TOHABcl-xL using Lipofectin (Invitrogen). The cells were cultured with DMEM containing 1.1 μg/mL zeocin, and zeocin-resistant clones were isolated. After examination of HA–Bcl-xL induction by doxycycline, two clones (Hela–Bcl-xL$^{Tet\text{-}on}$ clone A, clone B) were established and used for further experiments.

Mice. Conditional Bcl-xL knockout mice (*bcl-x$^{flox/flox}$ Alb-Cre* [albumin/cre recombinase]) and Mcl-1 knockout mice (*mcl-1$^{flox/flox}$ Alb-Cre*) were previously described.[15] Balb/c nude mice (CAnN.Cg-Foxn1^{nu}/CrlCrlj) were purchased from Charles River Laboratories (Yokohama, Japan). They were maintained in a specific pathogen–free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. The *in vitro* apoptosis assay, measurement of caspase-3/7 activity, and the water-soluble tetrazolium salt (WST) assay, were described previously.[16] The *in vivo* apoptosis assay, measurement of serum alanine aminotransferase (ALT) level, and caspase-3/7 activity and histological analyses were also previously described.[15]

Western Blot Analysis. Whole-cell extracts from cultured cells or tissues were prepared and subjected to western blot. For immunodetection, the following antibodies were used: anti–Bcl-xL antibody and anti-human Mcl-1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse Mcl-1 antibody from Rockland (Gilbertsville, PA); anti-Bid antibody, anti-Bax antibody, and anti-cleaved caspase-3 antibody from Cell Signaling Technology (Beverly, MA); anti-Bak antibody from Millipore (Billerica, MA); anti-Bim antibody from Assay Design (Ann Arbor, MI); anti-ubiquitin-specific peptidase 9, X-linked (USP9X) antibody from Abnova (Taipei, Taiwan); and anti–beta actin antibody from Sigma-Aldrich (St. Louis, MO) or Cell Signaling Technology.

Xenograft Tumor. To produce a xenograft tumor, 3×10^6 to 5×10^6 Hela–Bcl-xL$^{Tet\text{-}on}$ clone A or Huh7 cells were subcutaneously injected to Balb/c nude mice. For induction of HA–Bcl-xL, the mice that were injected with Hela–Bcl-xL$^{Tet\text{-}on}$ clone A cells were fed with water containing 100 μg/mL doxycycline. For anticancer therapy, ABT-737 was administered as described.[17] Sorafenib tablets were crushed and orally administered with water containing 12.5% Cremophor EL (Sigma-Aldrich) and 12.5% ethanol. We estimated the volume of the xenograft tumor using the following formula: tumor volume $= \pi/6 \times (\text{major axis}) \times (\text{minor axis})^2$.

Small RNA Interference. Hepatoma cell lines were transfected with Stealth select RNAi (set of three oligonucleotides, Invitrogen) RNA interference (RNAi) directed against Mcl-1 or USP9X. A Stealth RNAi negative control kit (set of three oligonucleotides, Invitrogen) was used as a control for sequence-independent effects following Stealth RNAi delivery. The transfections were carried out using Lipofectamine RNAiMAX (Invitrogen) according to the reverse transfection protocol.

Real-Time Reverse-Transcription Polymerase Chain Reaction. Real-time reverse-transcription PCR (RT-PCR) was performed as previously described.[15] Mcl-1 messenger RNA (mRNA) expressions were measured using TaqMan Gene Expression Assays (Assay ID: Hs03043899_m1) and were corrected with the quantified expression level of beta actin mRNA measured using TaqMan Gene Expression Assays (Assay ID: Hs99999903_m1).

Statistical Analysis. Data are presented as mean ± standard deviation. Differences between two groups were determined using the Student *t* test for unpaired observations unless otherwise noted. Multiple comparisons were performed by analysis of variance followed by Scheffe post hoc correction. $P < 0.05$ was considered statistically significant.

Results

Bcl-xL Overexpression Is a Molecular Mechanism of Rapid* In Vivo *Tumor Growth. Research has shown that Bcl-xL overexpression confers resistance to apoptosis in a variety of tumor cells. To examine its impact on tumor growth *in vivo*, we generated the Hela–Bcl-xL$^{Tet\text{-}on}$ cell line which expresses the modified tetracycline repressor molecule (rtTA) and Bcl-xL under control of tetracycline-responsive cis-elements. We chose Hela cells as a model because they expressed a relatively small amount of Bcl-xL in comparison with human hepatoma cells including Huh7, Hep3B, and HepG2 (Fig. 1A). Tetracycline analogue doxycycline treatment efficiently induced Bcl-xL in Hela–Bcl-xL$^{Tet\text{-}on}$ cells as expected (Fig. 1B) and conferred resistance to apoptosis as evidenced by significantly lower levels of caspase-3/7 activity in culture (Fig. 1C), although it did not have a significant effect on cell growth assay (Fig. 1D). Next, we subcutaneously injected Hela–Bcl-xL$^{Tet\text{-}on}$ cells into nude mice. When subcutaneous tumors grew to approximately 1 cm, the mice were randomly assigned to two groups: a doxycycline-drinking group and a water-drinking group. Subcutaneous tumors grew rapidly in the doxycycline-drinking group compared with the water-drinking group (Fig. 1E). As expected, xenograft tumors displayed higher levels of Bcl-xL expression than those in the water drinking group (Fig.

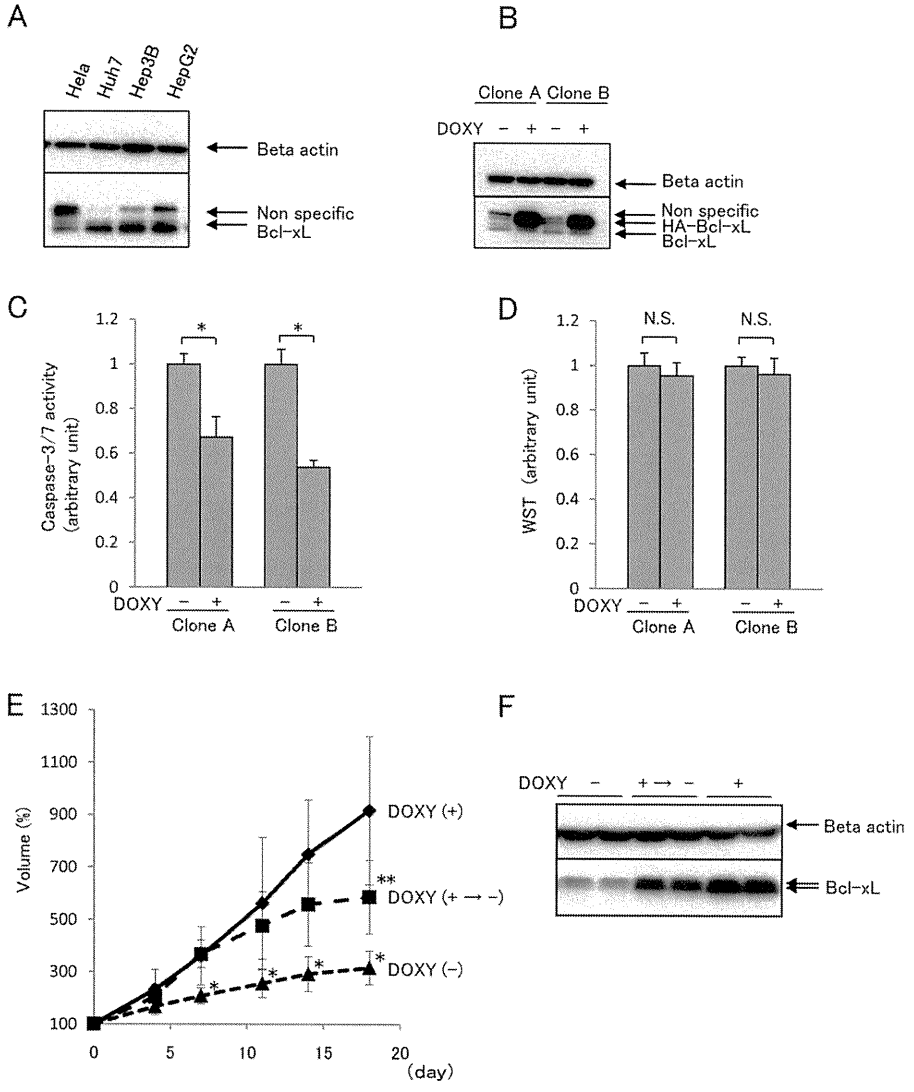

Fig. 1. Bcl-xL overexpression in vitro and in vivo by the Tet-on system. (A) Western blot analysis of Bcl-xL in human hepatoma cells and Hela cells. (B,C,D) Bcl-xL overexpression in vitro. Two independent clones of Hela-Bcl-xLTet-on cells were cultured with or without 1 μM doxycycline (DOXY) for 24 hours. (B) Western blot analysis of Bcl-xL. (C,D) Caspase-3/7 activity in culture supernatant and cell viability by the WST assay (N = 4). *P < 0.05. N.S., not significant. (E,F) Bcl-xL overexpression in vivo. Nude mice carrying xenograft tumors of Hela-Bcl-xL$^{Tet\text{-}on}$ clone A were randomly assigned to water given with or without 100 mg/mL doxycycline for 7 days. After 7 days, the mice of the doxycycline-drinking group were randomly assigned to two groups: one in which doxycycline drinking was continued and the other in which water was given instead (N = 5 or 6 per group). (E) The percentage of xenograft tumor volume. (F) Western blot analysis of xenograft tumor for the expression of Bcl-xL. *P < 0.05 versus the other two groups. **P < 0.05 versus the DOXY (+) group.

1F). In addition, switching the mice to water drinking at 7 days after doxycycline drinking decreased Bcl-xL expression and retarded tumor growth compared with continuing doxycycline drinking (DOXY + → − versus DOXY +, respectively; Fig. 1F). These results indicate that Bcl-xL overexpression was directly linked to rapid growth of tumors *in vivo* and suggest that Bcl-xL may be a therapeutic target for inhibiting tumor progression, especially for Bcl-xL–overexpressing tumors.

Bcl-xL Inhibitor ABT-737 Dose-Dependently Induces Apoptosis of Hepatoma Cells but Fails to Suppress Tumor Growth in a Xenograft Model. To examine the impact of pharmaceutical inactivation of Bcl-xL overexpressed in hepatoma cells, Huh7 and Hep3B hepatoma cells were cultured with escalating doses of ABT-737. ABT-737 dose-dependently activated caspase-3/7 in hepatoma cells and suppressed tumor growth at high dosages (Fig. 2A,B). To examine the *in vivo* effect of ABT-737, nude mice were subcutaneously injected with Huh7 cells to generate xenograft tumors and were randomly assigned into two groups when the diameter of the subcutaneous tumors reached approximately 1 cm: ABT-737 injection group and vehicle injection group. Administration of ABT-737 at 50 mg/kg body weight/day for 7 days failed to suppress tumor growth (Fig. 2C). In contrast, mild ALT elevation and thrombocytopenia were observed in ABT-737–injected mice (Fig. 2D). Previous research has demonstrated that both are observed in mice after ABT-737 administration,[17,18] confirming that the dose injected in the present experiment is sufficient for inducing a biological effect of ABT-737 *in vivo*.

ABT-737 Posttranscriptionally Increases Expression of Mcl-1. To examine the mechanisms underlying relative resistance of hepatoma cells to ABT-737, we examined the expression profile of the Bcl-2 family

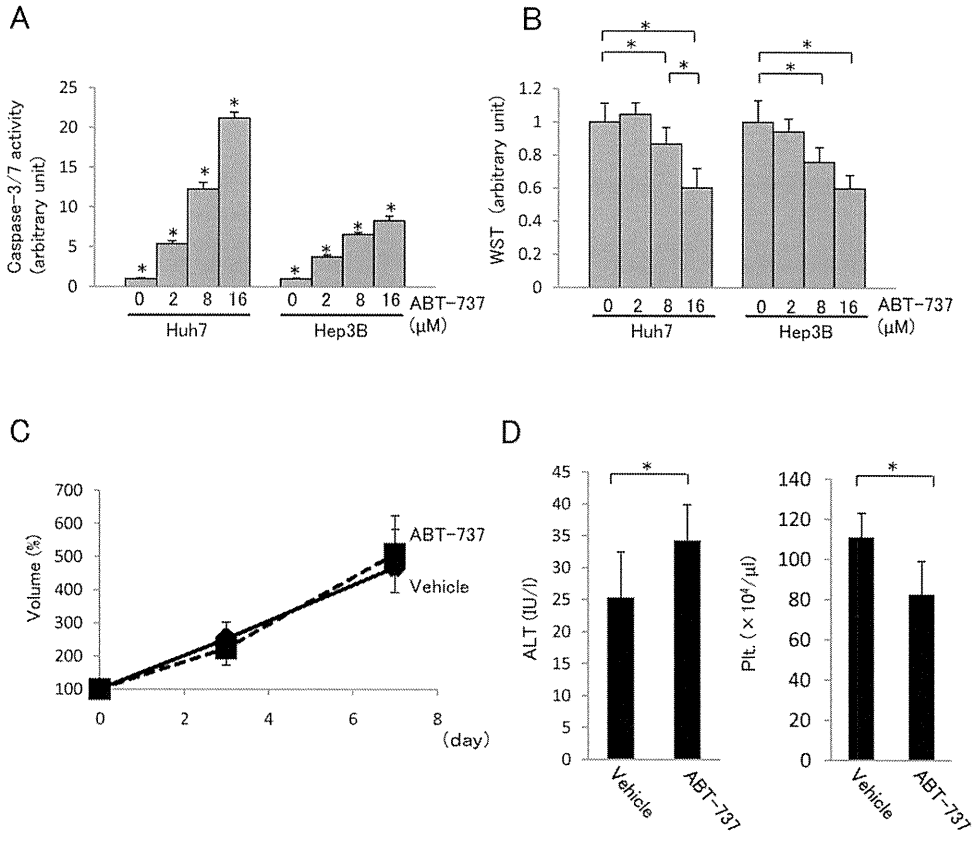

Fig. 2. Apoptosis and growth of hepatoma cells treated with ABT-737 *in vitro* and *in vivo*. (A,B) Huh7 and Hep3B cells were treated with indicated doses of ABT-737 for 24 hours (N = 4). (A) Caspase-3/7 activity of culture supernatant. *$P < 0.05$ versus all other groups. (B) Cell viability by the WST assay. *$P < 0.05$. (C,D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered 50 mg/kg ABT-737 or vehicle daily for 7 days. (N = 9 for each group.) (C) The percentage of xenograft tumor volume. (D) Serum ALT levels and circulating platelet count. *$P < 0.05$.

