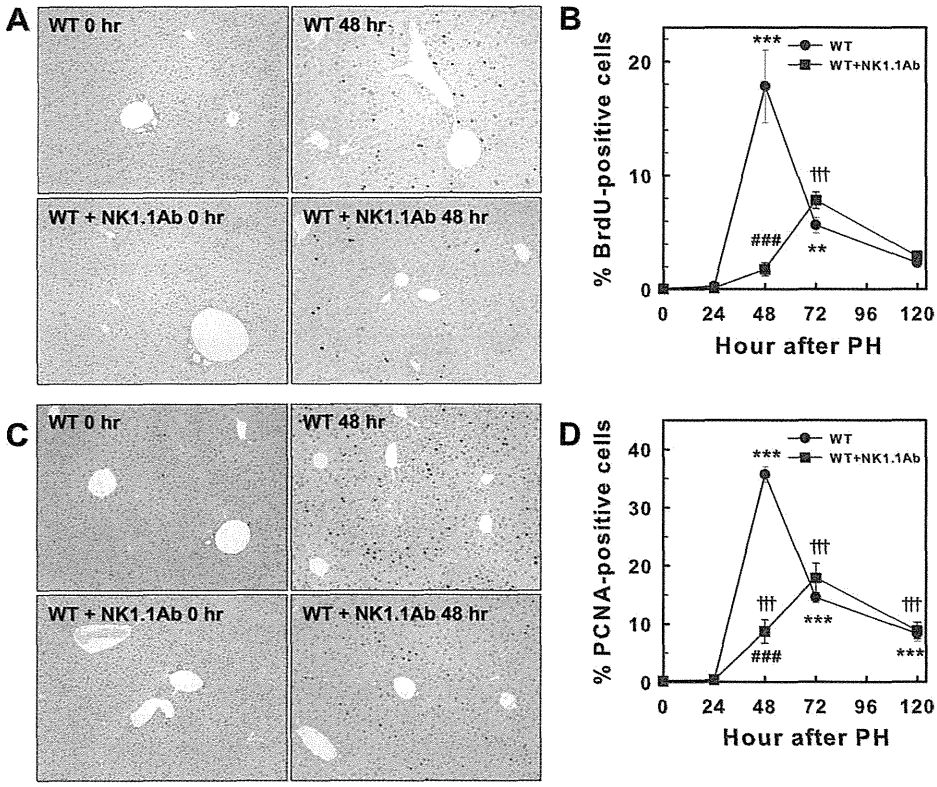

Fig. 1. Anti-NK1.1 antibody inhibits liver regeneration after partial hepatectomy (PH). Wild-type (WT) mice were given a single injection of an anti-NK1.1 antibody (Ab; 150 μg/body), 24 h before PH. Representative photomicrographs of immunohistochemistry for bromodeoxyuridine (BrdU; *A*) and proliferating cell nuclear antigen (PCNA; *C*), before and 48 h after PH, are shown (original magnification: ×100). The average labeling indices for BrdU (*B*) and PCNA (*D*) for WT controls (●) and anti-NK1.1 antibody-treated WT (■) 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).

RESULTS

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. 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 h 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 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. 2*A*). The hepatic expression levels of cyclin D1 were peaked at 48 h after PH in control mice as expected; however, the levels in mice pretreated with an anti-NK1.1 antibody only reached one-fifth of controls at the same time point (Fig. 2*B*). 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

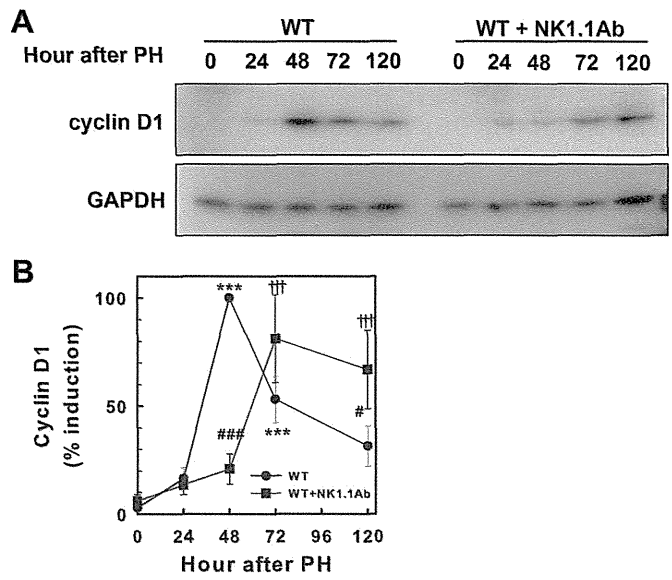

Fig. 2. Anti-NK1.1 antibody blunts induction of cyclin D1 after PH. Representative photographs of Western blotting for hepatic cyclin D1 (*A*) and densitometrical data (*B*) for WT controls (●) and anti-NK1.1 antibody-treated WT (■) are shown ($n = 5$; $^{***}P < 0.001$ vs. WT before PH; †††$P < 0.001$ vs. antibody-treated WT before PH; #$P < 0.05$; ###$P < 0.001$ vs. WT at the same time point).

