin D1, 48 h after PH. In mice given NK1.1 antibody, increases in hepatic TNF-α, IL-6/phospho-STAT3, and hepatocyte growth factor (HGF) levels following PH were also blunted significantly, whereas IFN-γ mRNA levels were not different. CD1d-KO mice per se showed normal liver regeneration; however, pretreatment with an anti-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 upregulate TNF-α, IL-6/STAT3, and HGF in a coordinate fashion, thus promoting normal regenerative responses in the liver.

lines of evidence have suggested that alteration in the 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 a considerable proportion of natural killer (NK) and NKT cell fractions (6). NK cells are defined as large, granular lymphocytes that exert cytotoxic activity against tumors and viral-infected cells through the perforin and granzyme systems (27). NK cells preferentially reside in the hepatic sinusoid, and these liver-specific NK cells are called 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 histocompatibility 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 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 intraperitoneal 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 anti-mouse antibody, 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).

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 μg) 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>
</tr>
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<tbody>
<tr>
<td>IFN-γ (NM_008337.3)</td>
<td>forward: 5′-CGGCAACATCATGAAAGCTTA-3′</td>
<td>199 bp</td>
</tr>
<tr>
<td></td>
<td>reverse: 5′-GTTGCTTAGGGCTGATTGTC-3′</td>
<td></td>
</tr>
<tr>
<td>TNF-α (NM_013693.2)</td>
<td>forward: 5′-AACCTCTTAGGCGACGTCTA-3′</td>
<td>122 bp</td>
</tr>
<tr>
<td></td>
<td>reverse: 5′-GGACACATCTTTGCTTCTTG-3′</td>
<td></td>
</tr>
<tr>
<td>HGF (NM_010427.4)</td>
<td>forward: 5′-AGAATGGCAGTCACGATCAGA-3′</td>
<td>179 bp</td>
</tr>
<tr>
<td></td>
<td>reverse: 5′-GATGGCAATCCACGACAGG-3′</td>
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</tr>
<tr>
<td>IL-4 (NM_021283.2)</td>
<td>forward: 5′-ACGGAGGATGGATGTGCCAAAC</td>
<td>150 bp</td>
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<tr>
<td></td>
<td>reverse: 5′-GCAAGTGGGACATCTGATACG-3′</td>
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<tr>
<td>IL-6 (NM_031168.1)</td>
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<td>112 bp</td>
</tr>
<tr>
<td></td>
<td>reverse: 5′-GCAAGTGGGAACTTGAGTTG-3′</td>
<td></td>
</tr>
<tr>
<td>SOCS-3 (NM_007707)</td>
<td>forward: 5′-GATATACCTTTTTGAAACGGACTTCG-3′</td>
<td>146 bp</td>
</tr>
<tr>
<td></td>
<td>reverse: 5′-GCAAGGAGGACATCTGATACG-3′</td>
<td></td>
</tr>
<tr>
<td>GAPDH (NM_008084.2)</td>
<td>forward: 5′-TTGCTCGTACTGGAATCAG-3′</td>
<td>150 bp</td>
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HGF, hepatocyte growth factor; SOCS-3, suppressor of cytokine signal-3.
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). To determine whether delayed regeneration occurs in the late phase, we observed an extended time course with the second injection of an anti-NK1.1 antibody at 48 h after PH. At day 5 after PH, all animals were surviving, and the liver/body wt 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 does not irreversibly impair the regenerating process.

Furthermore, we detected the hepatic expression of cyclin D1 following PH by Western blotting (Fig. 2A). The hepatic expression levels of cyclin D1 were increased in WT mice after PH, but were increased only slightly in anti-NK1.1 antibody-treated mice (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 indicate that NK and NKT cells play a role in liver regeneration.
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 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.
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 concert 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 and NKT 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-AV 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-AV 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