anti-Bak antibody (Millipore, Billerica, MA), and anti–β-actin antibody (Sigma-Aldrich, St. Louis, MO).

Isolation of Mitochondria-Rich and Cytosolic Fraction. After liver tissue was homogenized using isolation buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid, 1 mg/mL fatty acid–free bovine serum albumin, 1 × protein inhibitor cocktail, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–potassium hydroxide, pH 7.4), the lysate was centrifuged at 600*g* for 10 minutes, and the supernatant was centrifuged at 15,000*g* for 10 minutes. The pellet was regarded as a mitochondria-rich fraction and the supernatant as a cytosolic fraction.

Immunoprecipitation of Bcl-xL. Approximately 30 mg liver tissue was lysed with a TNE buffer (1% Nonidet P-40, 1 mM ethylenediaminetetra-acetic acid, 1 × protein inhibitor cocktail, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.8). Equal amounts of protein samples were rotated with protein G sepharose (GE Healthcare, Tokyo, Japan) and anti-Bcl-xL antibody (Abcam, Cambridge, MA) overnight at 4°C. After centrifugation, the pellet was collected as the immunoprecipitate protein.

Incubation of tBid or Bid for Immunoprecipitation. Liver tissue (90 mg) was lysed with 800 μL lysis buffer (2 mM ethylenediaminetetra-acetic acid, 10 mM ethylene glycol tetra-acetic acid, 50 mM NaF, 5 mM $Na_2P_4O_7$, 10 mM β-glycerophosphate, 0.1% 2-mercaptoethanol, 1% Triton X, 1 × protein inhibitor cocktail, 50 mM Tris-HCl, pH 7.5). Equal volumes of protein samples were incubated with or without recombinant mouse tBid or full-length Bid (R&D Systems, Minneapolis, MN).

Analysis of Cytochrome C Release. The mitochondria-rich fraction was diluted in a mitochondria dilution buffer (395 mM sucrose, 0.1 mM ethylene glycol tetra-acetic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–potassium hydroxide, pH 7.4). The diluted mitochondria were incubated with recombinant mouse tBid or full-length Bid diluted with a reaction buffer (125 mM KCl, 0.5 mM $MgCl_2$, 3.0 mM succinic acid, 3.0 mM glutamic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid–potassium hydroxide, 1 × protein inhibitor cocktail, 2.5 mM ethylenediaminetetra-acetic acid and BOC-Asp (OMe) CH_2F 20 μM, pH 7.4) for 30 minutes at 37°C. The levels of cytochrome c in the buffer were determined using an enzyme-linked immunosorbent assay kit (R&D Systems). The maximum or spontaneous release of cytochrome c was defined as the level of samples incubated with 0.1% Triton X-100 or medium alone, respectively. The percentage release of cytochrome c was calculated using the following formula:

% release = (experimental release − spontaneous release) × 100/(maximum release − spontaneous release).

ABT-737 Injection Study. ABT-737 was provided by Abbott Laboratories (Abbott, Park, IL). ABT-737 was dissolved with a mixture of 30% propylene glycol, 5% Tween 80, and 65% D5W (5% dextrose in water), final pH 4 to 5. Mice were given a single intraperitoneal injection of ABT-737 at 100 mg/kg and sacrificed 16 hours later. Platelets were counted using an automated cell counter (Sysmex, Kobe, Japan).

Statistical Analysis. Data are presented as mean ± standard deviation. Multiple comparisons of TUNEL-positive cells were performed by analysis of variance followed by Fisher's *post hoc* correction. The other multiple comparisons were performed by analysis of variance followed by Scheffe *post hoc* correction. $P < 0.05$ was considered statistically significant.

Results

Hepatocyte Apoptosis Caused by Bcl-xL Deficiency Is Completely Lost with Bid-Deficient Background. To examine the possibility of whether Bid is involved in apoptosis caused by Bcl-xL deficiency, hepatocyte-specific Bcl-xL KO mice were crossed with traditional Bid KO mice. After mating *bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice with *bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ mice, western blot analysis confirmed lack of Bcl-xL and Bid in the liver of Bcl-xL KO mice and Bid KO mice, respectively, and intermediate expression of Bid in the Bid+/- liver (Fig. 1A). Consistent with our previous findings,[8] Bcl-xL KO mice (*bid*$^{+/+}$ *bcl-x*$^{flox/flox}$ *AlbCre*) produced spontaneous hepatocyte apoptosis (Fig. 1B), which was associated with caspase-7 activation in the liver (Fig. 1C). Serum ALT levels (Fig. 1D), caspase-3/7 activity (Fig. 1E), and the frequency of TUNEL-positive hepatocytes (Fig. 1F) were significantly higher in Bcl-xL KO mice than in wild-type mice (*bid*$^{+/+}$ *bcl-x*$^{flox/flox}$). Bid KO mice (*bid*$^{-/-}$ *bcl-x*$^{flox/flox}$) did not produce any liver phenotypes under physiological conditions, in agreement with a previous report.[7] This was further confirmed by our additional analysis on Bid KO mice and control littermates, which showed no difference in serum ALT levels (Supporting Fig. 1A), caspase-3/7 activity (Supporting Fig. 1B), and the ratios of liver weight to body weight (Supporting Fig. 1C). Of importance is the finding that serum ALT levels were reduced to the normal levels in Bcl-xL/Bid double-KO mice (*bid*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*). Bcl-xL KO with Bid heterozygosity (*bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*) displayed intermediate ALT levels between Bcl-xL KO mice and double-KO mice. In agreement with this observation, the number of TUNEL-positive hepatocytes in Bcl-xL/Bid double-KO mice reached background levels. In addition, the levels of caspase-3/7 activity in serum were also normalized in Bcl-xL/Bid double-KO mice. Taken together, these observations indicated that