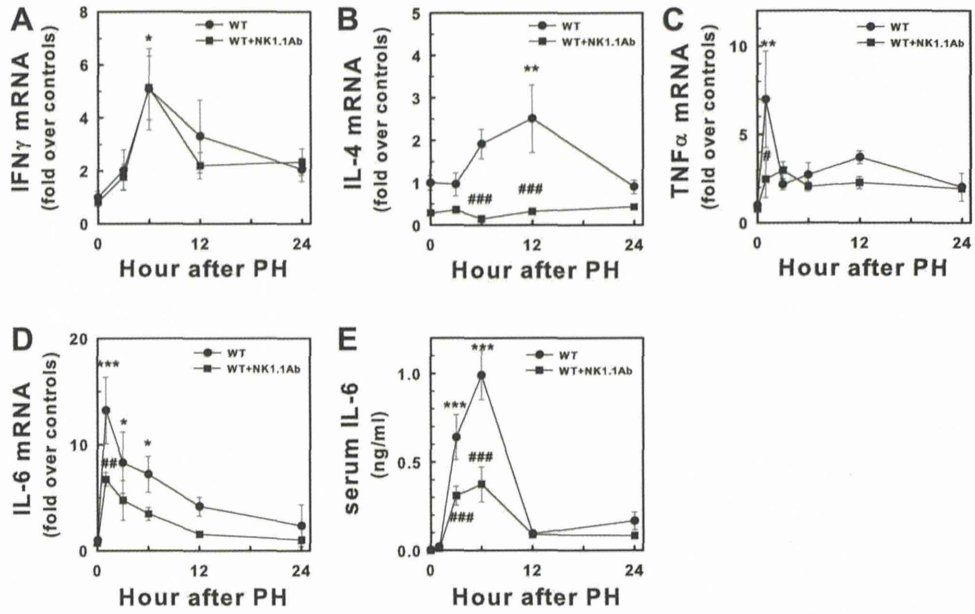

Fig. 3. Cytokine expression following PH in mice pretreated with an anti-NK1.1 antibody. Steady-state mRNA levels for IFN-γ, IL-4, TNF-α, and IL-6 in the liver were measured by real-time RT-PCR, and serum IL-6 levels were measured by ELISA. Average values of IFN-γ (*A*), IL-4 (*B*), TNF-α (*C*), and 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).

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. 3*A*). 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. 3*B*). Next, we measured TNF-α mRNA levels following PH (Fig. 3*C*). 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. 3, *D* and *E*). Furthermore, phosphorylation levels of STAT3 were peaked at 3 h after PH in WT controls; however, the levels were blunted markedly in mice pretreated with an anti-NK1.1 antibody (Fig. 4, *A* and *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 that IL-6 and JAK-STAT signaling following PH were thoroughly downregulated 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 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. 5, *A* 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. 5*C*). Collectively, these findings indicated that pretreatment with an anti-

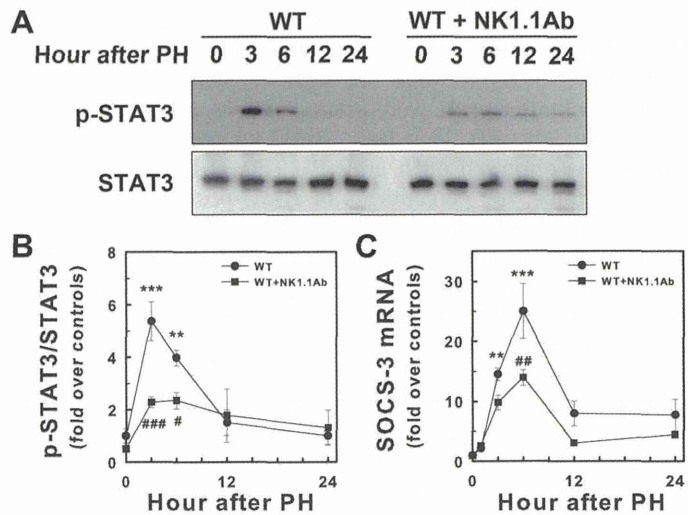

Fig. 4. Phosphorylation of STAT3 (p-STAT3) and expression of suppressor of cytokine signal (SOCS)-3 following PH in mice pretreated with an anti-NK1.1 antibody. p-STAT3 was detected by Western blotting. Steady-state mRNA levels for SOCS-3 were determined by real-time RT-PCR. Representative photographs 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).