proteins. Administration of ABT-737 did not affect expression of proapoptotic multidomain members Bak and Bax or BH3-only proteins Bid and Bim in cultured hepatoma cell lines Huh7 and Hep3B (Fig. 3A). Although the slower migrating species of Bim at 4 hours was increased, this change disappeared at 24 hours. In agreement with previous research,[19,20] Mcl-1 was constitutively expressed in hepatoma cells. Of importance is the finding that the levels of Mcl-1 expression were rapidly increased as early as 4 hours after ABT-737 exposure. Expression of mcl-1 mRNA did not differ between ABT-737–treated cells and vehicle-treated cells (Fig. 3B), suggesting the involvement of a posttranscriptional mechanism. Because Mcl-1 is a rapid-turnover protein, the levels of Mcl-1 may be regulated by protein degradation.[21] To examine this, we treated hepatoma cells with cycloheximide, a well-established protein synthesis inhibitor, in the presence or absence of ABT-737. Cycloheximide-induced rapid decline in Mcl-1 expression was substantially blocked in the presence of ABT-737, suggesting that ABT-737 significantly delays degradation and prolongs the stability of Mcl-1 (Fig. 3C). Recently, it was reported that the deubiquitinase USP9X is involved in stabilization of Mcl-1.[22] In this study, western blot analysis revealed that the levels of USP9X expression were not changed in Huh7 and Hep3B with ABT-737 (Supporting Fig. 1A). Furthermore, USP9X down-regulation by small interfering RNA (siRNA) could not block the Mcl-1 up-regulation induced by ABT-737 (Supporting Fig. 1B).These results suggest that USP9X was not involved in Mcl-1 up-regulation induced by ABT-737. Of importance is the finding that Mcl-1 expression was also up-regulated after administration of ABT-737 in our xenograft model (Fig. 3D). Because Mcl-1 is not a target of ABT-737, relative resistance to ABT-737 of hepatoma cells may be due, at least in part, to posttranscriptional induction of Mcl-1.

Mcl-1 Knockdown Sensitizes Hepatoma Cells to ABT-737. To examine the impact of Mcl-1 induction in hepatoma cell resistance to ABT-737, we silenced Mcl-1 expression through use of three different siRNAs. Western blot analysis revealed that Mcl-1 siRNA2 and siRNA3 completely knocked down Mcl-1 expression in Hep3B cells, whereas Mcl-1 siRNA1 did so only partially (Fig. 4A). Mcl-1 knockdown or a medium dose of ABT-737 (4 μM) modestly activated caspase-3/7 in Hep3B cells, whereas both substantially activated caspase-3/7 (Fig. 4B). In addition, Mcl-1 knockdown or ABT-737 alone failed to suppress the growth of tumor cells but caused significant suppression when used together (Fig. 4C). Caspase-3 activation was also confirmed by western blots (Fig. 4A). It should be noted that Mcl-1 siRNA1 reduced Mcl-1

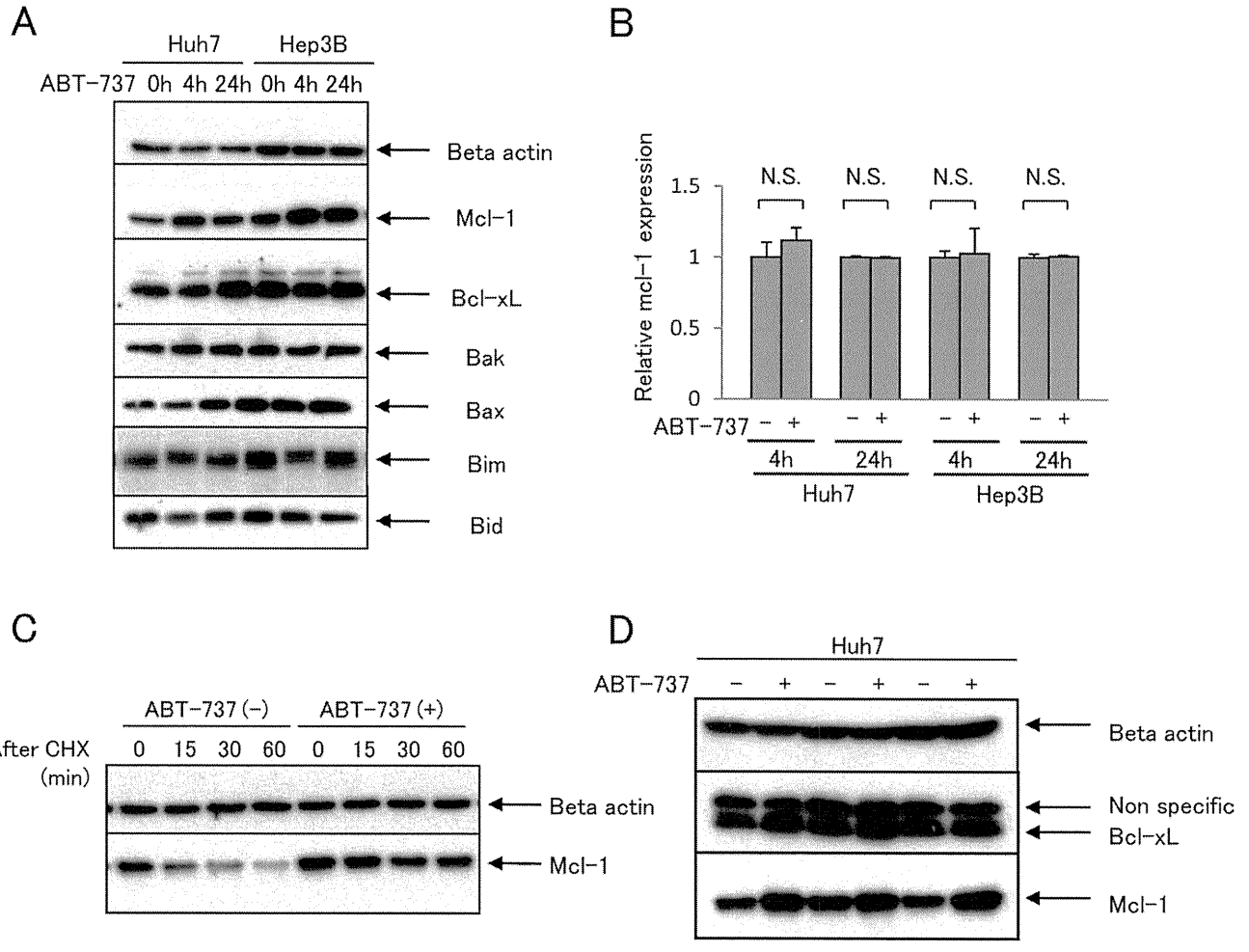

Fig. 3. Up-regulation of Mcl-1 in human hepatoma cells by ABT-737 in vitro and in vivo. (A,B) Huh7 and Hep3B cells were cultured with 4 μM ABT-737 for the indicated times. (A) Western blot analysis for the expression of Bcl-2 family proteins. (B) Real-time RT-PCR analysis for mcl-1 mRNA expression (N = 6). The levels were normalized to each group without ABT-737. N.S., not significant. (C) Huh7 cells were cultured with or without 4 μM ABT-737 for 4 hours and then further treated with 1 mM cycloheximide (CHX) for the indicated times. Western blot analysis for Mcl-1 expression. (D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered 50 mg/kg ABT-737 or vehicle daily for 7 days. Western blot analysis of xenograft tumor after 7-day treatment for the expression of Bcl-2 family proteins.

expression in ABT-737-treated cells to levels similar to those of untreated cells (Fig. 4A). Even in this case, mcl-1 knockdown enhanced caspase activation and growth suppression of Hep3B cells induced by ABT-737. Similar data were obtained with another hepatoma cell line, Huh7 (Fig. 4A and Supporting Fig. 2). These results indicate that Mcl-1 up-regulation induced by ABT-737 is involved in the resistance of hepatoma cells to ABT-737 and suggest that combination therapy with ABT-737 and Mcl-1 inhibitor may be predictably effective *in vivo*.

We previously reported that, similar to Bcl-xL, Mcl-1 plays an important role in apoptosis resistance of normal hepatocytes. In addition, knockdown of both Mcl-1 and Bcl-xL led to impaired liver development during embryogenesis.[15] Thus, the concern arises that simultaneous inactivation of both Bcl-xL and Mcl-1 may cause severe liver injury in adults. To examine this possibility, we injected ABT-737 into hepatocyte-specific Mcl-1 knockout mice or wild-type littermates. ABT-737 injection into wild-type mice led to mild liver apoptosis, which is consistent with our previous finding,[17] whereas injection into Mcl-1 knockout mice induced massive liver apoptosis leading to severe liver injury, and most animals died within 1 day (Fig. 4D,E). This result indicates that inactivation of both Bcl-xL and Mcl-1 may be hazardous and that Mcl-1 inactivation should be done in a tumor-specific manner.

Sorafenib Down-Regulates Mcl-1 Expression in Hepatoma Cells Much More Strongly than in Normal Liver Cells. Previous research has shown that sorafenib down-regulates Mcl-1 expression in hepatoma cells in a mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK)-independent manner.[16,23] In the present study, to examine whether Mcl-1

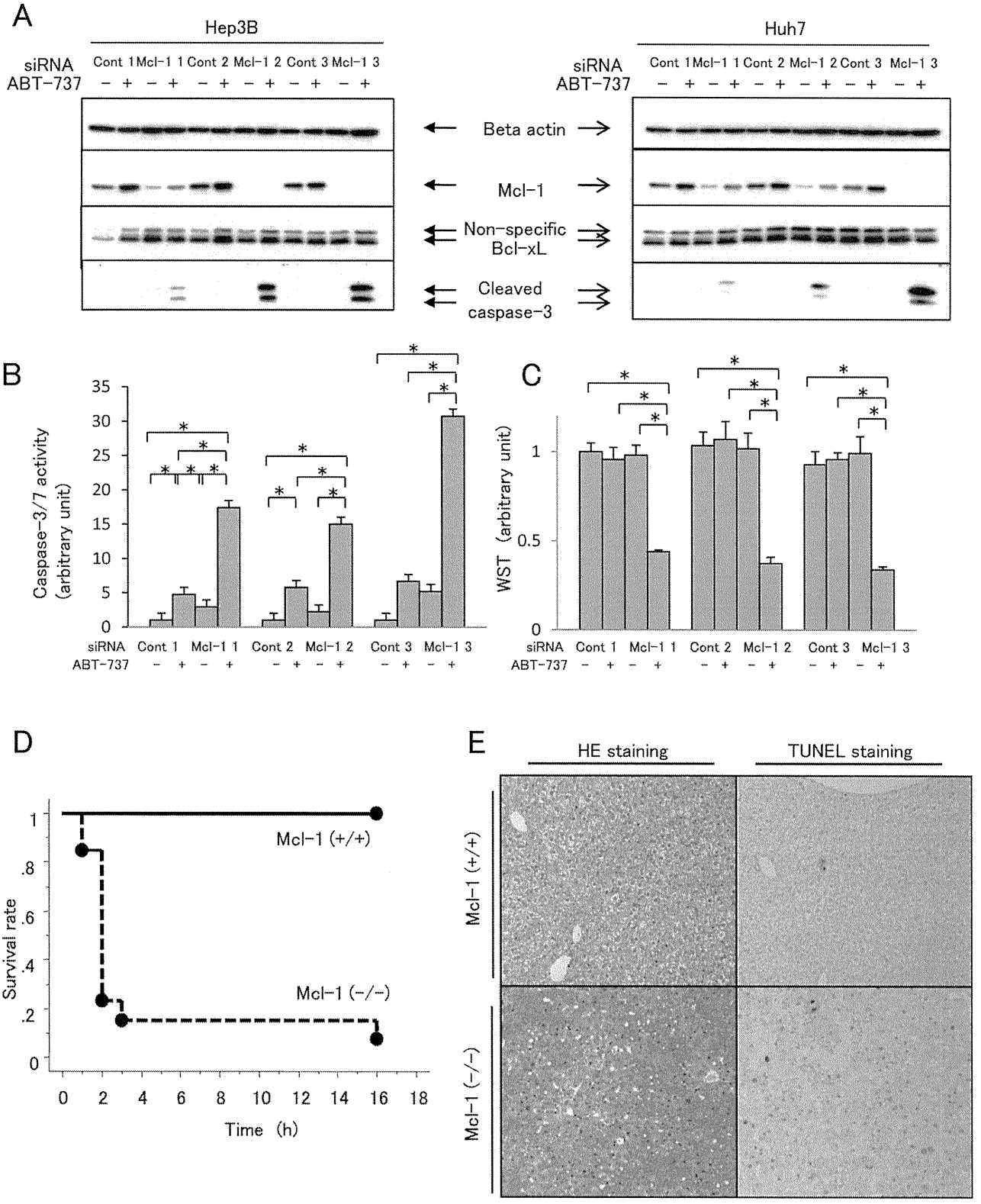

Fig. 4. Effects of ABT-737 under inhibition of Mcl-1 in vitro and in vivo. (A-C) Hep3B and Huh7 cells were transfected with Mcl-1 siRNAs (Mcl-1 1, Mcl-1 2, and Mcl-1 3) or control siRNAs (Cont 1, Cont 2, Cont 3). Forty-eight hours after transfection, they were treated with or without 4 μM ABT-737 for 24 hours (N = 4). (A) Western blot analysis for the expression of Mcl-1, Bcl-xL, and cleaved caspase-3. (B) Caspase-3/7 activities of supernatant in Hep3B culture dishes. *P < 0.05. (C) Cell viability of Hep3B cells by the WST assay. *P < 0.05. (D,E) Wild-type mice (Mcl-1 +/+) and hepatocyte-specific Mcl-1 knockout mice (Mcl-1 −/−) were intraperitoneally administered 50 mg/kg of ABT-737. (D) Survival curve of the mice (N = 13 or 15). (E) Hematoxylin and eosin (HE) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining of the liver sections 16 hours after administration of ABT-737 with wild-type mice and immediately after death with Mcl-1 knockout mice. Representative photographs are shown.