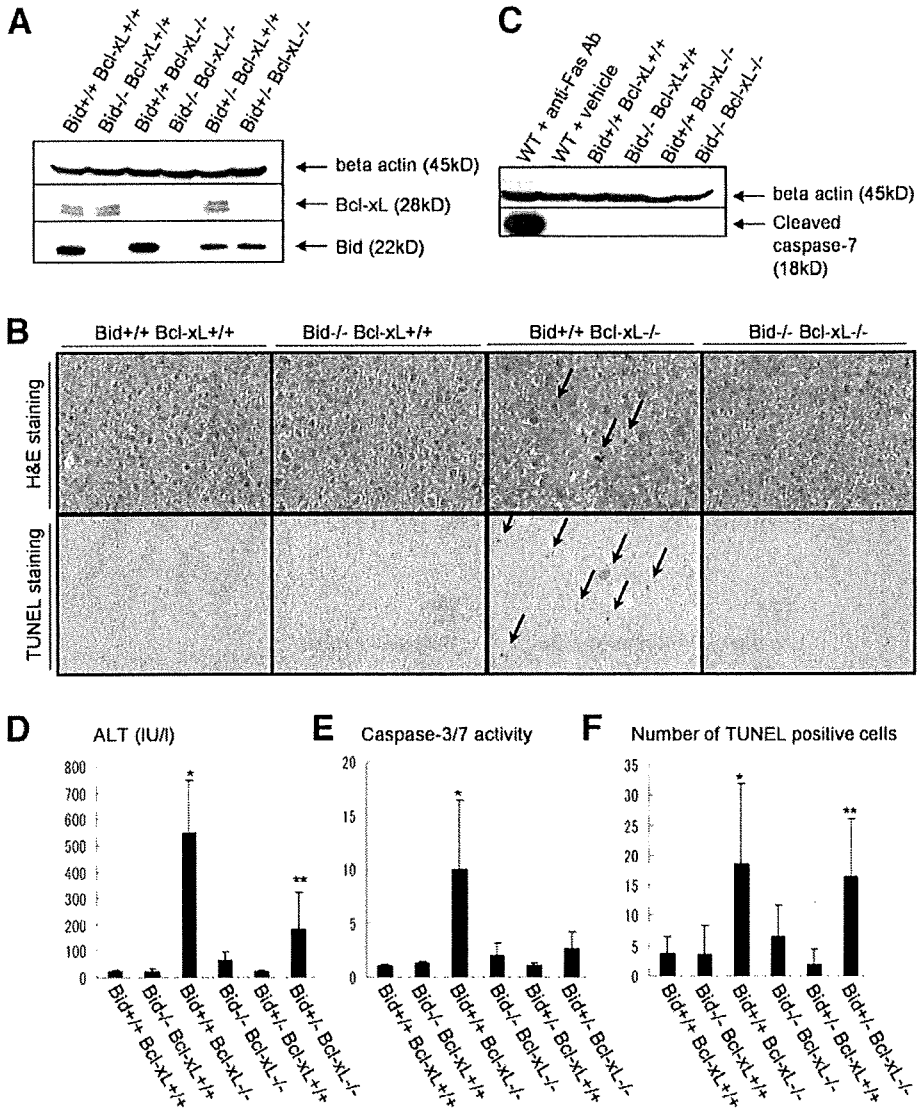

Fig. 1. Bcl-xL/Bid double-KO mice. Offspring from mating *bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice and *bid*$^{+/-}$ *bcl-x*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. Bcl-xL+/+ stands for *bcl-x*$^{flox/flox}$ without *AlbCre*, and Bcl-xL−/− stands for *bcl-x*$^{flox/flox}$ with *AlbCre*. (A) Western blot of whole liver lysate for the expression of Bcl-xL and Bid. (B) Representative pictures of liver histology stained with hematoxylin-eosin and TUNEL. Arrows indicate typical apoptotic cells. (C) Western blot of whole liver lysate for the expression of cleaved caspase-7. Live lysates from wild-type mice 3 hours after intraperitoneal injection of 30 μg anti-Fas antibody (clone Jo2) or vehicle were included as a positive and a negative control, respectively. (D) Serum ALT levels. N = 14 mice per group. *$P < 0.05$ versus the other five groups; **$P < 0.05$ versus the other four groups except Bid−/− Bcl-xL−/− group. (E) Serum caspase-3/7 activity. N = 13 mice per group. *$P < 0.05$ versus the other five groups. (F) Statistics of TUNEL- positive cells. N = 6 mice per group. * and **$P < 0.05$ versus all of Bcl-xL +/+ groups and Bid−/− Bcl-xL−/− group.

apoptosis caused by Bcl-xL deficiency is completely dependent on the BH3-only protein Bid. Bid is activated by tumor necrosis factor (TNF) receptor,[15] and TNF-α, which is a ligand of TNF receptor, is produced by Myd88 signal pathway.[16] To examine the possibility of involvement of Myd88 or TNF-α in this apoptosis, we generated Myd88 Bcl-xL double-KO mice by crossing myd88$^{-/-}$ mice with bcl-x$^{flox/flox}$ AlbCre mice and administered neutralizing anti–TNF-α antibody into Bcl-xL KO mice. Hepatocyte apoptosis caused by Bcl-xL deficiency was not ameliorated with Myd88 KO background or by administration of anti–TNF-α antibody (Supporting Fig. 2A, B).

Hepatocyte Apoptosis Caused by Bcl-xL Deficiency Requires Both Bak and Bax. To depict the precise relationships among core Bcl-2 family proteins in regulating liver homeostasis, hepatocyte-specific Bcl-xL–deficient mice were crossed with traditional Bak or Bax KO mice. The levels of serum ALT were slightly decreased with a Bak KO background (*bak*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*), whereas they did not change with a Bax KO background (*bax*$^{-/-}$ *bcl-x*$^{flox/flox}$ *AlbCre*) (Fig. 2A, B). To examine the contribution of both Bax and Bak, Bcl-xL KO mice were crossed with conditional Bak/Bax KO mice. The levels of serum ALT were completely normalized in Bak/Bax KO background (*bak*$^{-/-}$ *bax*$^{flox/flox}$ *bcl-x*$^{flox/flox}$ *AlbCre*) (Fig. 2C). Hepatocyte apoptosis determined by TUNEL staining of liver sections and caspase activation determined by caspase-3/7 activity in serum also returned to background levels (Fig. 2D, E). These observations clearly indicated that apoptosis caused by Bcl-xL deficiency was generated through the Bak/Bax-dependent mitochondrial cell death pathway. To clarify the background levels of hepatocyte apoptosis, we also analyzed the liver apoptosis in *bak*$^{-/-}$ and *bak*$^{-/-}$ *bax*$^{flox/flox}$ *AlbCre* mice. Similarly, in *bid*$^{-/-}$