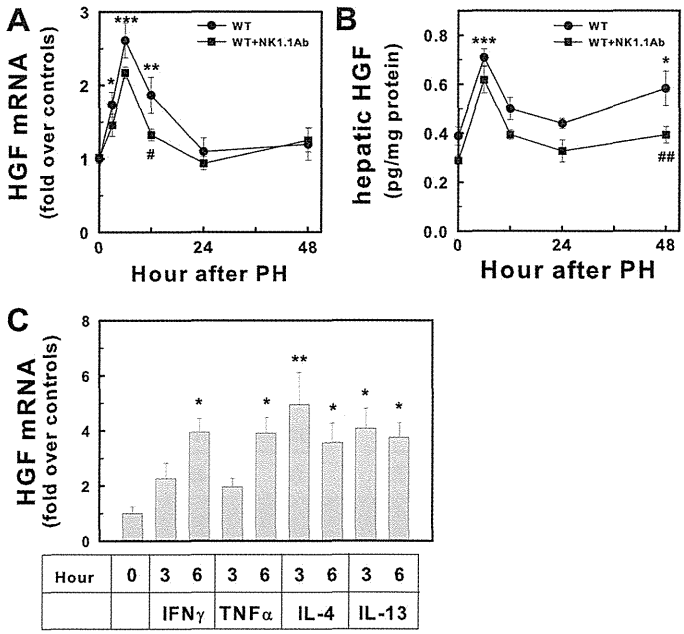

Fig. 5. Expression of hepatocyte growth factor (HGF) following PH in mice pretreated with an anti-NK1.1 antibody. Steady-state mRNA and protein levels for HGF in the liver were measured by real-time RT-PCR and ELISA, respectively. Average values of HGF mRNA levels (*A*) and HGF protein levels (*B*) in WT (●) and anti-NK1.1 antibody-treated WT (■) are plotted ($n = 5$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. WT before PH; $\#P < 0.05$; $\#\#P < 0.01$ vs. WT at the same time point). Hepatic stellate cell-T6 cells were incubated with IFN-γ, TNF-α, IL-4, or IL-13 (10 ng/ml each) for 3–6 h, 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).

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. 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.

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 corporation 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. 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 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. 5*C*).

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^{y} 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^{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 with respect to IL-6 and the