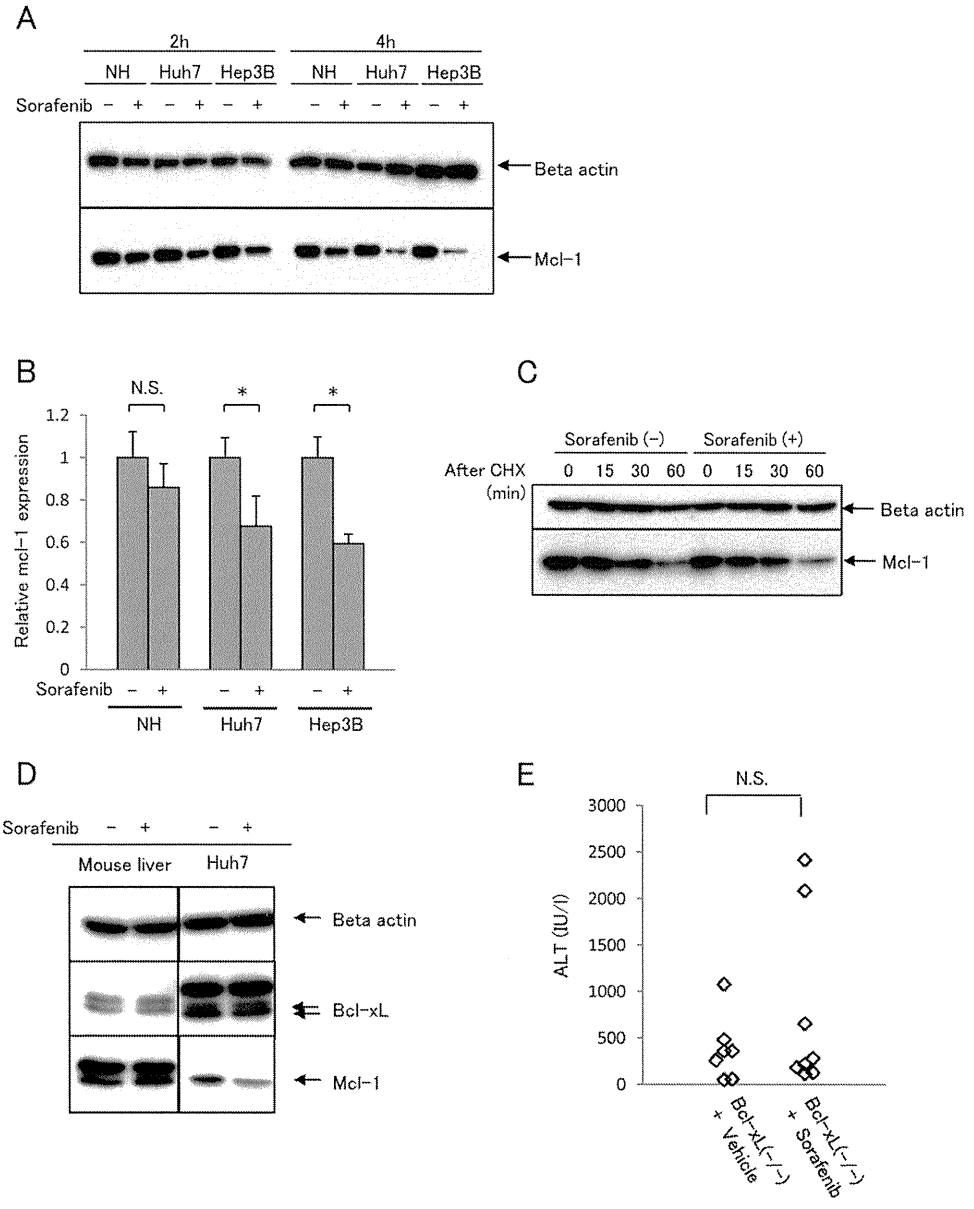

Fig. 5. Effects of sorafenib on the Mcl-1 expression in hepatoma cells and nontransformed cells in vitro and in vivo. (A,B) Normal hepatocytes (NH) and Huh7 and Hep3B cells were treated with 5 μM sorafenib. (A) Western blot analysis for the expression of Mcl-1 after treatment for 2 or 4 hours. (B) Real-time RT-PCR analysis for mcl-1 expression after treatment for 4 hours (N = 6). The levels were normalized to each group without sorafenib. N.S., not significant. *P < 0.05. (C) Huh7 cells were cultured with 1 mM cycloheximide (CHX) with or without 5 μM sorafenib for the indicated times. Western blot analysis for Mcl-1 expression. (D) Nude mice carrying xenograft tumors of Huh7 cells were orally administered 30 mg/kg sorafenib or vehicle daily for 3 days. Western blot analysis for the expression of Bcl-xL and Mcl-1 in the xenograft tumor of Huh7 cells and the liver of mice. (E) Hepatocyte-specific Bcl-xL knockout mice (Bcl-xL −/−) were orally administered daily 30 mg/kg sorafenib or vehicle daily for 3 days. The serum ALT levels are shown (N = 7 or 8). The difference between two groups was determined using Mann-Whitney's U test. N.S., not significant.

suppression of sorafenib is tumor-specific, nontransformed human hepatocytes and hepatoma cell lines were treated with sorafenib. Sorafenib down-regulated Mcl-1 expression in all hepatoma cell lines tested, but had a lesser effect on nontransformed human hepatocytes (Fig. 5A). Sorafenib down-regulated mcl-1 mRNA expression in Huh7 and Hep3B hepatoma cells but not in nontransformed hepatocytes (Fig. 5B). To examine the posttranscriptional effect of sorafenib for Mcl-1 expression, we treated Huh7 cells with cycloheximide in the presence or absence of sorafenib. Cycloheximide-induced decline in Mcl-1 expression was not accelerated by sorafenib (Fig. 5C). This result indicated that, in contrast to the case of ABT-737, sorafenib does not affect the degradation process of Mcl-1.

We also examined Mcl-1 expression in the liver as well as xenograft tumors. Administration of sorafenib significantly suppressed Mcl-1 expression in Huh7 xenograft tumors but not in the liver (Fig. 5D). To examine the safety of sorafenib in the absence of Bcl-xL *in vivo*, we administered sorafenib to hepatocyte-specific Bcl-xL knockout mice. These mice had elevated levels of serum ALT at baseline, as we reported previously,[8] but displayed neither further ALT elevation nor lethal liver failure upon sorafenib administration (Fig. 5E). Taken together, these results indicate that sorafenib did not affect Mcl-1 expression in the liver and therefore did not cause further liver injury even if Bcl-xL was inactivated.

ABT-737 Induced Apoptosis of Hepatoma Cells and Suppressed Growth of Xenograft Tumor with Sorafenib Coadministration. To examine the impact of coadministration of sorafenib and ABT-737 on

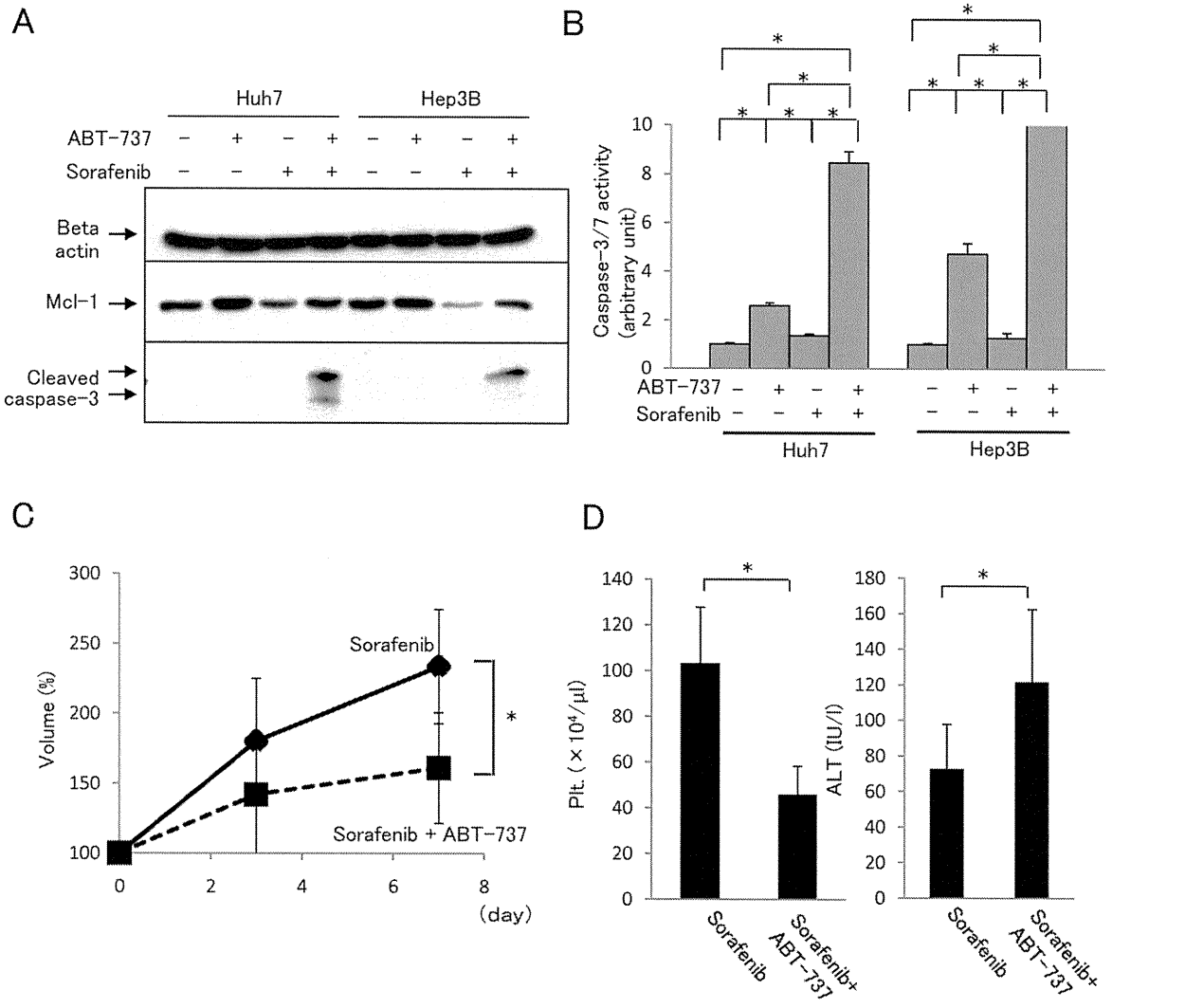

Fig. 6. Effects of ABT-737 with sorafenib treatment in vitro and in vivo. (A,B) Huh7 and Hep3B cells were treated with or without 4 μM ABT-737 together with or without 5 μM sorafenib. (A) Western blot analysis for the expression of Mcl-1 and cleaved caspase-3. (B) Caspase-3/7 activity of culture supernatants. *$P < 0.05$. (C,D) Nude mice carrying xenograft tumors of Huh7 cells were intraperitoneally administered daily 50 mg/kg ABT-737 or vehicle with daily oral administration of 30 mg/kg sorafenib for 7 days ($N = 8$ or 11). (C) The percentage of xenograft tumor volume. (D) Circulating platelet (Plt.) count and serum ALT levels. *$P < 0.05$.

inducing apoptosis, we treated Huh7 and Hep3B hepatoma cells with ABT-737 and/or sorafenib. Although ABT-737 up-regulated Mcl-1 expression in Huh7 and Hep3B cells, sorafenib abolished the Mcl-1 up-regulation induced by ABT-737; the levels of Mcl-1 expression of cells treated with both were similar to those of nontreated cells (Fig. 6A). Sorafenib failed to activate caspase-3/7 in hepatoma cells by itself, but efficiently activated it in the presence of ABT-737 (Fig. 6B). It was also confirmed by efficient cleavage of caspase-3 on western blot analysis (Fig. 6A).