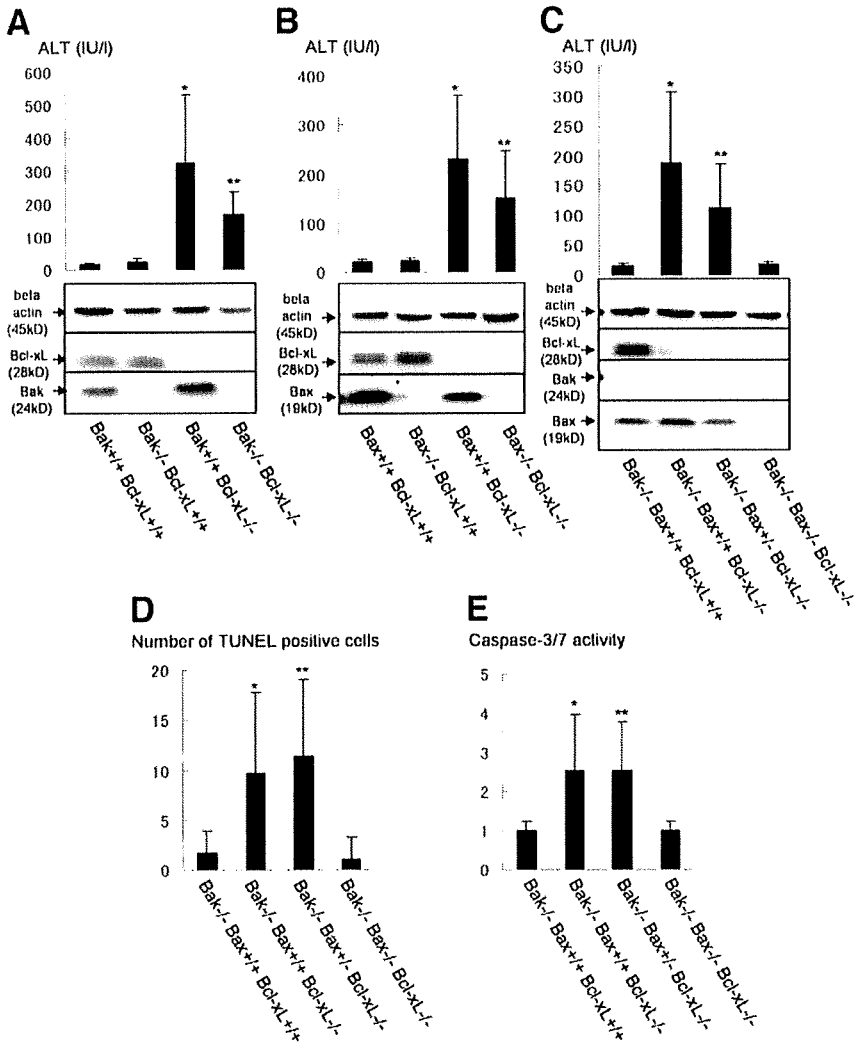

Fig. 2. Bcl-xL KO mice with Bak or Bax KO background. (A) Offspring from mating *bak*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice and *bak*$^{+/-}$ *bcl-x*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL and Bak are shown. N = 14 mice per group. * and **$P < 0.05$ versus the other three groups. (B) Offspring from mating *bax*$^{+/-}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice and *bax*$^{+/-}$ *bcl-x*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL and Bax are shown. N = 15 mice per group. * and **$P < 0.05$ versus the other two Bcl-xL+/+ groups. (C, D, and E) Offspring from mating *bak*$^{-/-}$ *bax*$^{flox/+}$ *bcl-x*$^{flox/flox}$ *AlbCre* mice and *bak*$^{-/-}$ *bax*$^{flox/+}$ *bcl-x*$^{flox/flox}$ mice were sacrificed at 6 weeks after birth. Bax+/+ stands for *bax*$^{flox/flox}$ without *AlbCre*, and Bax−/− stands for *bax*$^{flox/flox}$ with *AlbCre*. N = 8 or 10 mice per group. Serum ALT levels and western blot of whole liver lysate for the expression of Bcl-xL, Bak, and Bax are shown (C). *$P < 0.05$ versus Bak−/− Bax+/+ Bcl-xL+/+ and Bak−/− Bax−/− Bcl-xL−/− groups; **$P < 0.05$ versus Bak−/− Bax−/− Bcl-xL−/− group. Statistics of TUNEL-positive cells (D). * and **$P < 0.05$ versus Bak−/− Bax+/+ Bcl-xL+/+ and Bak−/− Bax−/− Bcl-xL−/− groups. Serum caspase-3/7 activity (E). * and **$P < 0.05$ versus Bak−/− Bax+/+ Bcl-xL+/+ and Bak−/− Bax−/− Bcl-xL−/− groups.

mice, there was no difference between two groups in serum ALT levels (Supporting Fig. 3A), caspase-3/7 activity (Supporting Fig. 3B), and the ratios of liver weight to body weight (Supporting Fig. 3C), which suggests that healthy hepatocytes in wild-type mice are completely protected from Bid or Bak/Bax-mediated apoptosis by Bcl-xL.

Bcl-xL Interacts with Cytosolic Bax and Mitochondrial Bak in the Liver. To examine the expression of a variety of Bcl-2–related molecules in the liver, cytosolic and mitochondrial fractions from liver lysate were subjected to western blot analysis (Fig. 3A). Anti-apoptotic Bcl-2 proteins, Bcl-xL and Mcl-1, were expressed at both the mitochondria and the cytosol. In contrast, Bak and Bax were exclusively expressed at the mitochondria and the cytosol, respectively. Full-length Bid was expressed mainly in the cytosol. To examine whether Bcl-xL physically interacts with those Bcl-2–related proteins, liver lysate was immunoprecipitated with Bcl-xL and identified using corresponding antibodies (Fig. 3B). At least a part of Bcl-xL was bound to Bak and Bax, but not to Mcl-1 or full-length Bid.

tBid, But Not Full-Length Bid, Displaces Bak and Bax from Bcl-xL by Binding to Bcl-xL. Bcl-2–like molecules have been shown to be capable of binding Bak or Bax, and through this interaction, to neutralize each activity.[17] Conversely, other research showed that Bcl-xL does not have to bind Bax-like molecules to protect against cell death.[18] To examine the impact of tBid on the association between Bcl-xL and Bak or Bax, we added tBid to the liver lysate and examined the interaction of each Bcl-2–related protein with Bcl-xL by immunoprecipitation. Addition of 500 nM tBid abolished the association between Bcl-xL and Bak or Bax (Supporting Fig. 4). Simultaneously, Bcl-xL binding of tBid was observed. Addition of 20 nM tBid also abolished, if not completely, the association between Bcl-xL and Bak or Bax (Fig. 4). In contrast, adding the same concentration of full-length Bid had little effect on Bcl-xL binding of Bak or Bax (Fig. 4). These results indicated that tBid can bind to Bcl-xL

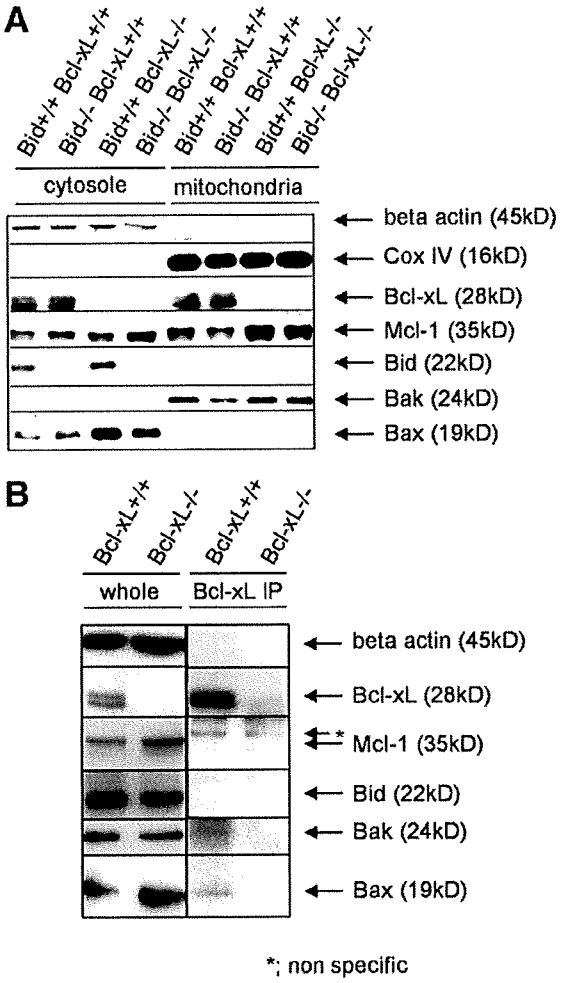

Fig. 3. Expression of Bcl-2-related molecules in the liver and their association with Bcl-xL. Bcl-xL+/+ stands for *bcl-x*$^{flox/flox}$ without *AlbCre*, and Bcl-xL−/− stands for *bcl-x*$^{flox/flox}$ with *AlbCre*. (A) Western blot after cellular fractionations of the liver lysate. Loading amounts of cytosolic and mitochondrial fractions were adjusted to be equivalent for the starting liver samples. (B) Western blot after anti-Bcl-xL immunoprecipitation. Whole cellular lysate and immunoprecipitates with anti-Bcl-xL were verified with the indicated antibodies. Samples from Bcl-xL−/− mice were included as a negative control.

and suggest that tBid binding of Bcl-xL unleashes Bak or Bax from Bcl-xL.

A Small But Significant Level of tBid Is Detected in the Healthy Liver. Genetic evidence that Bid is required for Bak/Bax-dependent apoptosis caused by Bcl-xL deficiency and biochemical evidence that full-length Bid is inactive for displacing Bak or Bax from Bcl-xL together suggest that tBid is produced in wild-type liver. To confirm this, we performed western blot analysis using antibody that can detect tBid (Fig. 5A). Liver lysate from Bid KO mice served as a negative control, whereas that from wild-type mice injected with anti-Fas antibody served as a positive control. A significant level of tBid was detected in wild-type liver, although the amount was smaller than in Fas-stimulated mice, which displayed massive live cell apoptosis.

Bcl-xL–Deficient Mitochondria Are Susceptible to a Trace Amount of tBid. To examine the impact of a small amount of tBid on Bcl-xL–deficient mitochondria, tBid or full-length Bid at various concentrations was incubated with mitochondria isolated from Bcl-xL–deficient liver or wild-type liver (Fig. 5B). In agreement with previous reports,[19] wild-type mitochondria efficiently released cytochrome c on exposure to tBid. Full-length Bid was far less effective at releasing cytochrome c. Importantly, Bcl-xL–deficient mitochondria were capable of releasing cytochrome c on exposure to a smaller amount of tBid than wild-type mitochondria. This agrees with the in vivo findings that Bcl-xL–deficient hepatocytes, but not wild-type hepatocytes, underwent apoptosis with a trace amount of tBid.