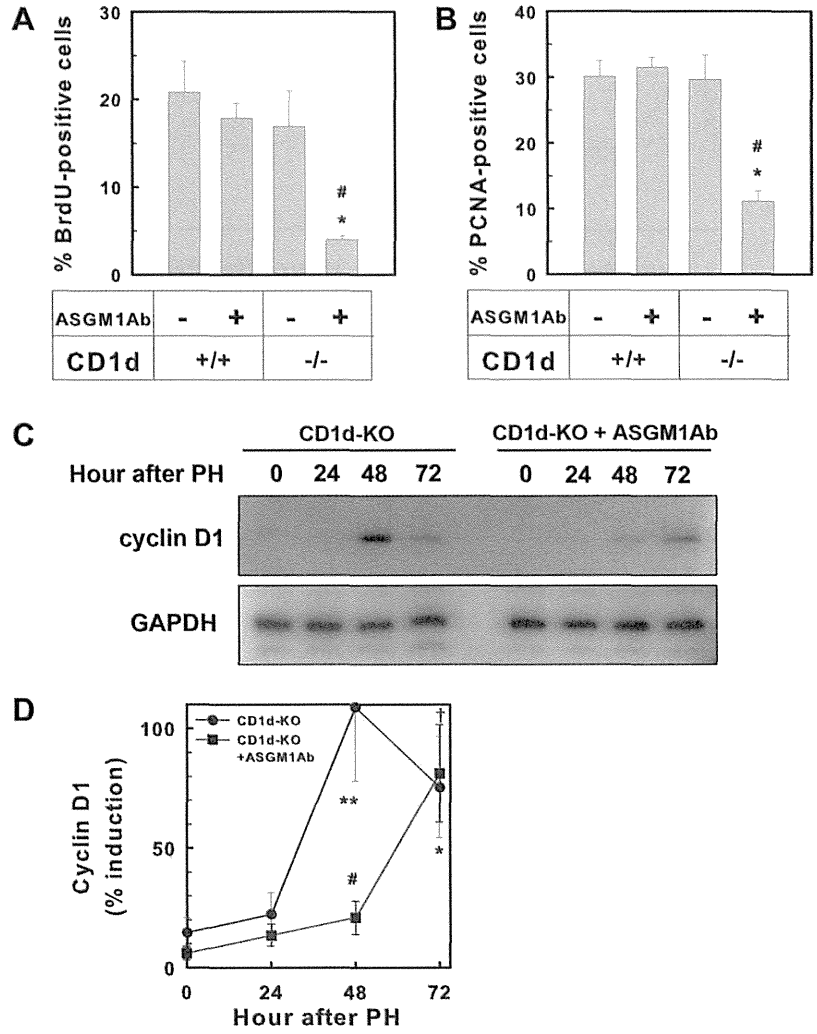

Fig. 6. Anti-asialo ganglio-*N*-tetraosylceramide (GM1) antibody (ASGM1Ab) impairs liver regeneration following PH in CD1d-knockout (KO) mice. WT and CD1d-KO mice were given a single injection of ASGM1Ab (200 μg/body), 24 h before PH. The average percentages of BrdU-positive hepatocytes (*A*) and PCNA-positive hepatocytes (*B*), 48 h after PH, are plotted ($n = 5$; $^*P < 0.05$ 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 (●) and CD1d-KO pretreated with ASGM1Ab (■) are shown ($n = 5$; $^*P < 0.05$; $^{**}P < 0.01$ vs. CD1d-KO before PH; $\dagger P < 0.05$ vs. ASGM1Ab-treated CD1d-KO before PH; $\#P < 0.05$ vs. WT at the same time point).

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.

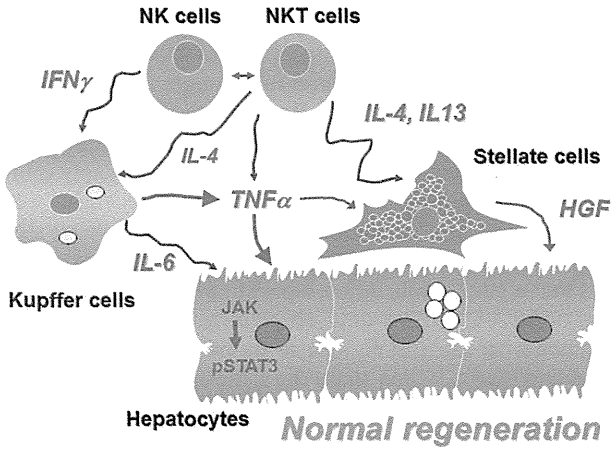

Fig. 7. Working hypothesis: natural killer (NK) and NKT cells contribute to the normal regenerative responses in the liver.

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).

GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: K.I. conception and design of research; S.H., K.A., S.I., H.Y., and T.A. performed experiments; S.H., K.I., K.K., and S.Y. analyzed data; S.H., K.I., K.T., K.K., and S.Y. interpreted results of experiments; S.H. and K.I. prepared figures; S.H. and K.I. drafted manuscript; K.I. and S.W. edited and revised manuscript; S.H., K.I., K.T., K.A., S.I., H.Y., T.A., K.K., S.Y., and S.W. approved final version of manuscript.

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A phase 1and pharmacological trial of OPB-31121, a STAT3 inhibitor, in patients with advanced hepatocellular carcinoma

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Running title: Safety, PK, and efficacy of OPB-31121 in HCC

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Abstract

Aim: To evaluate the safety, pharmacokinetics, and antitumor activity of OPB-31121, a signal transducer and activator of transcription-3 inhibitor, in patients with advanced hepatocellular carcinoma (HCC).

Methods: HCC patients with Child–Pugh A or B who progressed on or were intolerant to sorafenib were eligible for this Phase 1 trial. We used a standard 3+3 dose-escalation design with a 28-day cycle at dose levels of 50, 100, 200, and 400 mg/day. Tumor responses were assessed using the modified Response Evaluation Criteria In Solid Tumors.