To examine whether ABT-737 has an antitumor effect in the presence of sorafenib, we administered ABT-737 and sorafenib together to nude mice bearing Huh7 xenograft tumors. Although even sorafenib alone significantly suppressed tumor growth compared with the vehicle alone (Supporting Fig. 3), coadministration of ABT-737 and sorafenib led to significant further suppression of tumor growth compared to administration of sorafenib alone (Fig. 6C). Similar to administration of ABT-737 as a single agent, coadministration of sorafenib and ABT-737 also induced mild thrombocytopenia and ALT elevation (Fig. 6D). However, coadministration did not induce further morbidity or mortality in mice, suggesting that this regimen is safe and effective for controlling HCC progression.

Discussion

Tumor cells have two characteristic features: uncontrolled proliferation and apoptosis resistance. Uncontrolled proliferation, driven by activation of a variety

of oncogenes, is directly linked to tumor growth. Apoptosis resistance is believed to be required for the oncogene-induced aberrant proliferation, because without it, tumor cells tend to undergo apoptosis.[24] However, the direct link between apoptosis resistance and growth of solid tumors *in vivo* has not been well studied. Clarifying this point is very important, especially because a very recent study by Weber et al.[25] produced the contradictory finding that aged hepatocyte-specific Mcl-1 knockout mice develop HCC-like lesions, suggesting a link between hepatocarcinogenesis and increased proliferation resulting from increased apoptosis. In the present study, we used conditional expression of Bcl-xL in tumor cells to show that Bcl-xL overexpression, which is frequently found in human HCC, can be directly linked to tumor growth *in vivo*, although it did not promote significant cell growth *in vitro*. Our results suggest that tumor cells encounter a variety of cellular stresses and require antiapoptosis to survive *in vivo* rather than *in vitro*. Thus, we consider antiapoptosis to be an important mechanism for progression of a solid tumor, even if it may not be the case for tumor development as suggested by Weber et al.[25] Our finding also provides proof of the concept that Bcl-xL may be a target for therapy against HCC progression.

In the present study, we showed that, unlike hematopoietic malignancy, hepatoma cells are relatively resistant to ABT-737. Although ABT-737 dose-dependently induced apoptosis in hepatoma cells, a relatively high dose of ABT-737 (more than 8 μM) was required to suppress tumor growth *in vitro*. Importantly, administration of an *in vivo* effective dose of ABT-737 (50 mg/kg) failed to suppress xenograft tumors. We found increased expression of Mcl-1 in cultured hepatoma cells as well as xenograft tumors upon ABT-737 treatment. This may be part of the mechanism of their relative resistance to ABT-737 because hepatoma cells were highly sensitive to this agent if Mcl-1 expression levels were kept constant by an siRNA strategy. Previous articles have reported that Mcl-1 knockdown makes some tumor cells sensitive to ABT-737.[26,27] The present study showed that ABT-737 up-regulation of Mcl-1 rather than Mcl-1 expression itself may be a mechanism of tumor cell resistance to this agent.

A recent study demonstrated that long-term exposure to ABT-737 made initially sensitive lymphoma cell lines resistant to this agent via up-regulation of Mcl-1.[28] In this study, Mcl-1 up-regulation in the ABT-737–resistant lymphoma cells were reported to be mediated by transcriptional up-regulation. In the present study, hepatoma cells showed immediate, posttranscriptional up-regulation of Mcl-1. This rapid response may contribute to the difficulty of treating hepatoma cells with ABT-737 compared with lymphoma cells in which ABT-737 is reported to be effective not only *in vitro*[29] but also *in vivo*.[30] The mechanism by which hepatoma cells posttranscriptionally up-regulate Mcl-1 upon ABT-737 exposure is not clear at present. However, our study has shown that Mcl-1 up-regulation was mediated by delayed degradation of Mcl-1 protein in ABT-737–treated cells without involving the USP9X deubiquitinase. ABT-737 is a Bad mimetic small molecule and preferentially binds with the BH3-binding groove of Bcl-xL. This binding may release endogenous BH3-only proteins such as Bim and Bid and presumably Bak and Bax from Bcl-xL and these unleashed Bcl-2 proteins may then bind Mcl-1. The interaction between Mcl-1 and the unleashed Bcl-2 proteins may cause increased Mcl-1 stability. Because Bak/Bax and Bid/Bim function as effectors and activators for the mitochondrial pathway of apoptosis, respectively, their binding with Mcl-1 may also cause apoptosis resistance to ABT-737.

Not only efficacy but also safety is an important point when considering a therapeutic strategy for cancer. Tumor cells sometimes share similar mechanisms for survival with normal cells. Indeed, HCCs overexpress Bcl-xL, but this molecule also plays an important role in maintaining the integrity of normal hepatocytes.[8] In the present study, we administered ABT-737 to Mcl-1 knockout mice and demonstrated that inactivation of both Bcl-xL and Mcl-1 could induce lethal hepatitis. We previously reported that Bcl-xL and Mcl-1 are required for liver development during embryogenesis,[15] and the present study also revealed the critical importance of both molecules in the adult liver. Recently, the possibility of combination therapy for down-regulation of Bcl-xL and Mcl-1 has been reported *in vitro*.[26,27,31] The present study, for the first time, focused on the *in vivo* safety of this strategy.

Regarding safety concerns about the inactivation of both Mcl-1 and Bcl-xL, sorafenib is an attractive agent because as we have revealed in this study, it down-regulates Mcl-1 expression in a relatively specific manner in tumor cells. Experiments with sorafenib administration into Bcl-xL knockout mice confirmed the safety of coadministration of sorafenib and ABT-737. The underlying mechanisms by which sorafenib down-regulates Mcl-1 in a tumor-specific manner are not clear. Some reports have shown that the down-regulation of Mcl-1 by sorafenib is independent of MEK/ERK,[16,23,32] but is dependent on Raf, AKT (protein kinase B), and Tyr705 phosphorylation of signal transducer and activator of transcription 3 (STAT3).[33,34]

Together with the report that activation of Ras/Raf and STAT3 pathways was found in HCC,[35] these pathways in tumor cells may be more activated than in healthy cells and result in the specificity of Mcl-1 down-regulation in tumor cells by sorafenib. Further experiments are needed to clarify this point.

Sorafenib belongs to a recently approved new class of targeted therapeutics that inhibit the oncogenic kinase pathway for HCC. It has been found to significantly prolong survival of patients with advanced HCC, although its effect appeared to be one of maintaining a stable disease state rather than inducing tumor regression.[36,37] It is speculated that sorafenib produces anticancer effects through a variety of ways such as suppression of angiogenesis and cell cycle arrest of tumor cells. Although it down-regulates Mcl-1,[16,23,32-34] its effect on apoptosis has not been clearly understood. In the present study, we found that sorafenib could not efficiently induce apoptosis in hepatoma cells by itself. This might explain why this agent elicits predominantly disease-stabilizing, cytostatic responses rather than tumor regression. Adding ABT-737 efficiently induced apoptosis of hepatoma cells, clearly indicating that the target of ABT-737, presumably Bcl-xL, blocks the apoptosis-inducing potency of sorafenib. Furthermore, coadministration of ABT-737 and sorafenib led to stronger suppression of xenograft tumor growth than did administration of sorafenib alone. These results suggest that combining sorafenib with ABT-737 may be an attractive strategy for producing durable clinical responses to combat HCC.

In conclusion, we have demonstrated that the inhibition of Bcl-xL by ABT-737 under sorafenib administration was safe and effective for anti-HCC therapy in preclinical models. ABT-737, a direct activator of apoptosis machinery, may unlock the potent antitumor potential of oncogenic kinase inhibitors such as sorafenib.

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References

1. Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. J Cell Physiol 2003;195:158-167.
2. Yip KW, Reed JC. Bcl-2 family proteins and cancer. Oncogene 2008; 27:6398-6406.
3. Takehara T, Liu X, Fujimoto J, Friedman SL, Takahashi H. Expression and role of Bcl-xL in human hepatocellular carcinomas. HEPATOLOGY 2001;34:55-61.
4. Watanabe J, Kushihata F, Honda K, Mominoki K, Matsuda S, Kobayashi N. Bcl-xL overexpression in human hepatocellular carcinoma. Int J Oncol 2002;21:515-519.
5. Takehara T, Takahashi H. Suppression of Bcl-xL deamidation in human hepatocellular carcinomas. Cancer Res 2003;63:3054-3057.
6. Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, et al. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. Cancer Res 2008;68:9167-9175.
7. Watanabe J, Kushihata F, Honda K, Sugita A, Tateishi N, Mominoki K, et al. Prognostic significance of Bcl-xL in human hepatocellular carcinoma. Surgery 2004;135:604-612.
8. Takehara T, Tatsumi T, Suzuki T, Rucker EB 3rd, Hennighausen L, Jinushi M, et al. Hepatocyte-specific disruption of Bcl-xL leads to continuous hepatocyte apoptosis and liver fibrotic responses. Gastroenterology 2004;127:1189-1197.
9. Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, et al. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 2005;435:677-681.
10. Vogler M, Dinsdale D, Dyer MJ, Cohen GM. Bcl-2 inhibitors: small molecules with a big impact on cancer therapy. Cell Death Differ 2009;16:360-367.
11. Mott JL, Gores GJ. Piercing the armor of hepatobiliary cancer: Bcl-2 homology domain 3 (BH3) mimetics and cell death. HEPATOLOGY 2007;46:906-911.
12. Del Gaizo Moore V, Schlis KD, Sallan SE, Armstrong SA, Letai A. BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. Blood 2008;111:2300-2309.
13. Hann CL, Daniel VC, Sugar EA, Dobromilskaya I, Murphy SC, Cope L, et al. Therapeutic efficacy of ABT-737, a selective inhibitor of BCL-2, in small cell lung cancer. Cancer Res 2008;68:2321-2328.
14. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, et al. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer Res 2008;68:3421-3428.
15. Hikita H, Takehara T, Shimizu S, Kodama T, Li W, Miyagi T, et al. Mcl-1 and Bcl-xL cooperatively maintain integrity of hepatocytes in developing and adult murine liver. HEPATOLOGY 2009;50:1217-1226.
16. Shimizu S, Takehara T, Hikita H, Kodama T, Miyagi T, Hosui A, et al. The let-7 family of microRNAs negatively regulates Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. J Hepatol 2010;52:698-704.
17. Hikita H, Takehara T, Kodama T, Shimizu S, Hosui A, Miyagi T, et al. BH3-only protein bid participates in the Bcl-2 network in healthy liver cells. HEPATOLOGY 2009;50:1972-1980.
18. Mason KD, Carpinelli MR, Fletcher JI, Collinge JE, Hilton AA, Ellis S, et al. Programmed anuclear cell death delimits platelet life span. Cell 2007;128:1173-1186.
19. Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, et al. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006;44:151-157.
20. Fleischer B, Schulze-Bergkamen H, Schuchmann M, Weber A, Biesterfeld S, Müller M, et al. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. Int J Oncol 2006;28:25-32.
21. Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell 2005;121:1085-1095.
22. Schwickart M, Huang X, Lill JR, Liu J, Ferrando R, French DM, et al. Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. Nature 2010;463:103-107.
23. Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851-11858.
24. Cotter TG. Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer 2009;9:501-507.

25. Weber A, Boger R, Vick B, Urbanik T, Haybaeck J, Zoller S, et al. Hepatocyte-specific deletion of the antiapoptotic protein myeloid cell leukemia-1 triggers proliferation and hepatocarcinogenesis in mice. HEPATOLOGY 2010;51:1226-1236.
26. van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, et al. The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 2006;10:389-399.
27. Keuling AM, Felton KE, Parker AA, Akbari M, Andrew SE, Tron VA. RNA silencing of Mcl-1 enhances ABT-737-mediated apoptosis in melanoma: role for a caspase-8-dependent pathway. PLoS One 2009;4: e6651.
28. Yecies D, Carlson NE, Deng J, Letai A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. Blood 2010;115:3304-3313.
29. Vogler M, Dinsdale D, Sun XM, Young KW, Butterworth M, Nicotera P, et al. A novel paradigm for rapid ABT-737-induced apoptosis involving outer mitochondrial membrane rupture in primary leukemia and lymphoma cells. Cell Death Differ 2008;15:820-830.
30. Mason KD, Vandenberg CJ, Scott CL, Wei AH, Cory S, Huang DC, et al. In vivo efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. Proc Natl Acad Sci U S A 2008;105: 17961-17966.
31. Lin X, Morgan-Lappe S, Huang X, Li L, Zakula DM, Vernetti LA, et al. 'Seed' analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-XL inhibitor ABT-737. Oncogene 2007;26:3972-3979.
32. Yu C, Bruzek LM, Meng XW, Gores GJ, Carter CA, Kaufmann SH, et al. The role of Mcl-1 down-regulation in the proapoptotic activity of the multikinase inhibitor BAY 43-9006. Oncogene 2005;24: 6861-6869.
33. Ulivi P, Arienti C, Amadori D, Fabbri F, Carloni S, Tesei A, et al. Role of RAF/MEK/ERK pathway, p-STAT-3 and Mcl-1 in sorafenib activity in human pancreatic cancer cell lines. J Cell Physiol 2009;220: 214-221.
34. Blechacz BR, Smoot RL, Bronk SF, Werneburg NW, Sirica AE, Gores GJ. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. HEPATOLOGY 2009;50:1861-1870.
35. Calvisi DF, Ladu S, Gorden A, Farina M, Conner EA, Lee JS, et al. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. Gastroenterology 2006;130:1117-1128.
36. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008; 359:378-390.
37. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 2009;10:25-34.