Administration of ABT-737 Produces ALT Elevation in Wild-Type Mice But to a Lesser Extent in Bid KO Mice. Bcl-2–like molecules have been receiving attention as a target for inducing apoptosis, especially in cancer cells.[20] A variety of BH3 mimetics that interact with the hydrophobic groove of anti-apoptotic Bcl-2 proteins has been developed. They inhibit binding of anti-apoptotic Bcl-2–like molecules with BH3-only proteins and presumably with Bak and Bax. ABT-737, a prototype of this class of agents, was designed to mimic the BH3-only protein Bad and can inhibit the function of Bcl-2, Bcl-xL, or Bcl-w but not that of Mcl-1.[21] Our data on Bcl-xL KO mice raised the possibility that pharmacological inhibition of Bcl-xL may cause hepatocyte apoptosis. To examine this possibility, we injected ABT-737 and examined the liver injury. As expected, the levels of ALT

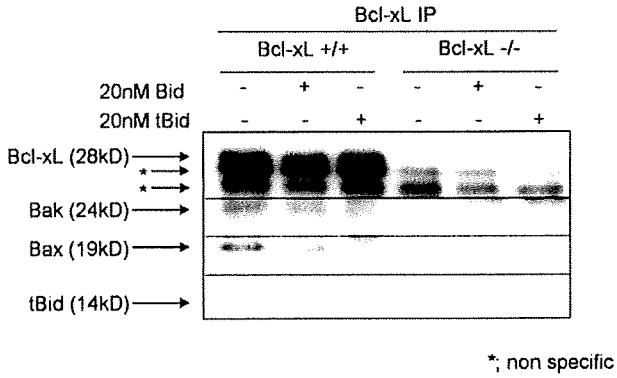

Fig. 4. tBid binds to Bcl-xL and displaces Bak or Bax from Bcl-xL. Liver lysate from *bcl-x*$^{flox/flox}$ without *AlbCre* (Bcl-xL+/+) and *bcl-x*$^{flox/flox}$ with *AlbCre* (Bcl-xL−/−) were incubated with or without 20 nM recombinant tBid or recombinant full-length Bid at 37°C for 20 minutes. After immunoprecipitation with Bcl-xL, immunoprecipitates are verified with indicated antibodies. Immunoprecipitated lysate from Bcl-xL−/− mice was loaded as a negative control.

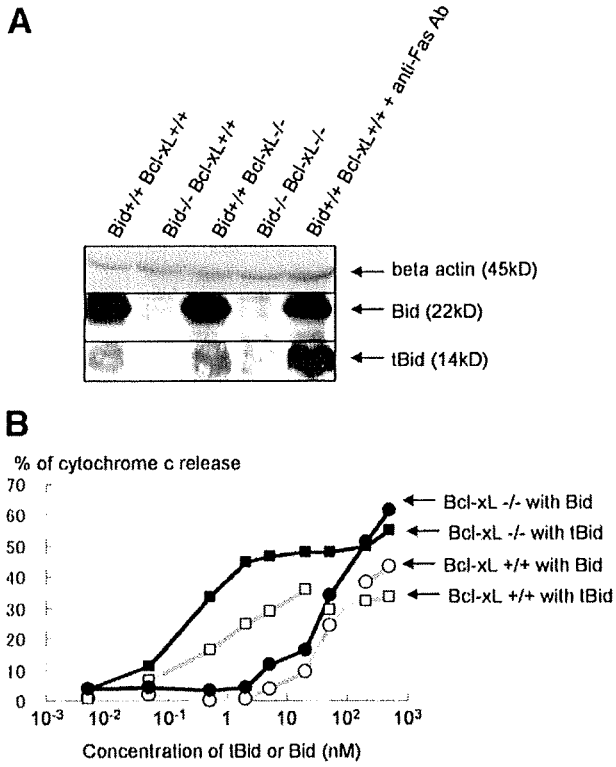

Fig. 5. A small amount of tBid is expressed in wild-type liver and is sufficient for producing cytochrome c release from Bcl-xL–deficient mitochondria. (A) Western blot of liver lysate for Bid and tBid expression. Lysate from wild-type (Bid+/+ Bcl-xL+/+) mice 1 hour after intravenous injection of 10 μg anti-Fas antibody (clone Jo2) and from Bid−/− mice were included as a positive and a negative control of tBid, respectively. (B) Mitochondrial release of cytochrome c to tBid. Mitochondria were isolated from Bcl-xL–deficient or wild-type liver and incubated with recombinant tBid or recombinant full-length Bid at various concentrations for 30 minutes. Similar results were obtained in three times repeated experiments.

were clearly elevated in wild-type mice (Fig. 6A). TUNEL staining of the liver section showed apoptosis in hepatocytes scattered in the liver lobule (Fig. 6B). Importantly, no significant elevation of serum ALT levels was observed with a Bak/Bax double-KO background. The data indicated that genetic and pharmacological ablation of Bcl-xL led to a similar apoptosis phenotype in the liver.

To examine the impact of Bid in ABT-737–induced hepatocyte apoptosis, ABT-737 was administered to wild-type mice and Bid KO mice. Elevation of serum ALT levels was ameliorated with a Bid KO background (Fig. 6C). It has been well established that administration of ABT-737 led to acute thrombocytopenia.[22] This was explained by the fact that Bcl-xL is a critical apoptosis antagonist in platelets.[10] In our experiment, the counts of circulating platelets declined significantly in the wild-type mice (Fig. 6D), which is in the agreement with previous studies.[10] Interestingly, a similar degree of thrombocytopenia was observed even in Bid KO mice, suggesting that Bid does not play a significant role in regulating platelet homeostasis, unlike in hepatocytes. The data imply that the impact of Bid in the Bcl-2 network in healthy cells is cell-type specific.

Discussion

One of the important findings of the current study is that the BH3-only protein Bid is an essential molecule for apoptosis of differentiated hepatocytes caused by Bcl-xL deficiency. This is surprising, because differentiated hepatocytes are generally considered to be quiescent cells. Organ homeostasis may be ensured in two ways: one is through turnover of cells, and the other is by the quies-

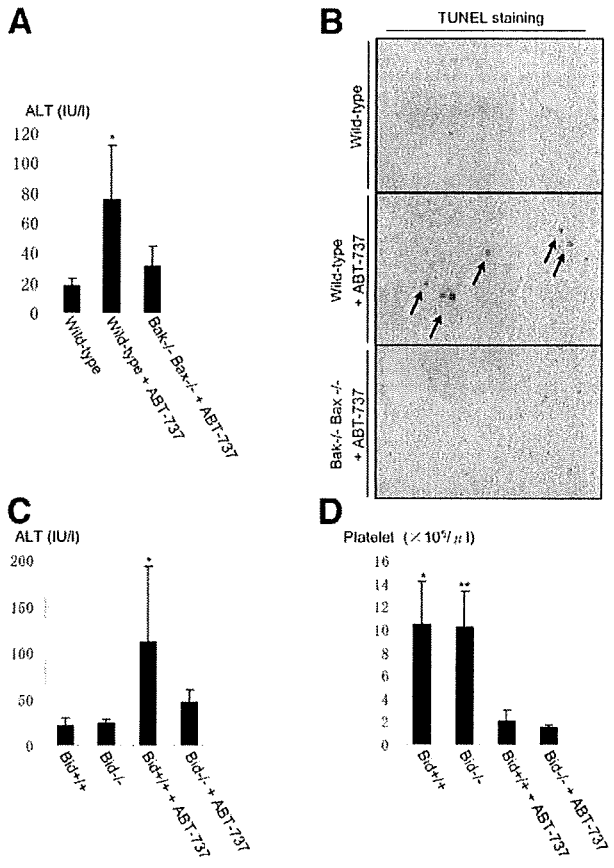

Fig. 6. ABT-737 administration in wild-type, Bak/Bax double-KO, and Bid KO mice. (A and B) Wild-type mice or hepatocyte-specific Bak/Bax double-KO mice were challenged with intraperitoneal injection of ABT-737 at 100 mg/kg or vehicle alone and sacrificed 16 hours later. Serum ALT levels (A) and representative pictures of TUNEL staining in the liver (B) are shown. N = 5 or more than 5 mice per group. *$P < 0.05$ versus the other two groups. (C and D) Wild-type mice or Bid KO mice were challenged with intraperitoneal injection of ABT-737 at 100 mg/kg or vehicle alone and sacrificed 16 hours later. Serum ALT levels (C) and circulating platelet counts (D) were determined. N = 5 or more than 5 mice per group. *$P < 0.05$ versus the other three groups for (C); * and **$P < 0.05$ versus the other two groups, with ABT-737 for (D).