Results: Twenty-four patients were enrolled, of which 23 received OPB-31121 (20 males; median age: 65 years). The most common adverse drug reactions were nausea (87.0%), vomiting (82.6%), diarrhea (69.6%), fatigue/malaise (52.2%), anorexia (47.8%), and peripheral sensory neuropathy (26.1%). The recommended dose for OPB-31121 was determined to be 200 mg. Six patients had stable disease for ≥8 weeks, resulting in disease control rates of 25.0–42.9%. In the 200-mg dose cohort, 3/7 patients had stable disease and a median time to progression of 61.0 days. The maximum concentration and area under the plasma concentration–time curve of OPB-31121 were dose-proportional.

Conclusions: OPB-31121 demonstrated insufficient antitumor activity for HCC. Furthermore, peripheral nervous system-related toxicities may negatively affect long-term administration of OPB-31121. Therefore, it was deemed difficult to continue the clinical development of

OPB-31121 for treating advanced HCC and further investigation is expected in the agent with favorable profile in this category.

Key words: advanced hepatocellular carcinoma, dose escalation, OPB-31121, Phase 1 trial, recommended dose, STAT3 inhibitor

Clinical trial registration: Clinicaltrials.gov, NCT01406574

INTRODUCTION

Liver cancer is the fifth highest cause of cancer-related death following lung cancer, gastric cancer, and colorectal cancer.[1] Like lung cancer, biliary cancer and pancreatic cancer, liver cancer is a solid tumor with a low survival rate.[2] The most common type of liver cancer is hepatocellular carcinoma (HCC). Although systemic chemotherapy with sorafenib has been established as a first-line therapy for unresectable HCC,[3,4] there are currently no treatment options for second-line therapy.

HCC often progresses to an irreversible phase.[5–7] In addition, the effects of sorafenib on tumor shrinkage (complete response [CR] and partial response [PR]) are often discouraging, and a large proportion of patients stop administration because of adverse drug reactions (ADRs). Therefore, new therapeutic agents that are safe, effective, and usable for extended periods are in high demand.

Signal transducer and activator of transcription-3 (STAT3) is a transcription factor activated by a series of tyrosine kinases.[8] Upon activation, STAT3 forms homo- or heterodimers, which translocate to the nucleus to regulate gene expression. STAT3 is a promising candidate target for the development of anticancer therapies because it is constitutively activated in various human malignancies.[9-11] Of note, activated STAT3 promotes carcinogenesis through signal transduction and immune evasion, and is expressed in the majority of HCCs with poor prognosis.[9,10,12–14]

OPB-31121 is a novel, orally administered STAT3 inhibitor with potent antitumor effects against various human liver cancer cell lines.[15,16] In a preclinical, antiproliferation study using liver cancer cell lines, OPB-31121 inhibited the proliferation of cell lines with median inhibitory concentrations (IC_{50}) of 8.16–162.00 nM. In HepG2 cells stimulated with interleukin-6, OPB-31121 inhibited intracellular STAT3 phosphorylation at Tyr705, and decreased the nuclear level of dimeric phosphorylated STAT3 in a dose-dependent manner with an IC_{50} of 12.3 nM. Furthermore, an *in vivo* study in which HepG2 cells were subcutaneously inoculated into severe combined immunodeficiency (SCID) mice showed that orally administered OPB-31121 significantly suppressed tumor volume. Based on these findings, OPB-31121 was determined to have potential as a therapeutic drug with a novel mode of action against hematopoietic and solid tumors, including HCC. Several phase 1 clinical trials for OPB-31121 targeting advanced solid tumors and hematological malignancies were conducted in Asian countries and the United States. This time, we performed a multicenter Phase 1 study to evaluate the safety, recommended dose (RD), pharmacokinetics (PK), and antitumor activity of OPB-31121 in patients with advanced HCC.

METHODS

Ethics

This clinical trial was conducted in compliance with the ethical principles of the Declaration of Helsinki. The institutional review board at each study site verified the appropriateness of the

trial from ethical, scientific, and medical perspectives. Before the screening, the investigators gave each patient a full explanation of the study and the patients provided written informed consent.

Study design

We performed a multicenter, Phase 1, open-label, non-randomized, dose-escalation trial to evaluate the safety and RD of OPB-31121 administered orally once daily for 4 weeks (28 days) in patients with advanced HCC. The PK characteristics of OPB-31121 and its metabolite were examined after single and multiple doses of OPB-31121. In addition, we examined the antitumor effects of OPB-31121 after once daily administration for 4 weeks (28 days), as well as the safety and efficacy of long-term administration of OPB-31121 from Week 5 (Day 32) onwards.