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ORIGINAL ARTICLE

Natural killer cell is a major producer of interferon γ that is critical for the IL-12-induced anti-tumor effect in mice

Akio Uemura · Tetsuo Takehara · Takuya Miyagi · Takahiro Suzuki · Tomohide Tatsumi · Kazuyoshi Ohkawa · Tatsuya Kanto · Naoki Hiramatsu · Norio Hayashi

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Abstract Although the anti-tumor effect of IL-12 is mediated mostly by IFNγ, which cell types most efficiently produce IFNγ and therefore initiate or promote the anti-tumor effect of IL-12 has not been clearly determined. In the present study, we demonstrated hydrodynamic injection of the IL-12 gene led to prolonged IFNγ production, NK-cell activation and complete inhibition of liver metastasis of CT-26 colon cancer cells in wild-type mice, but not in IFNγ knockout mice. NK cells expressed higher levels of STAT4 and upon IL-12 administration displayed stronger STAT4 phosphorylation and IFNγ production than non-NK cells. Adoptive transfer of wild-type NK cells into IFNγ knockout mice restored IL-12-induced IFNγ production, NK-cell activation and anti-tumor effect, whereas transfer of the same number of wild-type non-NK cells did not. In conclusion, NK cells are predominant producers of IFNγ that is critical for IL-12 anti-tumor therapy.

Keywords IFNγ · Innate immunity · Liver tumor · IL-12 · NK

A. Uemura and T. Takehara contributed equally to this work.

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A. Uemura · T. Takehara · T. Miyagi · T. Suzuki · T. Tatsumi · K. Ohkawa · T. Kanto · N. Hiramatsu · N. Hayashi (✉)
Department of Gastroenterology and Hepatology,
Osaka University Graduate School of Medicine,
2-2 Yamada-oka, Suita, Osaka 565-0871, Japan
e-mail: hayashin@gh.med.osaka-u.ac.jp

A. Uemura
e-mail: akioue@gh.med.osaka-u.ac.jp

Introduction

IL-12 is a 70-kDa heterodimer protein, composed of p35 and p40 subunits, mainly produced by antigen-presenting cells. IL-12 was originally found as a "natural killer-stimulating factor" and a "cytotoxic lymphocyte maturation factor" [1, 2]. IL-12 has multi-potent effects, inducing a Th1 response, enhancing the CD8 T-cell response, activating natural killer cells and inducing production of IFNγ [3, 4]. Therapeutic use of IL-12, either using its recombinant protein or gene, can induce an efficient anti-tumor effect on primary or metastatic tumors in various murine models and humans [5, 6].

Research has shown that IL-12 mediates anti-tumor effects in a variety of ways. They include anti-proliferative effects, anti-angiogenic effects [7, 8] and cytotoxic effects of effector lymphocytes. A variety of effector cells has been reported to be required for IL-12-mediated anti-tumor effects: they include CD8 T cells [9], NKT cells [10], CD4 T cells [11] and NK cells [12]. The relative contribution of these cells may differ among IL-12 doses and types of tumor models [13]. Endogenous IFNγ production is required for most, if not all, of the anti-tumor effects of IL-12 administration [14, 15]. IL-12 stimulates a variety of immune cells, such as T cells [16], B cells [17] and NK cells [18], to produce IFNγ. However, which cell types are most critical for producing IFNγ during IL-12 therapy is not clearly known.

In the present study, we used a murine model of liver metastasis of CT-26 colon cancer cells and found that NK cells highly expressed the IL-12 signaling molecule STAT4 and most efficiently produced IFNγ. IFNγ was essential for the anti-tumor effect of IL-12, and NK-cell production of IFNγ sufficed to produce the full-blown anti-tumor effects. These results demonstrated that NK cells

serve not only as an effector but also as an important mediator producing IFNγ that is critical for the anti-tumor effects of IL-12.

Materials and methods

Mice

Specific pathogen-free female Balb/c mice were purchased from Clea Japan, Inc (Tokyo, Japan). Rag2 knockout (Rag2 KO) mice with a Balb/c background were purchased from Taconic (Germantown, NY). IFNγ knockout (GKO) mice with a Balb/c background were kindly provided by Dr. Yoichiro Iwakura (Institute of Medical Science, University of Tokyo). All mice used were at the age of 6 to 10 weeks. They were housed under conditions of controlled temperature and light with free access to food and water at the Institute of Experimental Animal Science, Osaka University Graduate School of Medicine. All animals received humane care, and the study protocol complied with the institution's guidelines.

Tumor models

Intra-splenic injection of tumor cells was used to establish micro-disseminated liver tumors in mice [19]. CT-26 colon cancer cells originating from Balb/c mice were maintained in RPMI1620 supplemented with 10% FCS. Syngeneic mice were anesthetized with pentobarbital and given a cut on the left side flank. CT-26 cells (1×10^5) were suspended in 200 μl of PBS and injected into the spleen.

Injection of naked plasmid DNA

A plasmid coding the murine IL-12 gene, pCMV-IL-12, was generously provided by Dr. M Watanabe (Laboratory of Experimental Immunology, Division of Basic Sciences, National Cancer Institute-Frederick Cancer Research and Development Center) [20]. Plasmid DNA was prepared using an EndoFree plasmid system (Qiagen, Hilden, Germany,) according to the manufacturer's instructions. Hydrodynamic injection of plasmid DNA was performed as previously described [21]. In brief, 25 μg of plasmid DNA was diluted with 2.0 ml of lactated Ringer's solution and injected into the tail vein, using a syringe with a 26-gauge needle. DNA injection was completed within 5 to 8 s.

ELISA

Blood samples were serially obtained from the venous plexus in the retro-orbita under light anesthesia. The levels of serum IL-12 p70, IFNγ (BD Biosciences-Pharmingen, San Diego, CA), IFNγ-inducible protein 10 (IP-10) and monokine induced by IFNγ (MIG) (R&D Systems, Inc, Minneapolis, MN) were measured using commercially available ELISA kits in accordance with the manufacturer's instructions.

Mononuclear cells

Mononuclear cells were isolated from the liver or spleen as previously described. The NK activity of mononuclear cells was assessed by a standard 4-h ^{51}Cr-releasing assay using Yac1 cells as targets. In some experiments, mononuclear cells were separated into $DX5^+$ cells (NK cells) and $DX5^-$ cells (non-NK cells) using the MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the isolated NK-cell population was found to be greater than 90% by FACS analysis.

Flow cytometric analysis

Liver mononuclear cells were isolated 2 days after pCMV-IL-12 injection. Cytokine secretion was then blocked by the addition of brefeldin A for 4 h. Next, liver mononuclear cells were stained with FITC-conjugated anti-TCRβ antibody and biotin-conjugated anti-CD49b antibody (DX5), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), and stained with PE-conjugated anti-INFγ antibody or corresponding isotype controls. Analysis was performed using a FACSCalibur (Becton Dickinson), with the resulting data analyzed using the CELLQuest program (Becton Dickinson). NK cells were identified as $DX5^+/TCR\beta^-$ lymphocytes, NKT cells as $DX5^+/TCR\beta^+$ lymphocytes and T cells as $DX5^-/TCR\beta^+$ lymphocytes.

Adoptive transfer

For adoptive transfer experiments, GKO mice were injected intravenously 1 day before plasmid DNA injection with 2.0×10^8 whole mononuclear cells or 4.0×10^6 NK cells, or non-NK cells or whole mononuclear cells, all of which had been harvested from wild-type mice that can produce IFNγ.

Western blotting

Mouse recombinant IL-12 was purchased from R&D Systems, Inc (Minneapolis, MN). Mononuclear cells were treated with or without IL-12. Whole cell lysate was prepared from mononuclear cells from mice, and 20 μg of protein was separated by SDS-PAGE and transferred to the PVDF membrane. The membrane was stained with anti-STAT4 antibody (BD biosciences),

anti-phospho-specific STAT4 (pY693) antibody (BD biosciences), anti-STAT1 antibody (Cell Signaling), anti-phospho-specific STAT1 antibody (Cell Signaling) and visualized by chemiluminescence.

NK-cell depletion

For depletion of NK cells in vivo, anti-asialoGM1 antibody (WAKO, Osaka, Japan) was intraperitoneally administered. We determined the appropriate dosing to be 500 μg/mouse (50 μl when dissolved according to the manufacturer's instructions) based on FACS analysis of hepatic mononuclear cells. The percentage of $DX5^+/TCR\beta^-$ cells (NK cells) is 12.6 ± 2.4% in IgG-injected liver, whereas it decreased to 0.76 ± 0.04% one day after anti-asialo GM1 antibody injection ($N = 3$/group). This effect remained at least 3 days after anti-asialo GM1 antibody injection. NKT cells were less affected than NK cells, because 90% of $DX5^+/TCR\beta^+$ cells (NKT cells) still remained in the liver after the treatment. Anti-asialoGM1 antibody was injected 1 day after tumor inoculation and then every 5 days. For the control, the same amount of normal rabbit immunoglobulin (DAKO, Copenhagen, Denmark) was intraperitoneally administered.

Histology

The formalin-fixed livers were paraffin-embedded, and liver sections were analyzed by hematoxylin-eosin staining. Acetone-fixed fresh frozen liver sections were immunostained with anti-mouse CD4 (H123.19), anti-mouse CD8α (53-6.7) or anti-CD31 (390) monoclonal antibody (all from BD Biosciences), using a VECSTAIN ABC kit (Vector Laboratories, Burlingame, California, USA).

Statistics

Data are represented as mean ± SD. Comparisons between groups were analyzed by unpaired t-test with Welch's correction. $p < 0.05$ was considered statistically significant.

Results

Hydrodynamic injection of IL-12-expressing plasmid led to prolonged production of IFNγ

Hydrodynamics-based gene delivery into mice establishes efficient foreign gene expression predominantly in the liver, especially in hepatocytes. Serial measurement of serum IL-12 demonstrated that pCMV-IL-12 injection led to substantial IL-12 production on day 1. The levels of serum IL-12 then rapidly declined (Fig. 1a). We also measured IFNγ production in serum, since IL-12 is known to activate IFNγ production. pCMV-IL-12 and, to a lesser extent, pCMV injection increased serum IFNγ on day 1. In contrast to the pCMV injection group, high levels of serum IFNγ were maintained at later time points in the pCMV-IL-12 injection group (Fig. 1a). Thus, hydrodynamic injection of pCMV-IL-12 led to prolonged production of IFNγ. Transient IFNγ production followed by control plasmid may be an indirect effect of liver injury caused by bolus injection of saline or DNA injection.

IL-12 therapy induced NK activation and anti-metastatic effects, both of which are critically dependent on IFNγ

To examine the biological effects of the produced IL-12, we evaluated the NK activity of mononuclear cells from the liver. pCMV-IL-12 injection, but not control pCMV injection, increased Yac1 lytic activity of hepatic mononuclear cells (Fig. 1b). When GKO mice were injected with pCMV-IL-12 or pCMV, the hepatic mononuclear cells did not display any lytic ability to Yac1 cells, suggesting that IL-12-mediated NK-cell activation required IFNγ.

To examine the anti-metastatic effect of IL-12, pCMV-IL-12 or pCMV was injected into wild-type mice 2 days after intrasplenic injection of CT-26 cells. At 14 days after tumor injection, the mice were killed for evaluation of liver tumor (Fig. 1c). While pCMV-injected mice displayed huge liver tumors, pCMV-IL-12-injected mice did not show any macroscopic or microscopic tumor (Fig. 1d). Liver weight was significantly higher in pCMV-injected mice than pCMV-IL-12-injected mice, reflecting liver tumor formation. To examine the involvement of IFNγ in the IL-12-induced anti-tumor effect, we injected pCMV or pCMV-IL-12 into GKO mice 2 days after CT-26 injection. At 14 days after CT-26 injection, both groups showed similar degrees of tumor formation and there was no significant difference in liver weight between the two. This indicated that IL-12-induced anti-metastatic effect was strictly dependent on IFNγ.