cence of matured cells. Typical examples for the former are hematopoietic organs, intestine and skin, whereas those for the latter are a variety of solid organs, such as the liver, lung, pancreas, heart, and brain. Because hematopoietic cells die at particular time points to maintain host homeostasis, it would not be surprising that their life span may be controlled by a variety of death signals. Indeed, Bim KO mice have excess hematopoietic cells, particularly lymphocytes, suggesting that Bim strictly controls homeostasis of hematopoietic cells.[23] In contrast, healthy cells in the solid organs are usually considered to not suffer from apoptotic stimuli. Although interaction between core Bcl-2 proteins and BH3-only proteins is important for understanding apoptosis regulation, little work has been done by generating mice simultaneously deficient in molecules of both groups. To the best of our knowledge, the only example clearly using this approach is a study on Bim/Bcl-2 double-KO mice that showed that growth retardation, skin abnormality, and lymphoid cell reduction found in Bcl-2 KO mice were ameliorated with a Bim-deficient background.[24] This suggested that lymphoid cells constitutively sense Bim-mediated killing signals, and, without Bcl-2, decrease in number. The current study is the first demonstration that parenchymal cells in a solid organ such as differentiated hepatocytes also suffer from Bid-mediated BH3 stress.

Bid is ubiquitously expressed in many cell types. Generally, Bid is inactive for death induction and is activated on proteolytic cleavage by caspase-8 or other proteases. In the current study, we found that not only full-length Bid but also tBid could be detected in wild-type liver. Administration of tBid, but not that of full-length Bid, at 20 nM in wild-type liver lysate or mitochondria was sufficient for unleashing Bak or Bax from Bcl-xL and releasing cytochrome c. Conversely, a lesser amount of tBid (for example, at 2 nM) was sufficient for inducing cytochrome c release from Bcl-xL–deficient mitochondria. These results are consistent with the idea that a small amount of tBid produced in the liver could activate cytochrome c release and apoptosis in hepatocytes of the Bcl-xL–deficient mice. What mechanisms are involved in the production of tBid from full-length Bid in the healthy liver is not known yet. Our results suggest that Myd88 and TNF-α may not be involved in the activation of tBid under physiological conditions. However, other ligation of death receptors such as Fas, and TNF-related apoptosis-inducing ligand receptor, can cause caspase-8 activation followed by Bid cleavage.[15,25] Bile salts, which are consistently produced in and secreted from hepatocytes, are capable of inducing hepatocyte apoptosis through Fas activation.[26] Natural killer cells are predominant lymphocytes accumulating in the liver and constitutively express TNF-related apoptosis-inducing ligand.[27] Further study is needed to examine what kinds of stresses activate the Bid pathway in a physiological setting.

Adult differentiated hepatocytes express at least two anti-apoptotic Bcl-2 proteins, Bcl-xL and Mcl-1, but not prototype Bcl-2.[8] Recently, Vick et al.[28] reported that hepatocyte-specific Mcl-1 KO mice developed naturally occurring apoptosis in hepatocytes. We also independently generated hepatocyte-specific Mcl-1 KO mice and obtained an apoptosis phenotype that could not be distinguished from that of hepatocyte-specific Bcl-xL KO mice.[29] Thus, Mcl-1, like Bcl-xL, plays a critical role in maintaining integrity of differentiated hepatocytes. There are two major models regarding how BH3-only proteins mediate Bak/Bax-dependent apoptosis: a direct model and an indirect model.[30] From the viewpoint of the indirect model, our data would mean a small amount of tBid is sequestered by Bcl-xL and Mcl-1 and, without Bcl-xL, is sufficient for neutralizing Mcl-1 to promote apoptosis. Conversely, from the viewpoint of the direct model, both Bcl-xL and Mcl-1 are needed to completely sequester a small amount of tBid, and without Bcl-xL, unleashed tBid would directly activate Bak and Bax. In the current study, we observed that tBid when administered in liver lysate could bind to Bcl-xL. This observation seems to agree with the indirect model, although we could not exclude the possibility of the direct model. Further study will be needed by developing Bid/Bcl-xL/Mcl-1 KO mice to examine the underlying mechanisms of how activated Bid regulates the mitochondrial pathway of apoptosis in the liver.

Malignant tumors frequently overexpress one or more members of the anti-apoptotic Bcl-2 family, which confers the resistance of tumor cells to apoptosis.[31,32] Recently, small molecules targeting specific anti-apoptotic Bcl-2 family proteins have been developed for treatment of cancer therapy.[33,34] The underlying concept of this strategy is the difference in addiction to anti-apoptotic Bcl-2 family proteins between normal cells and transformed cells. In general, normal cells are not considered to suffer from apoptotic stimuli or to have activated BH3-only proteins. In contrast, transformed cells suffer from a variety of apoptotic stimuli such as genotoxic *p53* activation and environmental stresses, and possess activated BH3-only molecules. If a single anti-apoptotic Bcl-2 protein is neutralized by a small molecule, it could release BH3-only molecules, which then neutralize other anti-apoptotic Bcl-2 proteins or directly activate Bax-like molecules, leading to cell death. However, the current study clearly indicated that normal hepatocytes could be under activation of Bid, raising concern that hepatocyte injury may be produced if Bcl-xL function is completely knocked down. Indeed, we have shown that administra-

tion of a high dose of ABT-737, which is an antagonist for Bcl-xL/Bcl-2, not for Mcl-1,[21] induced Bak/Bax-dependent hepatocyte apoptosis in wild-type mice but to a lesser extent in Bid KO mice. Therefore, special caution should be paid to hepatotoxicity when systemically administering a high dose of Bcl-xL–targeting molecules, because hepatocytes are suffering from Bid-mediated stresses.

In conclusion, we have demonstrated here that the BH3-only protein Bid is activated and antagonized by anti-apoptotic Bcl-2 family proteins under physiological conditions. BH3 stress or Bcl-2 addiction is not a unique characteristic of tumor cells. Even in healthy cells, cellular integrity is not controlled by a simple rheostat between Bax-like molecules and Bcl-2–like molecules. The current study reveals a previously unrecognized complicated network of Bcl-2 family proteins controlling the integrity of healthy cells. Dissection of the Bcl-2 network will be important for further understanding of liver pathophysiology.