OPB-31121 was administered on Day 1 and from Day 4 to Day 31 but not on Days 2 or 3, when blood samples were collected for PK analyses. A standard 3+3 dose-escalation design was used.

Patients

Twenty-four patients participated in this study across four study sites. Twenty-three patients were actually administered OPB-31121 and were included in the analyses. Patients who met the following criteria were enrolled: histopathological or clinical diagnosis of HCC; refractory

disease on, intolerance to, or ineligible for treatment with sorafenib, or absence of appropriate treatment methods; Child–Pugh class A or B; capable of oral ingestion; age 20–79 years; and Eastern Cooperative Oncology Group performance status (ECOG PS) of 0–2.

Safety assessments

The details and severities of adverse events (AEs) were evaluated in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. Dose-limiting toxicities (DLT) were defined as AEs for which a causal relationship with OPB-31121 could not be ruled out and that fell under any of the following categories: neutrophil count decrease of Grade 4 that persisted for ≥8 days, or Grade 3 or 4 febrile neutropenia, or infection concurrent with neutrophil count decrease; platelet count decrease of Grade 4 or platelet count decrease of Grade 3 that persisted for ≥8 days; Grade 3 or 4 nausea, vomiting, or diarrhea that occurred despite the use of anti-emetic or anti-diarrheal agents; Grade ≥3 AEs other than the AEs described above; AEs requiring interruption of OPB-31121 administration for a period of ≥8 consecutive days; and the same AEs that caused OPB-31121 administration to be interrupted twice. The RD was defined as the highest doses at which DLT occurred at an incidence of <30%, while considering the safety and efficacy of the results.

Efficacy assessments

Tumor responses were assessed by investigator in each site using the modified Response Evaluation Criteria In Solid Tumors.[17] Hepatic lesions were assessed using dynamic computed tomography or dynamic magnetic resonance imaging. Lesions were continuously scanned with a slice thickness of ≤5 mm. Time to progression was assessed by determining the length of time from the day on which OPB-31121 was started to the day of assessment as the overall response of progressive disease (PD). The tumor markers α-fetoprotein (AFP), AFP-L3, and protein induced by vitamin K absence-II were measured.

PK assessments

Blood samples were collected at 0 (predose), 1, 2, 4, 6, 8, 10, 24, 34, 48, 58, and 72 h on Day 1, before each dose on Days 11, 18, 25, and 32, and every 4 weeks after Day 32 (Days 60, 88, and every 4 weeks afterwards). The plasma concentrations of OPB-31121 and its major active metabolite were determined at a bioanalytical laboratory using a locally established method.

Statistical methods

PK parameters were calculated using the non-compartmental method. Descriptive statistics (number of patients, arithmetic mean, standard deviation, coefficient of variation, geometric mean, minimum, median, and maximum) were calculated for each parameter and plasma drug/metabolite concentration at each time point. The number of patients who experienced AEs/ADRs after OPB-31121 administration and the incidence of AEs/ADRs were summarized

for all patients combined and for each dose cohort. The *n* (%) of patients for each ECOG PS class and overall response were summarized for all patients combined and for each dose cohort. Kaplan–Meier survival curves were plotted to estimate the time to progression in the total cohort and in the individual dose cohorts.

RESULTS

Patient disposition and demographics

Of 24 patients enrolled in the study, 23 were treated with OPB-31121 (safety population). Of the 23 treated patients, 20 were male and their ages ranged from 46 to 79 years (median: 65 years) (Table 1). The ECOG PS was 0 and 1 in 16 and 7 patients, respectively. At baseline, the primary disease was stage II in one patient (4.3%), stage III in four patients (17.4%), and stage IVB in 18 patients (78.3%). The Child–Pugh class was A in 22 patients (95.7%) and B in one patient (4.3%). All 23 patients had previously received systemic chemotherapy with a median of two regimens, and 22 patients (95.7%) had previously received sorafenib.

Twenty patients of the safety population who received OPB-31121 were included in the evaluation of DLT, with six, three, six, and five patients in the 50-, 100-, 200-, and 400- dose cohorts, respectively. Three patients were excluded from the DLT evaluation; one patient had early withdrawn due to progression of primary disease in 50 mg cohort, one patient withdrew consent in 100 mg cohort, and one patient had withdrawn due to occurrence of an AE that made

continuation of OPB-31121 administration difficult. The mean ± standard deviation duration of treatment with OPB-31121 was 48.7 ± 44.7 days in all patients combined, and was 50.4 ± 27.5, 44.5 ± 33.1, 73.3 ± 65.0, and 15.0 ± 13.9 days, in the 50-, 100-, 200-, and 400-mg dose cohorts, respectively.