NK cells were the most potent producer of IFNγ during IL-12 therapy

To evaluate which cell types most efficiently produced IFNγ, we isolated hepatic mononuclear cells from mice 2 days after plasmid injection and then stained cell surface TCRβ and DX5 as well as intracellular IFNγ (Fig. 2). $TCR\beta^-/DX5^+$ NK cells, $TCR\beta^+/DX5^+$ NKT cells and $TCR\beta^+/DX5^-$ T cells from pCMV-IL-12-injected mice showed significant levels of IFNγ production compared

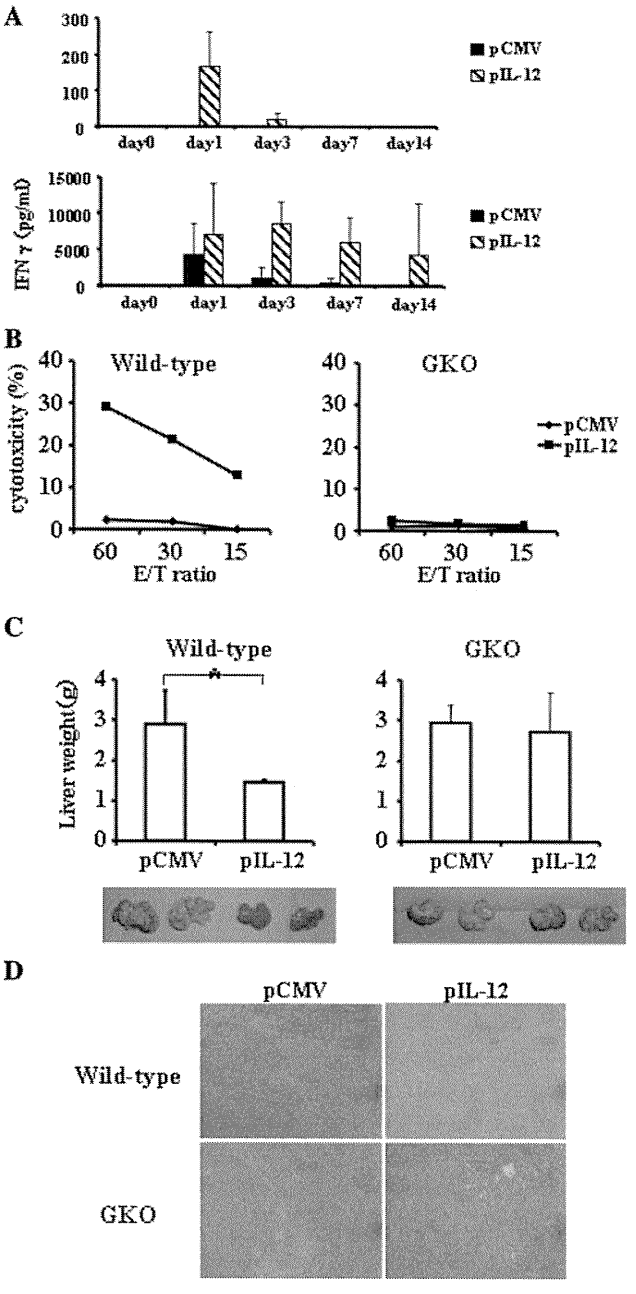

Fig. 1 Effects of hydrodynamic injection of IL-12-encoding plasmid. **a** Wild-type mice were hydrodynamically injected with either pCMV-IL-12 (*hatched bars*) or pCMV (*closed bars*) and bled at the indicated time points to measure the levels of serum IL-12 and IFNγ. Results are indicated as mean and SD ($n = 6$/group). **b** NK-cell activation after IL-12 administration. Hepatic mononuclear cells were isolated from wild-type mice (*left*) or GKO mice (*right*) which had been injected with pCMV-IL-12 (*closed squares*) or pCMV (*closed diamonds*) 4 days earlier. Yac1 lytic ability was measured by a standard ^{51}Cr-release assay at the indicated effector and target ratios (E/T ratio). All experiments were performed at least 3 times and representative data are shown. **c** and **d** Anti-metastatic effects of IL-12 therapy. Wild-type mice (*left*) or GKO mice (*right*) were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. At 14 days after the plasmid injection, the mice were killed to examine liver tumor development. **c** Data are indicated as mean and SD of the liver weight at the top ($n = 6$/group) and a representative picture of the liver in each group is shown at the bottom. *$p < 0.001$. **d** Representative histology of liver sections

with those from naive mice or pCMV-injected mice. The levels of IFNγ production were highest in NK cells among those cells. Even at a later time point, 7 days after plasmid injection, NK cells were found to produce the highest levels of IFNγ (data not shown).

IL-12-induced STAT4 signaling and IFNγ production increased in NK cells

IL-12 activates Janus kinases Tyk2 and Jak2, STAT4 as well as other STATs. To examine the activation of STAT1 and STAT4, we isolated splenocytes from wild-type mice and GKO mice and stimulated them with IL-12 and/or IFNγ in the presence or absence of anti-IFNγ Ab (Fig. 3a). IL-12 led to phosphorylation of both STAT1 and STAT4 in wild-type splenocytes. In contrast, the same treatment led to phosphorylation of STAT4, but not of STAT1, in GKO splenocytes. Addition of IFNγ restored STAT1 phosphorylation in GKO splenocytes. Furthermore, adding anti-IFNγ inhibited STAT1 phosphorylation in wild-type cells. These findings demonstrated that phosphorylation of STAT4 is a direct effect of IL-12 but phosphorylation of STAT1 is indirect, via an autocrine or paracrine IFNγ-dependent manner.

To examine STAT1 and STAT4 activation and IFNγ production in NK cells and non-NK cells, we prepared whole mononuclear cells as well as NK and non-NK populations from wild-type spleens and stimulated the cells with IL-12 (Fig. 3b). NK cells expressed higher levels of STAT4 than non-NK cells. Upon IL-12 treatment, STAT4 was rapidly phosphorylated in NK cells, but to a lesser extent in non-NK cells. In contrast, NK cells expressed lesser levels of STAT1 than non-NK cells. STAT1 was similarly phosphorylated in NK cells and non-NK cells upon IL-12 treatment. Both NK cells and non-NK cells produced significant levels of IFNγ, but the levels were much higher in NK cells than non-NK cells (Fig. 3c). These results indicated that compared with non-NK cells, NK cells possessed higher levels of STAT4, a direct signaling molecule of IL-12, and produced higher levels of IFNγ than non-NK cells.

NK cells were sufficient for IL-12-mediated anti-tumor effects

The above observation indicated that NK cells are a predominant producer of IFNγ, which was critical for the IL-12-induced anti-tumor effects. To examine whether NK

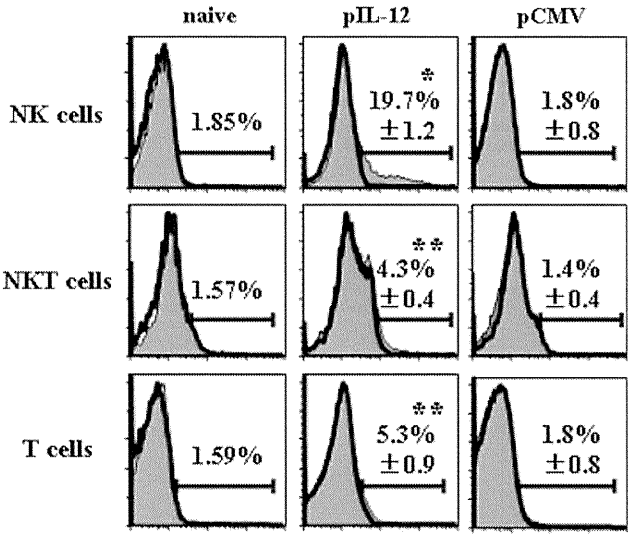

Fig. 2 IFNγ expression of mononuclear cells after IL-12 administration. Wild-type mice were injected with pCMV-IL-12 or pCMV, or were untreated (naive). Mononuclear cells were isolated from the liver 2 days after plasmid injection and stained with anti-TCRβ mAb, anti-DX5 mAb and anti-IFNγ mAb. *Closed histograms* show the IFNγ expression in the gated populations (TCRβ^+/DX5$^+$ cells for NK cells, TCRβ^+/DX5$^+$ cells for NKT cells and TCRβ^+/DX5$^-$ cells for T cells). Isotype control stainings are shown by *open histograms*. Numbers in histograms represent averages ± SD of percentages of positive cells ($n = 3$ mice/group). *$p < 0.0001$ vs. mock in NK populations. **$p < 0.05$ vs. mock in each population

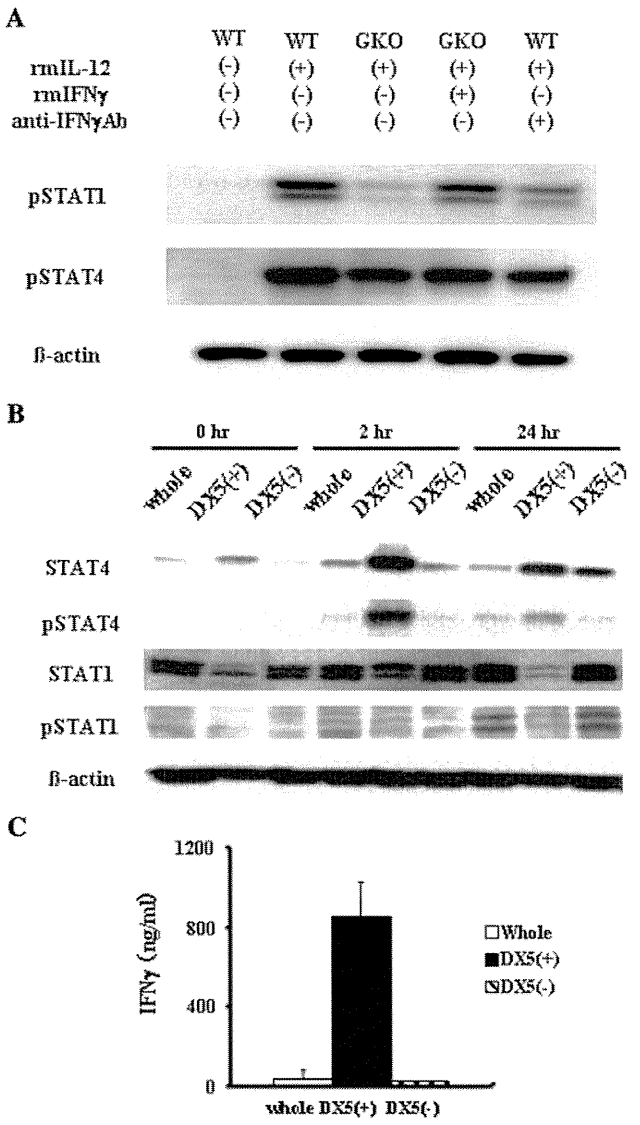

Fig. 3 STAT signaling and IFNγ production of mononuclear cells in vitro treated with IL-12. **a** STAT1 and STAT4 activation of splenocytes in vitro treated with IL-12. Splenocytes were isolated from wild-type mice or GKO mice and treated with or without recombinant IL-12 (20 ng/mL) in the presence or absence of recombinant IFNγ (500 ng/mL) or anti- IFNγ antibody (20 μg/mL) for 24 h. Cellular lysates were analyzed by Western blot for the expression of phospho-STAT1, phospho-STAT4 and β-actin. **b** and **c** STATs expression and signaling of NK cells and non-NK cells. Splenocytes were isolated from wild-type mice. Whole splenocytes were further purified into DX5$^+$ cells and DX5$^-$ cells. Each cell population was cultured with recombinant IL-12 (20 ng/mL) for the indicated times. **b** The cells were lysed to examine expression of whole STAT and phospho-STAT by Western blot. **c** The levels of IFNγ in the culture supernatant at 24 h were determined by ELISA. Data are expressed as mean and SD ($n = 3$)

cells are sufficient for the anti-metastatic effects of IL-12, we examined the anti-metastatic effect in Rag2 KO mice which lack T cells, B cells and NKT cells. pCMV-IL-12 injection enhanced the Yac1 lytic ability of hepatic mononuclear cells in Rag2 KO mice higher than in wild-type mice (Fig. 4a). To examine whether NK cells are sufficient for IL-12-mediated rejection of hepatic metastasis, we injected pCMV-IL-12 or pCMV into mice that had been intra-splenically injected with CT-26 cells 2 days earlier. Serum IFNγ levels of Rag2 KO mice were about 4 times higher than those of wild-type mice (Fig. 4b). pCMV-IL-12 completely suppressed hepatic metastasis in Rag2 KO mice (Fig. 4c).

Adoptive transfer of wild-type NK cells into GKO mice restored the anti-tumor effects of IL-12

Since NK cells were sufficient for producing IL-12-induced anti-tumor effects, we postulated that their production of IFNγ may play an important role in these effects. To test this, we performed adoptive transfer experiments with GKO mice. First, whole mononuclear cells isolated from the spleens of wild-type mice (2.0×10^8 cells) were adoptively transferred to GKO mice 1 day before plasmid injection. pCMV-IL-12 injection increased Yac1 lytic activity of hepatic mononuclear cells in the adoptively transferred group, but not in the untreated group (Fig. 5a). pCMV-IL-12 induced significant increase in serum IFNγ levels 4 days after plasmid injection in the adoptive transferred group, but not in the other groups (Fig. 5b). The

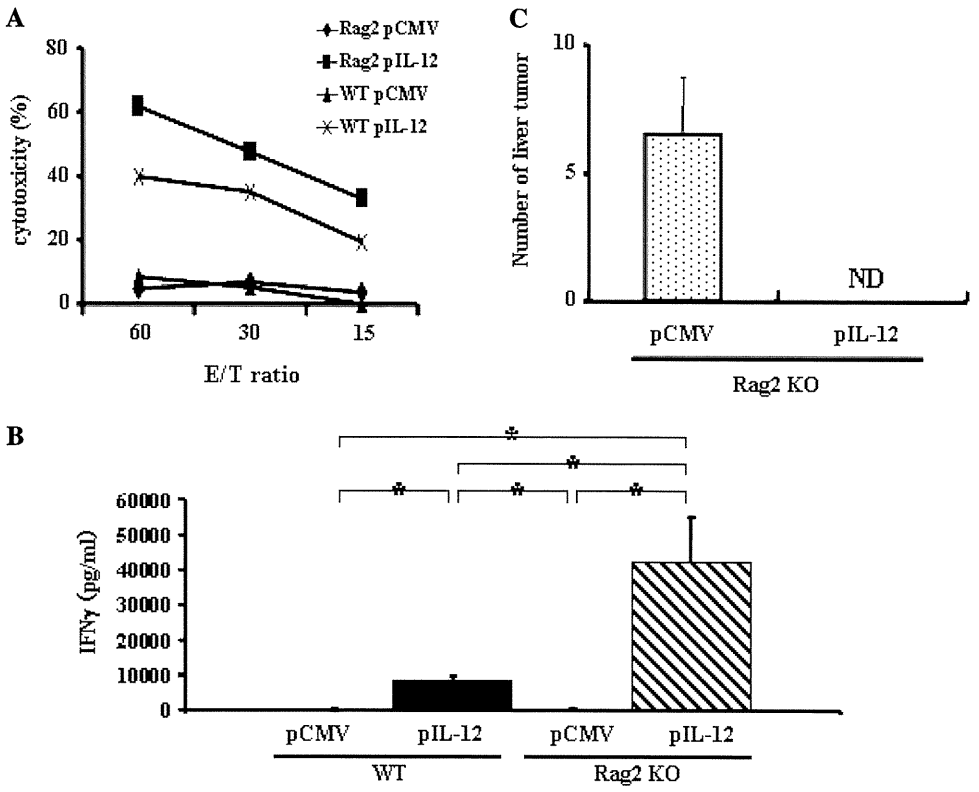

Fig. 4 Anti-tumor effects of IL-12 in Rag2 KO mice. Serum IFNγ levels and NK-cell activation. Wild-type or Rag2 KO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV and killed at 4 days. **a** Yac1 lytic ability of hepatic mononuclear cells was determined by Cr releasing assay as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ were determined by ELISA. Data are expressed as mean and SD ($n = 7$/group). $^*p < 0.0001$. **c** Anti-metastatic effect. Rag2 KO mice were intrasplenically injected with CT-26 cells and, 2 days later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Fourteen days after plasmid injection, mice were killed to examine tumor development in the liver. The numbers of hepatic tumors in each group are expressed as mean and SD ($n = 7$/group). *ND* not detectable

anti-metastatic effect of IL-12 was restored in GKO mice when whole mononuclear cells from wild-type mice were adoptively transferred (Fig. 5c).