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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

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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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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

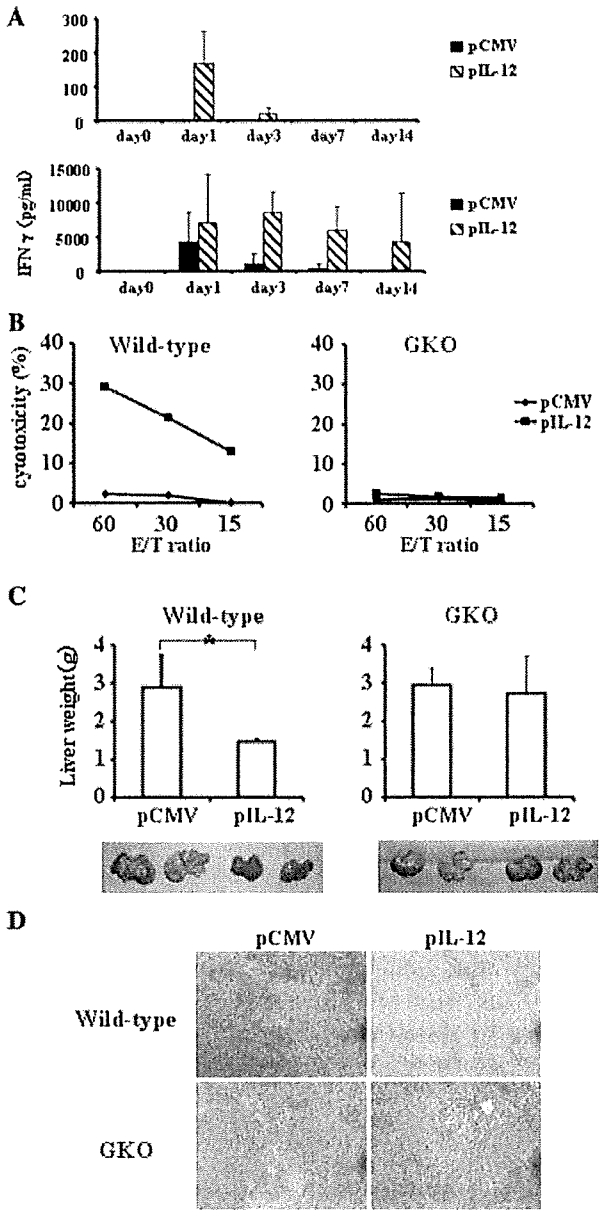

Fig. 1 Effects of hydrodynamic injection of IL-12-encoding plasmid. **a** Wild-type mice were hydrodynamically injected with either pCMV-IL-12 (*hatched bars*) or pCMV (*closed bars*) and bled at the indicated time points to measure the levels of serum IL-12 and IFNγ. Results are indicated as mean and 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

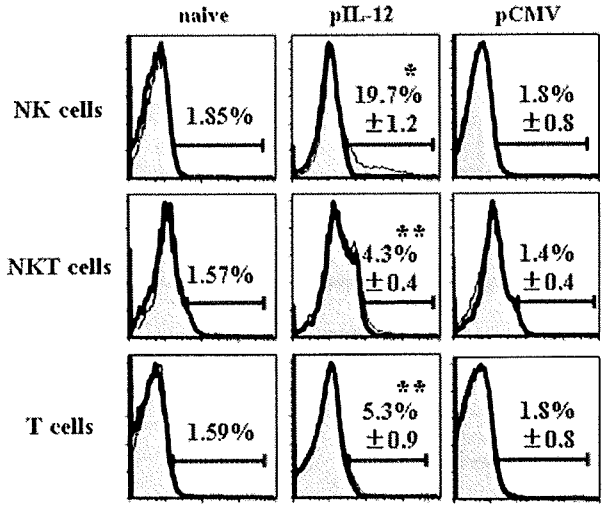

Fig. 2 IFNγ expression of mononuclear cells after IL-12 administration. Wild-type mice were injected with pCMV-IL-12 or pCMV, or were untreated (naive). Mononuclear cells were isolated from the liver 2 days after plasmid injection and stained with anti-TCRβ mAb, anti-DX5 mAb and anti-IFNγ mAb. *Closed histograms* show the IFNγ expression in the gated populations ($TCR\beta^-/DX5^+$ cells for NK cells, $TCR\beta^+/DX5^+$ cells for NKT cells and $TCR\beta^+/DX5^-$ 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

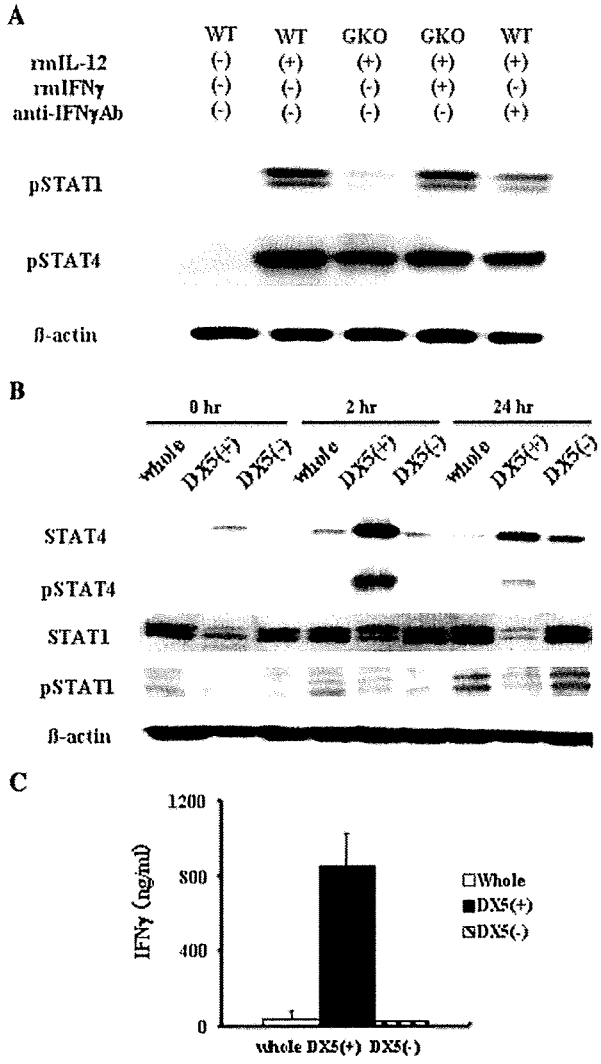

Fig. 3 STAT signaling and IFNγ production of mononuclear cells in vitro treated with IL-12. **a** STAT1 and STAT4 activation of splenocytes in vitro treated with IL-12. Splenocytes were isolated from wild-type mice or GKO mice and treated with or without recombinant IL-12 (20 ng/mL) in the presence or 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

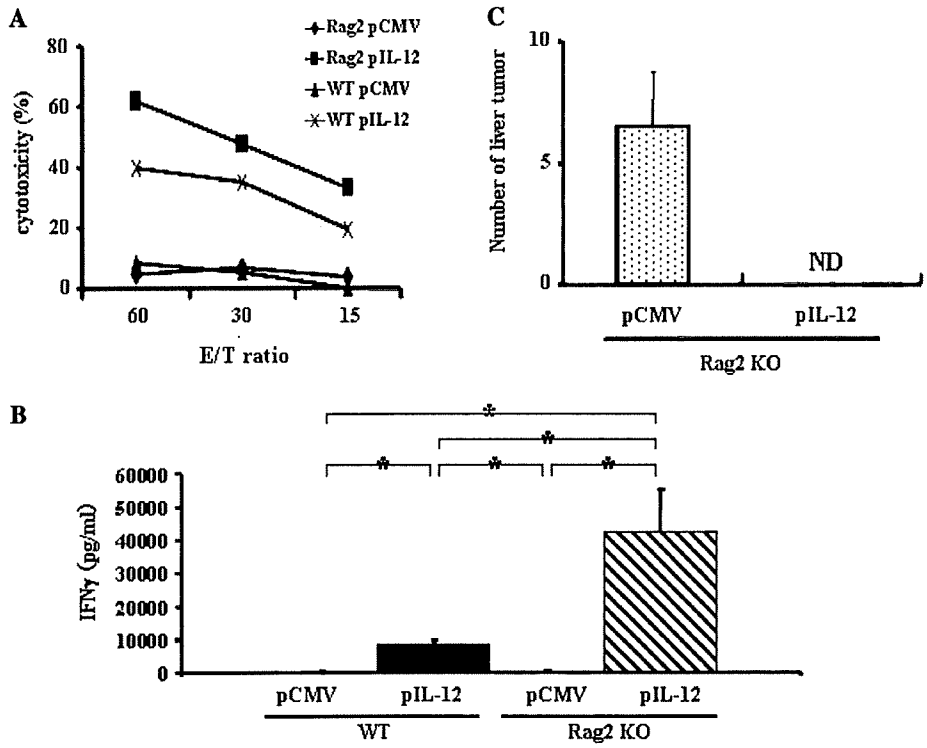

Fig. 4 Anti-tumor effects of IL-12 in Rag2 KO mice. Serum IFNγ levels and NK-cell activation. Wild-type or Rag2 KO mice were hydrodynamically injected with either pCMV-IL-12 or pCMV and killed at 4 days. **a** Yac1 lytic ability of hepatic mononuclear cells was determined by Cr releasing assay as the 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,