Safety evaluation

In this study, the OPB-31121 was initially administered at a starting dose of 400 mg/day in five patients; this dose is one dose-level lower than the RD of 600 mg for patients with advanced solid tumors determined in a previous Phase 1 study in Korea[18]. Further registration at this dose was terminated because four of the five patients developed DLTs: Grade 3 diarrhea in one patient, Grade 3 nausea in one patient, and Grade 3 peripheral sensory neuropathy and gait disturbance in two patients (both events occurred in both patients). After considering the incidence of these AEs and because the patients enrolled in this study had HCC and were likely to have impaired hepatic function, we decided to lower the dose of OPB-31121. Accordingly, the protocol was amended and subsequent patients were initially administered with OPB-31121 at 50 mg/day as the starting dose.

All 23 patients administered OPB-31121 reported ≥1 treatment-emergent AEs during the study period. ADRs were reported in 100% of patients (seven, four, seven, and five patients in the 50-, 100-, 200-, and 400-mg dose cohorts, respectively). Table 2 lists the ADRs that

occurred in >10% of patients. Most ADRs were of Grade 1 or 2 in severity. ADRs that occurred in ≥20% of patients included nausea (20 patients, 87.0%), vomiting (19 patients, 82.6%), diarrhea (16 patients, 69.6%), fatigue/malaise (12 patients, 52.2%), anorexia (11 patients, 47.8%), and peripheral sensory neuropathy (six patients, 26.1%). No deaths occurred during the study. Peripheral sensory neuropathy and gait were reported in one patient, 14 days after starting OPB-31121. Both events were determined severe and were deemed causally related to OPB-31121.

During the entire study period, four patients (one and three patients in the 200- and 400-mg dose cohorts, respectively), withdrew because of ADRs, which included diarrhea (two patients), nausea (two patients), vomiting (one patient), gait disturbance (one patient), and peripheral sensory neuropathy (one patient).

Grade 2 nausea was reported as a DLT in one of seven patients in the 200-mg dose cohort. In the 400-mg dose cohort, four patients developed the following DLTs: Grade 3 diarrhea in one patient, Grade 3 nausea in one patient, and Grade 3 peripheral sensory neuropathy and gait disturbance in two patients. Based on these results, OPB-31121 was considered tolerable at doses of up to 200 mg, but the maximum tolerated dose was not determined and further dose studies were not performed. Therefore, the RD of OPB-31121 for treating HCC was determined to be 200 mg/day.

Efficacy evaluation

A best overall response of CR or PR was not observed in any of the patients who received OPB-31121 (Table 3). Six patients maintained stable disease (SD) for ≥8 weeks, resulting in an overall disease control rate of 26.1% (28.6%, 25.0%, 42.9%, and 0% in the 50-, 100-, 200-, and 400-mg dose cohorts, respectively). A reduction in the size of viable (contrast enhancement in the arterial phase) target lesions was observed in the 50-mg (two patients), 100-mg (one patient), and 200-mg (one patient) dose cohorts (Fig. 1). The median time to progression was 61.0 days in all patients, and was 60.0, 61.0, and 89.0 days in the 50-, 100-, and 200-mg dose cohorts, respectively (Table 4, Fig. 2).

None of the patients reported an AFP response (best on-study decrease from baseline) of >50%. One patient in the 100-mg dose cohort experienced an AFP-L3 response of >50%. Five patients (50- (two patients), 100- (one patient), and 200-(two patients) mg dose cohorts) experienced a protein induced by vitamin K absence-II response of >50%.

PK of OPB-31121 and its major active metabolite

We determined the plasma concentrations of OPB-31121 and its major active metabolite after single and repeated doses of OPB-31121 (Fig. 3). The mean plasma concentration of the major active metabolite was greater than that of OPB-31121. The mean plasma concentrations of OPB-31121 and its major active metabolite increased rapidly, reaching a peak level at 1.9–10.0 h after each dose, and then decreased gradually in a dose-dependent manner.