To evaluate the contribution of IFNγ production from each subset of mononuclear cells to the anti-metastatic effect of IL-12, we adoptively transferred the same number of whole mononuclear cells, NK cells or non-NK cells from wild-type mice (4.0×10^6 cells) 1 day before pCMV-IL-12 injection and analyzed liver tumor formation. Only in the NK-cell-transferred group, pCMV-IL-12 injection induced NK cytolytic ability in the liver and IFNγ elevation in serum 4 days after plasmid injection, but not in the other groups (Fig. 5d, e). No liver tumor formed in the NK-cell-transferred group. In contrast, livers in other groups had massive tumors, and the liver weights were significantly heavier than those in the NK-cell-transferred group (Fig. 5f). These results clearly demonstrated the strong impact of IFNγ produced from NK cells on IL-12-induced anti-tumor effects compared with that from non-NK cells.

Anti-tumor effects of IL-12 deteriorated slightly in mice depleted of NK cells

To examine the involvement of NK cells in the tumor deletion by IL-12 therapy, we induced depletion of NK cells by repeatedly injecting anti-asialoGM1 antibody. The cytolytic ability of NK cells was completely abolished in the anti-asialoGM1 antibody-injected group (Fig. 6a). Serum IFNγ induction by IL-12 in the NK depletion group was about half of that in the control immunoglobulin injected group (Fig. 6b). Unexpectedly, pCMV-IL-12 injection inhibited macroscopic liver metastasis of CT-26 cells in NK cell-depleted mice (Fig. 6c). However, a number of microscopic tumor regions were observed after IL-12 therapy in NK cell-depleted mice but not in control IgG-injected mice (Fig. 6d). This finding indicated that NK cells are required for a full-blown IL-12 anti-tumor effect, but IL-12's anti-tumor effect was still observed even if the NK cells were knocked down. To examine the underlying mechanisms of anti-tumor effect in NK cell-depleted mice,

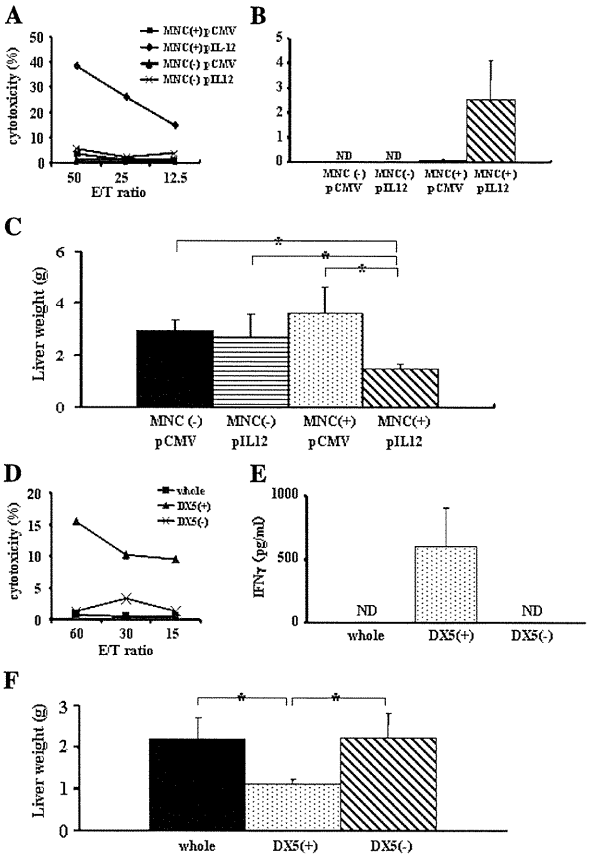

Fig. 5 Adoptive transfer of wild-type cells into GKO mice. Adoptive transfer of wild-type splenocytes restored anti-tumor effects of IL-12 in GKO mice. **a** GKO mice were intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. Yac1 lytic ability of hepatic mononuclear cells was expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **b** and **c** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice. Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **b** The levels of serum IFNγ 4 days after plasmid injection are expressed as mean and SD (n = 6/group). **c** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are indicated as mean and SD (n = 6/group). *ND* not detectable. *$p < 0.01$. Adoptive transfer of wild-type NK cells, but not non-NK cells, restored anti-tumor effects of IL-12 in GKO mice. **d** Wild-type splenocytes were purified into $DX5^+$ cells and $DX5^-$ cells. GKO mice were intravenously injected with 4.0×10^6 whole mononuclear cells or $DX5^+$ cells or $DX5^-$ cells and, 1 day later, hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after hydrodynamic injection. Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 3 times and representative data are shown. **e** and **f** GKO mice were intrasplenically injected with CT-26 cells and, 1 day later, intravenously injected with whole mononuclear cells, $DX5^+$ cells or $DX5^-$ cells (4.0×10^6/mouse). Two days after CT-26 injection, mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. **e** The levels of serum IFNγ are expressed as mean and SD (n = 6/group). **f** Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. The results are expressed as mean and SD (n = 6/group). *ND* not detectable. *$p < 0.001$

serum levels of IP-10 and MIG, chemokines downstream of IFNγ, were measured after IL-12 therapy (Fig. 6e). pCMV-IL-12-injected mice showed significant increase in both levels compared with pCMV-injected mice. Significant increase after pCMV-IL-12 injection was also found in NK cell-depleted mice, but not in GKO mice. This result suggests that production of these chemokines was not completely suppressed in NK cell-depleted mice in our experimental condition. Immunohistochemical analysis revealed that tumoral accumulation of CD4-positive cells and CD8-positive cells was observed in pCMV-IL-12-injected mice but not in pCMV-injected mice. On the other hand, similar levels of CD31 expression were observed in tumors of pCMV-injected mice and pCMV-IL-12-injected mice (Fig. 6d). These results suggest that IL-12's anti-tumor effects might be mediated by T-cell accumulating in the tumor rather than anti-angiogenesis.

Discussion

IL-12 is recognized as a master regulator of adaptive type 1, cell-mediated immunity. One major action of IL-12 is its induction of other cytokines, particularly IFNγ. A large amount of evidence has indicated that IL-12 administration leads to IFNγ production from a variety of immune cells, such as T cells [16], B cells [17], NK cells [18] and NKT cells [22]. The relative impact of each immune cell as the source of IFNγ has been controversial. The present study highlighted NK cells as a most efficient producer of IFNγ that is critical for IL-12-induced anti-tumor effects.

Flow cytometric analysis revealed higher in vivo production of IFNγ of NK cells than that of other cell types. The levels of serum IFNγ were around fourfold higher in Rag2 KO mice which only possess NK cells than in wild-type mice. On the other hand, NK-cell depletion in wild-type mice led to twofold reduction of serum IFNγ levels. These data indicate substantial contribution of NK cells in IFNγ production in vivo. Previous research has demonstrated that the specific cellular effects of IL-12 are due mainly to activation of STAT4 [23, 24]. IL-12-induced STAT4 phosphorylation leads to the production of IFNγ [25]. In agreement with these reports, our in vitro analysis showed that, in contrast to STAT1, STAT4 was directly phosphorylated upon IL-12 stimulation, being independent of IFNγ. Of interest is the finding that NK cells express higher levels of STAT4 than non-NK cells, suggesting that NK cells possess an ideal expression profile of STATs for producing IFNγ upon IL-12 stimulation. Indeed, in vitro analysis revealed that NK cells, upon IL-12 exposure, displayed higher levels of IFNγ production as well as STAT4 phosphorylation than non-NK cells. These in vitro

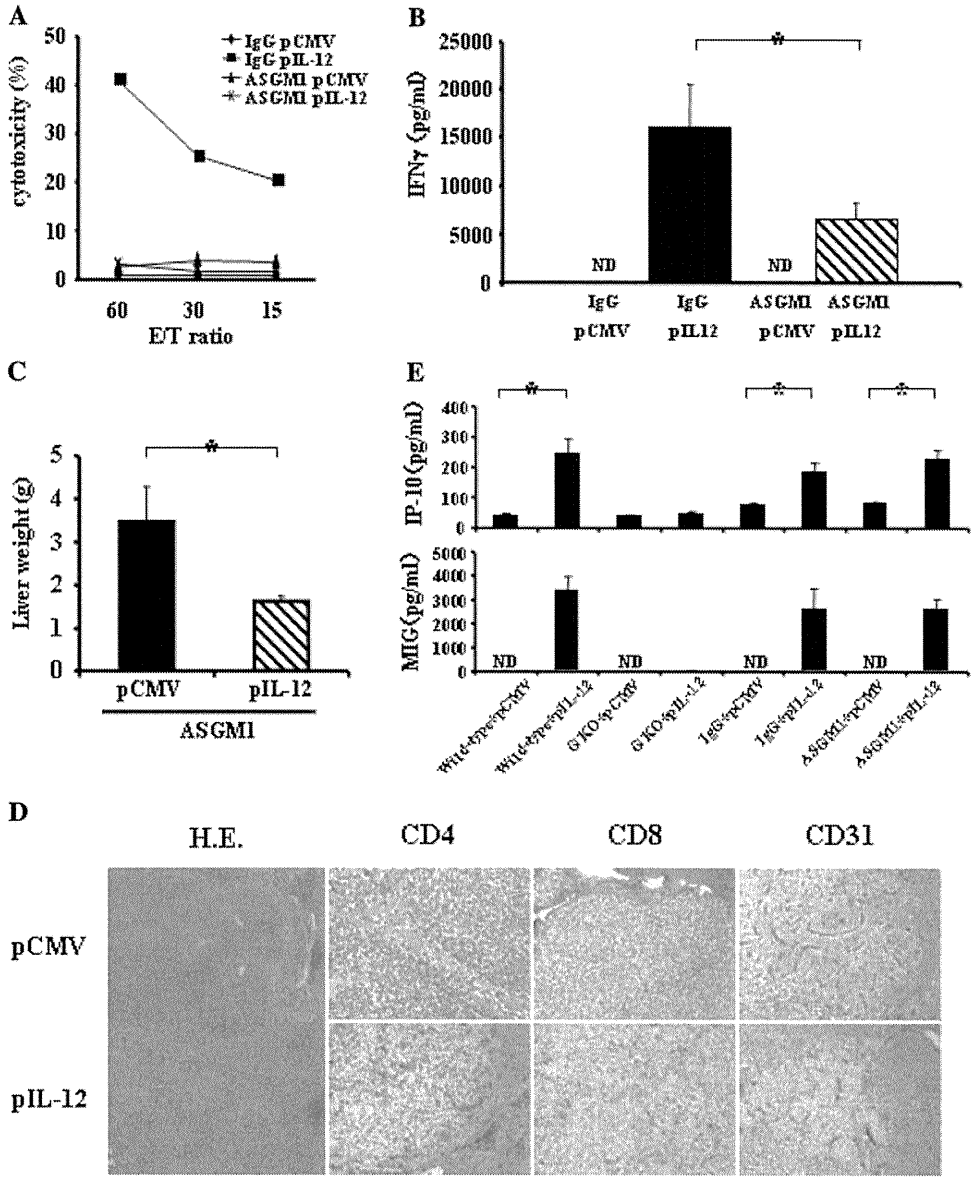

Fig. 6 Anti-tumor effects of IL-12 in NK-cell-depleted mice. Serum IFNγ levels and NK-cell activation. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and, 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 4 days after plasmid injection. **a** Yac1 lytic ability of hepatic mononuclear cells is expressed as the indicated effector and target ratios (E/T ratio). Experiments were done 2 times and representative data are shown. **b** The levels of serum IFNγ are expressed as mean and SD ($n = 6$/group). *ND* not detectable. *$p < 0.005$. Anti-metastatic effects. Wild-type mice were intrasplenically injected with CT-26 cells and, 1 day later and then every 5 days, intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and hydrodynamically injected with either pCMV-IL-12 or pCMV 2 days after CT-26 injection. Fourteen days after plasmid injection, mice were killed to examine liver tumor development by measuring liver weight. **c** The results are indicated as mean and SD ($n = 6$/group). *$p < 0.001$. **d** Representative histology of liver sections analyzed by hematoxylin-eosin staining and immunohistochemistry of CD4, CD8 and CD31. **e** Serum levels of IP-10 and MIG. Wild-type or GKO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Four days later, each mice were bled to measure the levels of serum IP-10 and MIG. Results are expressed as mean and SD ($n = 6$/group). *ND* not detectable. *$p < 0.001$

data are consistent with the in vivo observation that NK cells are efficient producers of IFNγ during IL-12 therapy.