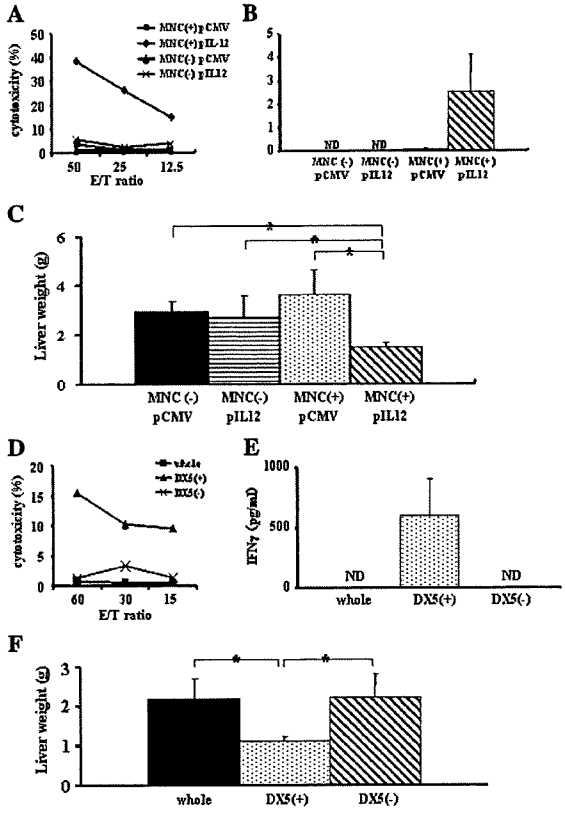

Fig. 5 Adoptive transfer of wild-type cells into GKO mice. Adoptive transfer of wild-type splenocytes restored anti-tumor effects of IL-12 in GKO mice. **a** GKO mice were intravenously injected with or without 2.0×10^8 splenocytes from wild-type mice and, 1 day later, hydrodynamically injected with 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

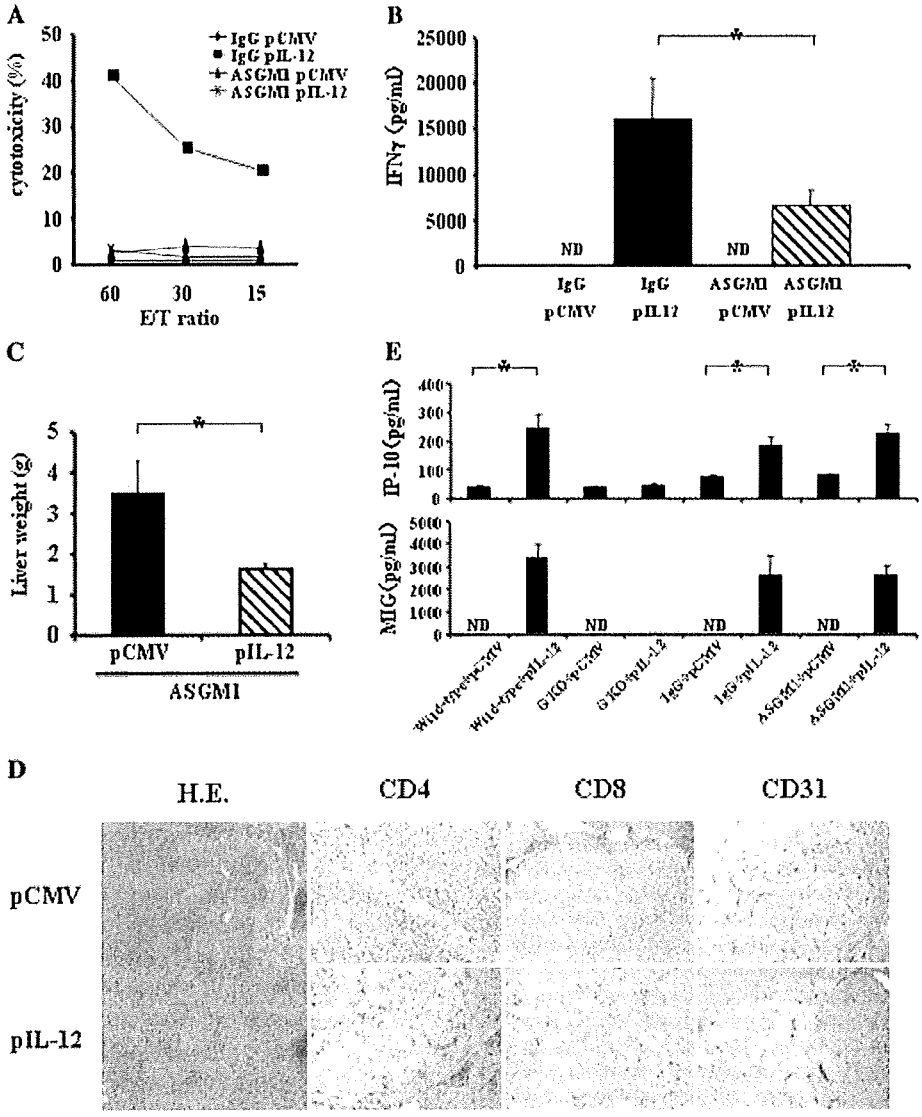

Fig. 6 Anti-tumor effects of IL-12 in NK-cell-depleted mice. Serum IFNγ levels and NK-cell activation. Wild-type mice were intraperitoneally injected with either anti-asialoGM1 antibody (ASGM1) or control IgG, and, 1 day later hydrodynamically injected with either pCMV-IL-12 or pCMV. Mice were killed 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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Activated liver dendritic cells generate strong acquired immunity in α-galactosylceramide treatment☆

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***Background/Aims*: α-Galactosylceramide (α-GalCer) presented by dendritic cells (DCs) activates NKT cells that in turn drive DC maturation. However, the potential of generating acquired immunity of liver DCs in α-GalCer treatment remains unclear.**

***Methods*: We examined the activation of acquired immunity in the α-GalCer treatment against liver or spleen tumor and the ability of liver and spleen DCs in the generation of acquired immunity.**

***Results*: Administration of α-GalCer resulted in generation of p53 peptide-specific cytotoxic T lymphocytes (CTLs) in mice bearing liver CMS4 tumor, aberrantly expressing p53, but not in mice bearing spleen CMS4 tumor. The growth of rechallenged CMS4 subcutaneous tumor was inhibited in α-GalCer-treated mice against liver CMS4 tumor, but not in α-GalCer-treated mice against CMS4 spleen tumor. The antigen presenting related functions of liver DCs were significantly higher than those of spleen DCs in α-GalCer-treated mice. Vaccination of normal mice with p53 peptide pulsed liver DCs isolated from α-GalCer treated mice resulted in generation of p53 peptide-specific CTLs, but that with p53 peptide pulsed spleen DCs did not.**

***Conclusions*: These results demonstrated that α-GalCer treatment induced unique immunologic activation of liver DCs in comparison with spleen DCs, which might be favorable to generate liver acquired immunity.**

***Keywords*: α-Galactosylceramide; Liver dendritic cells; Acquired antitumor immunity**

1. Introduction

α-Galactosylceramide (α-GalCer) presented by CD1d molecules expressing on dendritic cells (DCs) efficiently stimulates NKT cells implicated in innate immunity [1,2]. Recently, *in vivo* animal studies have shown that systemic administration of α-GalCer can lead to anti-tumor effects against metastatic liver tumor [3,4], suggesting that α-GalCer treatment might be promising for clinical application against liver tumor. Metastatic liver tumors resist conventional chemotherapy and radiotherapy, and present with a poor prognosis. Thus novel and more effective immunotherapy is needed, especially for metastatic liver cancer. Several phase I clinical studies have been done in cancer immunotherapy using intravenous administration of α-GalCer, but with limited clinical responses [5,6]. For further development of α-GalCer treatment in liver cancer patients, the antitumor effect of α-GalCer should be more precisely examined in the liver.