The PK characteristics of single doses of 50, 100, 200, and 400 mg OPB-31121 are shown in Table 5. The mean ± standard deviation maximum concentration (C_{max}) of OPB-31121 after single oral doses of 50, 100, 200, and 400 mg were 2.5 ± 1.3, 11.7 ± 13.4, 15.1 ± 9.3, and 19.0 ± 12.2 ng/mL, respectively. OPB-31121 was rapidly absorbed with median times to the maximum concentration of 3.9, 6.0, 6.0, and 9.9 h for 50, 100, 200, and 400 mg, respectively. The mean ± standard deviation of the area under the concentration–time curve from time 0 to infinity (AUC_{inf}) was 36.0 ± 16.4, 99.4 ± 120.0, 156.0 ± 107.0, and 437.0 ± 358.0 ng·h/mL for 50, 100, 200, and 400 mg, respectively. The mean ± standard deviation terminal half-life was 64.8 ± 53.1, 25.4 ± 13.7, 34.8 ± 34.8, and 31.9 ± 18.9 h for 50, 100, 200, and 400 mg, respectively. The coefficient of variation for the C_{max} and AUC_{inf} of OPB-31121 ranged from 52.6% to 114.1% and from 45.5% to 121.3%, respectively.

The accumulation of OPB-31121 and its major active metabolite was observed after 28 days of administration. Exposure (i.e. AUC_{inf} and C_{max}) to OPB-31121 and its major active metabolite tended to increase with increasing dose, but a possible relationship between C_{max} and the dose of OPB-31121 could not be determined because of the large inter-patient variability.

DISCUSSION

In the present study, we assessed the safety, tolerability, and efficacy of orally administered

OPB-31121 in patients with advanced HCC treated with doses ranging from 50 mg/day to 400 mg/day for 4 weeks.

It has been reported that STAT3 is consecutively activated in many solid tumors, including HCC, and in hematopoietic tumors.[8–11] Because OPB-31121 is a strong inhibitor of STAT3, it was considered a promising target molecule for the treatment of HCC.[15] Additionally, the results of preclinical studies in the liver fibrosis and hepatitis model indicated that OPB-31121 has activities to improve each marker of fibrosis, hepatitis and liver function. Thus, OPB-31121 was also considered likely to be effective for Child–Pugh B patients with high unmet needs, and therefore Child-Pugh B is included in this HCC-targeted study.

OPB-31121 was initially administered at a starting dose of 400 mg/day, one dose-level lower than the RD of 600 mg determined in patients with advanced solid tumors in a previous Phase 1 study in Korea[18]. In the Korean Phase 1 study, DLT was reported: Grade 3 vomiting in the 600-mg dose cohort, Grade 3 diarrhea and vomiting in the 800-mg dose cohort. Based on these results, OPB-31121 was considered tolerable at doses of up to 600 mg and the maximum tolerated dose, and RD for solid tumors was determined to be 600 mg/day.

However, the present results showed that this dose of OPB-31121 was poorly tolerated in patients with advanced HCC. Regarding the different tolerability for OPB-31121 between HCC and other solid tumors, although it is possible that the PK characteristics of OPB-31121 were affected in these patients because preclinical data indicated that OPB-31121 is

metabolized by hepatic drug-metabolizing enzymes mainly cytochrome P450 (CYP) 3A4 and 2C9 and patients with advanced HCC often show impaired liver function, there is no significant difference found in the concentration or proportion of OPB-31121 and metabolites in comparison between the PK results of this study and the Korean Phase 1 study. Accordingly, we amended the study protocol and subsequent patients were initially given a starting dose of 50 mg/day. We then found that OPB-31121 is relatively well tolerated at the dose of up to 200 mg. Therefore, OPB-31121 appeared to be safe and well tolerated in patients with advanced HCC at doses of 50–200 mg/day.

Regarding the efficacy of OPB-31121, none of the patients in this study had an objective antitumor response. In a preclinical, *in vivo* study, the antitumor effects of OPB-31121 were evaluated using SCID mice that were subcutaneously inoculated with the HuH-7 and HepG2 liver cancer cell lines. OPB-31121 was orally administered for 14 consecutive days. Tumor growth was significantly inhibited at doses of ≥60 mg/kg/day against HuH-7 cells and at doses of ≥10 mg/kg/day against HepG2 cells. Complete tumor stasis was observed at a dose of 300 mg/kg/day for HuH-7 cells. Tumor regression was observed at doses of ≥100 mg/kg/day against HepG2 cells. Although the doses used in this study and in the preclinical study appear to be similar, there were no responses (CR or PR) with an apparent reduction in tumor size in this study. These insignificant results are likely due to relatively low plasma OPB-31121 concentration, compared with that found effective in mice: i.e. the mean C_{max} of OPB-31121