Many studies have demonstrated that IFNγ production is required for the anti-tumor effects of IL-12 [14, 26, 27]. In fact, we have demonstrated that deletion of IFNγ abolished NK cytotoxicity and the anti-metastatic effect of IL-12 therapy in the liver. A large amount of evidence supports the concept that a major action of IL-12 is to promote the differentiation of naïve CD4 + T cells into Th1 cells, which produce IFNγ. Previous research reported that CD4

T-cell depletion caused inhibition of anti-tumor effects. More recent studies have supported a critical role of IFNγ as a third signal for CD8 T-cell differentiation. There have been many reports focusing on IFNγ production from T cells induced by IL-12 for the anti-tumor effect of IL-12 [28]. Segal et al. performed an elegant study showing a critical role of T-cell production of IFNγ in the anti-tumor effect by adoptively transferring T cells into GKO mice in a subcutaneous tumor model [29]. However, apart from this study, little is known about the contribution of each immune cell as a producer of IFNγ in terms of an anti-tumor effect. In our model, T-cell mediated adaptive responses were not required for the anti-metastatic effect of IL-12. More importantly, the anti-metastatic effects of IL-12 were restored in GKO mice by an adoptive transfer of wild-type NK cells. The same number of non-NK cells could not provoke IL-12-induced anti-tumor effects in GKO mice. The present study demonstrated for the first time a potent effect of NK cells on producing IFNγ that was critical for anti-metastatic effect during IL-12 therapy.

Our study showed that the main IFNγ producer of IL-12 was NK cells. So we focused on NK cells which were activated by IL-12 in an IFNγ-dependent manner to examine the cellular mechanism of protection against hepatic metastasis. Many studies have shown the importance of each subset (NK- [12], NKT- [10] and T [9, 30] cells) for anti-tumor effects of IL-12. In the present study, NK cells were sufficient while T cells, B cells, NKT cells were dispensable for IL-12-mediated NK-cell activation and anti-metastatic effects as IL-12 therapy showed Yac1 lytic ability and antimetastatic effects in Rag2 KO mice. On the other hand, NK-cell depletion by a repeated injection of anti-aialoGM1 antibody protected wild-type mice from macroscopic liver metastasis, but did not from microscopic liver metastasis. Thus, although NK cells were required for a full-blown IL-12 anti-tumor effect, other anti-tumor pathways are activated by IL-12 in the absence of NK cells. Serum levels of IP-10 and MIG suggest that production of these chemokines downstream of IFNγ was not suppressed in NK-cell-depleted mice in our experimental condition. When compared with the experiment on GKO mice, accumulation of CD4-positive cells and CD8-positive cells were more evident in NK-cell-depleted mice than in GKO mice (Supplementary Figure). On the other hand, there was no remarkable difference in the expression of CD31 between pCMV injection and pCMV-IL-12 injection. These results suggested that in NK-cell-depleted mice IL-12 may exert anti-tumor effect via T-cell accumulation rather than anti-angiogenesis.

Since the liver contains an abundance of immune cells (especially NK cells) [31], the cytokine-mediated activation of these cells may be a promising approach toward anti-tumor therapy in this organ [32]. IL-12 is a cytokine known to elicit a potent anti-tumor effect in mouse experimental models. However, clinical trials attempted to date were interrupted by fatal adverse effects. Systemic IL-12 therapy has been associated with dose-limiting toxicity [33]. IL-12 induces activation of the pro-inflammatory pathway which causes the complications of high dose cytokine, independent of the action of IFNγ [34]. On the other hand, the levels of immunosuppressive cytokine, for example, TGF-β1 or IL-10 were significantly higher in patients with hepatocellular cancer and colon cancer [35–38]. In particular, TGF-β1 in serum can limit NK-cell IFNγ production [39]. Thus, in patients with advanced disease, IL-12 may not be able to exert its potent anti-tumor immune-effects because IFNγ, which is an important mediator of the IL-12-induced immune response, is less effective in a tumor environment. In the present study, we demonstrated that NK-cell IFNγ production induced by IL-12 was sufficient for the anti-metastatic effect of IL-12 in the liver. Thus, a strategy of efficiently producing IFNγ from NK cells may be important for avoiding toxicity of IL-12 therapy.

IL-12 gene therapy has an advantage to allow local production of the cytokine at the tumor sites with low serum concentration. Studies demonstrated that intratumoral administration of adenovirus encoding IL-12 to animals with different types of carcinoma caused complete tumor eradication and increased long-term survival [40, 41]. Moreover, injection of IL-12-encoding adenovirus in one nodule of liver tumor resulted in regression of distant nodules in the liver [41]. However, in a clinical trial anti-tumor activity of IL-12-encoding adenovirus was only observed in the injected tumor sites, but not in distant tumors [42]. The present study shed light on hydrodynamic transfection of hepatocytes as a promising strategy to eradicate disseminated tumors from whole liver.

In summary, NK cells are not just an effector for innate immunity but a mediator producing IFNγ that is critical for the IL-12 anti-tumor effects. Extremely higher expression of STAT4 may be a basis for efficient production of IFNγ from NK cells.

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References

1. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G (1989) Identification and purification of natural killer cell stimulatory factor

(NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med 170(3):827–845
2. Stern AS, Podlaski FJ, Hulmes JD, Pan YC, Quinn PM, Wolitzky AG, Familletti PC, Stremlo DL, Truitt T, Chizzonite R, Gately MK (1990) Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc Natl Acad Sci USA 87(17):6808–6812
3. Watford WT, Moriguchi M, Morinobu A, O'Shea JJ (2003) The biology of IL-12: coordinating innate and adaptive immune responses. Cytokine Growth Factor Rev 14(5):361–368
4. Trinchieri G (2003) Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3(2):133–146
5. Colombo MP, Trinchieri G (2002) Interleukin-12 in anti-tumor immunity and immunotherapy. Cytokine Growth Factor Rev 13(2):155–168
6. Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, Anichini A (2007) Interleukin-12: biological properties and clinical application. Clin Cancer Res 13(16):4677–4685
7. Wigginton JM, Gruys E, Geiselhart L, Subleski J, Komschlies KL, Park JW, Wiltrout TA, Nagashima K, Back TC, Wiltrout RH (2001) IFN-gamma and Fas/FasL are required for the antitumor and antiangiogenic effects of IL-12/pulse IL-2 therapy. J Clin Invest 108(1):51–62
8. Lee JC, Kim DC, Gee MS, Saunders HM, Sehgal CM, Feldman MD, Ross SR, Lee WM (2002) Interleukin-12 inhibits angiogenesis and growth of transplanted but not in situ mouse mammary tumor virus-induced mammary carcinomas. Cancer Res 62(3):747–755
9. Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, Wolf SF, Gately MK (1993) Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J Exp Med 178(4):1223–1230
10. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M (1997) Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 278(5343):1623–1626
11. Zilocchi C, Stoppacciaro A, Chiodoni C, Parenza M, Terrazzini N, Colombo MP (1998) Interferon gamma-independent rejection of interleukin 12-transduced carcinoma cells requires CD4 + T cells and Granulocyte/Macrophage colony-stimulating factor. J Exp Med 188(1):133–143
12. Kodama T, Takeda K, Shimozato O, Hayakawa Y, Atsuta M, Kobayashi K, Ito M, Yagita H, Okumura K (1999) Perforin-dependent NK cell cytotoxicity is sufficient for anti-metastatic effect of IL-12. Eur J Immunol 29(4):1390–1396
13. Takeda K, Hayakawa Y, Atsuta M, Hong S, Van Kaer L, Kobayashi K, Ito M, Yagita H, Okumura K (2000) Relative contribution of NK and NKT cells to the anti-metastatic activities of IL-12. Int Immunol 12(6):909–914
14. Ogawa M, Yu WG, Umehara K, Iwasaki M, Wijesuriya R, Tsujimura T, Kubo T, Fujiwara H, Hamaoka T (1998) Multiple roles of interferon-gamma in the mediation of interleukin 12-induced tumor regression. Cancer Res 58(11):2426–2432
15. Subleski JJ, Hall VL, Back TC, Ortaldo JR, Wiltrout RH (2006) Enhanced antitumor response by divergent modulation of natural killer and natural killer T cells in the liver. Cancer Res 66(22):11005–11012
16. Kubin M, Kamoun M, Trinchieri G (1994) Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. J Exp Med 180(1):211–222
17. Yoshimoto T, Okamura H, Tagawa YI, Iwakura Y, Nakanishi K (1997) Interleukin 18 together with interleukin 12 inhibits IgE production by induction of interferon-gamma production from activated B cells. Proc Natl Acad Sci USA 94(8):3948–3953
18. Lauwerys BR, Renauld JC, Houssiau FA (1999) Synergistic proliferation and activation of natural killer cells by interleukin 12 and interleukin 18. Cytokine 11(11):822–830
19. Takehara T, Uemura A, Tatsumi T, Suzuki T, Kimura R, Shiotani A, Ohkawa K, Kanto T, Hiramatsu N, Hayashi N (2007) Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNalpha gene to mice. Int J Cancer 120(6):1252–1260
20. Watanabe M, Fenton RG, Wigginton JM, McCormick KL, Volker KM, Fogler WE, Roessler PG, Wiltrout RH (1999) Intradermal delivery of IL-12 naked DNA induces systemic NK cell activation and Th1 response in vivo that is independent of endogenous IL-12 production. J Immunol 163(4):1943–1950
21. Takehara T, Suzuki T, Ohkawa K, Hosui A, Jinushi M, Miyagi T, Tatsumi T, Kanazawa Y, Hayashi N (2006) Viral covalently closed circular DNA in a non-transgenic mouse model for chronic hepatitis B virus replication. J Hepatol 44(2):267–274
22. Shin T, Nakayama T, Akutsu Y, Motohashi S, Shibata Y, Harada M, Kamada N, Shimizu C, Shimizu E, Saito T, Ochiai T, Taniguchi M (2001) Inhibition of tumor metastasis by adoptive transfer of IL-12-activated Valpha14 NKT cells. Int J Cancer 91(4):523–528
23. Thierfelder WE, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DA, Doherty PC, Grosveld GC, Ihle JN (1996) Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature 382(6587):171–174
24. Kaplan MH, Sun YL, Hoey T, Grusby MJ (1996) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. Nature 382(6587):174–177
25. Morinobu A, Gadina M, Strober W, Visconti R, Fornace A, Montagna C, Feldman GM, Nishikomori R, O'Shea JJ (2002) STAT4 serine phosphorylation is critical for IL-12-induced IFN-gamma production but not for cell proliferation. Proc Natl Acad Sci USA 99(19):12281–12286
26. Comes A, Di Carlo E, Musiani P, Rosso O, Meazza R, Chiodoni C, Colombo MP, Ferrini S (2002) IFN-gamma-independent synergistic effects of IL-12 and IL-15 induce anti-tumor immune responses in syngeneic mice. Eur J Immunol 32(7):1914–1923
27. Hafner M, Falk W, Echtenacher B, Mannel DN (1999) Interleukin-12 activates NK cells for IFN-gamma-dependent and NKT cells for IFN-gamma-independent antimetastatic activity. Eur Cytokine Netw 10(4):541–548
28. Komita H, Homma S, Saotome H, Zeniya M, Ohno T, Toda G (2006) Interferon-gamma produced by interleukin-12-activated tumor infiltrating CD8 + T cells directly induces apoptosis of mouse hepatocellular carcinoma. J Hepatol 45(5):662–672
29. Segal JG, Lee NC, Tsung YL, Norton JA, Tsung K (2002) The role of IFN-gamma in rejection of established tumors by IL-12: source of production and target. Cancer Res 62(16):4696–4703
30. Nastala CL, Edington HD, McKinney TG, Tahara H, Nalesnik MA, Brunda MJ, Gately MK, Wolf SF, Schreiber RD, Storkus WJ, Lotze MT (1994) Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. J Immunol 153(4):1697–1706
31. Doherty DG, O'Farrelly C (2000) Innate and adaptive lymphoid cells in the human liver. Immunol Rev 174:5–20
32. Seki S, Habu Y, Kawamura T, Takeda K, Dobashi H, Ohkawa T, Hiraide H (2000) The liver as a crucial organ in the first line of host defense: the roles of Kupffer cells, natural killer (NK) cells and NK1.1 Ag + T cells in T helper 1 immune responses. Immunol Rev 174:35–46
33. Car BD, Eng VM, Lipman JM, Anderson TD (1999) The toxicology of interleukin-12: a review. Toxicol Pathol 27(1):58–63
34. Biber JL, Jabbour S, Parihar R, Dierksheide J, Hu Y, Baumann H, Bouchard P, Caligiuri MA, Carson W (2002) Administration of