DCs effectively elicit immune responses to self and foreign antigens [7,8]. These specialized antigen-presenting cells (APCs) can induce the generation of both

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Abbreviations: DC, dendritic cell; APC, antigen-presenting cells; CTLs, cytotoxic T lymphocytes; α-GalCer, α-galactosylceramide; M-NC, mononuclear cells.

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antigen-specific cytotoxic T lymphocytes (CTLs) and T helper cells. α-GalCer administration resulted in maturation of spleen DCs and activation of the CD8+ T cell immune response via costimulatory molecules expressed on the spleen DCs [9,10]. However, in contrast to well-characterized spleen DCs, the details of activation of liver DCs by α-GalCer treatment remains to be clarified because of the difficulty of procuring adequate numbers of isolated liver DCs for functional analysis [11]. Although most previous studies reported that α-GalCer treatment induces early activation of liver NKT and NK cells [3,4,12], which were the main effector cells to eradicate metastatic tumor cells, little is known regarding the induction of liver acquired immunity after early rejection of liver tumor. Nakagawa et al. reported that CD122+CD8+ memory T cells play critical roles in metastatic liver tumor rejection by α-GalCer treatment [13]. However, the ability of α-GalCer to activate liver DCs and generate acquired immunity remains to be clarified.

In the current study, we evaluated the induction of acquired immunity by α-GalCer activated liver DCs in comparison with spleen DCs. We demonstrated that α-GalCer treatment resulted in generating strong acquired immunity after liver tumor treatment, but not after spleen tumor treatment. We also show that α-GalCer treatment activated liver DCs more strongly with respect to the antigen-presenting function and antigen-specific CTL induction than spleen DCs. Thus, α-GalCer treatment resulted in unique immunologic activation of liver DCs, which might contribute to induction of acquired immunity in the liver.

2. Materials and methods

2.1. Mice and cell lines

Six-to-ten-week-old female BALB/c mice and C57BL/6 mice were purchased from Shizuoka Experimental Animal Laboratory (Shizuoka, Japan). The animals were handled under aseptic conditions. Procedures were performed according to approved protocols and in accordance with recommendations for the proper care and use of laboratory animals. CMS4 sarcomas (H-2^d) express mutated p53 and present the wild-type $p53_{232-240}$ epitope recognized by H-$2K^d$-restricted CTLs [14,15], and MC38 colon cancer cell lines were maintained as previously described [16]. α-Galactosylceramide (α-GalCer) was kindly provided by Kirin Pharma (Gunma, Japan) and prepared as previously described [15].

2.2. IFN-γ ELISPOT assays for p53 peptide-reactive CD8+ T cells responses after α-GalCer treatment for CMS4 tumor and animal experiments

To examine the induction of the acquired antitumor immunity, BALB/c mice were injected intrahepatically or intrasplenically with 5×10^5 CMS4 cells on day 0 and treated intraperitoneally (i.p.) with α-GalCer (2 μg/100 μl) or 100 μl of vehicle on day 1. Fourteen days after α-GalCer treatment, CD8+ T cells were isolated from the spleen of immunized mice by using magnetic beads (MACS, Miltenyi Biotec, Gladbach, Germany). Next, CD8+ T cells (1×10^5 cells/well) and syngeneic bone marrow derived DCs (BMDCs) generated from normal BALB/c mice (2×10^4 cells/well) were cocultured with $p53_{232-240}$ peptide in ELISPOT culture plate. We used mouse IFN-γ ELISPOT kit (R & D Systems, Minneapolis, MN) to detect the $p53_{232-240}$ peptide-specific CD8+ T cell responses, as previously described [16]. To assess the systemic acquired immunity due to α-GalCer treatment, mice were injected in the liver or the spleen with 5×10^5 CMS4 cells or MC38 cells on day 0 and were injected i.p. with α-GalCer on day 1. On day 14 after α-GalCer treatment, 1×10^6 CMS4 cells or MC38 cells were injected as a rechallenge into the right flank of treated mice, respectively. Tumor size was assessed every 7 days.

2.3. Preparation of liver and spleen DCs and flow cytometry

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, hepatic mononuclear cells (MNC) and splenic MNC were prepared as previously described [15]. CD11c+ dendritic cells were isolated from liver MNC and spleen MNC by magnetic cell sorting using MACS (Miltenyi Biotec) according to the manufacturer's protocol. For phenotypic analysis of liver and spleen DCs, PE- or FITC- or APC-conjugated monoclonal antibodies against mouse cell surface molecules [CD11c (Miltenyi Biotec), CD40, CD80, CD86, MHC class II, CD8α and CD11b (all from BD-Pharmingen, San Diego, CA)] were used, and flow cytometric analysis was performed using a FACS Calibur (Becton Dickinson, San Jose, CA) flow cytometer. We defined DCs with CD11c+ MHC class II+ cells by flow cytometry and evaluated the expressions of these antigen presenting related molecules. Data were analyzed using FlowJo software (Tree Star, Ashland, OR) and reported as the mean fluorescence intensity (MFI).

2.4. Cytokine measurement

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. To assess cytokine production, we cultured 2×10^5 DCs in 1 ml of complete medium with LPS (R & D Systems Inc., 10 μg). After 48 h, cell culture supernatants were harvested and tested using a species-specific enzyme linked immunosorbent assay (ELISA) kit for IL-12, IFN-γ and TNF-α (BD-Pharmingen) according to the manufacturer's protocols.

2.5. T cell proliferation assay

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. The DCs were added in various numbers to 5×10^5 allogeneic T lymphocytes (purified using Thy-1.2 immunomagnetic microbeads from C57BL/6 mice) in 96-well U-bottom plates and then pulsed with [^{3}H] thymidine (1 μ Ci/well) on day 3 for an additional 20 h as previously described [17].

2.6. Immunization of p53 peptide-pulsed liver or spleen DCs from α-GalCer-treated mice

Twenty-four hours after i.p. treatment with α-GalCer or vehicle, liver and spleen DCs were prepared as above. Isolated DCs were incubated with $p53_{232-240}$ peptide at concentration of 10 μg/mL per 10^6 DCs/mL for 2 h as previously described [14]. 1×10^6 $p53_{232-240}$ peptide pulsed liver or spleen DCs were injected i.p. into normal BALB/c mice. Five days after i.p. immunization, CD8+ T cells were isolated from the spleen of immunized mice by using magnetic beads (MACS) and were subjected to mouse IFN-γ ELISPOT assay as above described.

2.7. Statistical analyses

The statistical significance of differences between the groups was determined by applying Student's *t*-test with Welch correction after each group had been tested with equal variance and Fisher exact probability test. The statistical significance of the differences in more than three groups was determined by applying one-way ANOVA. Statistical significance was defined as $p < 0.05$.