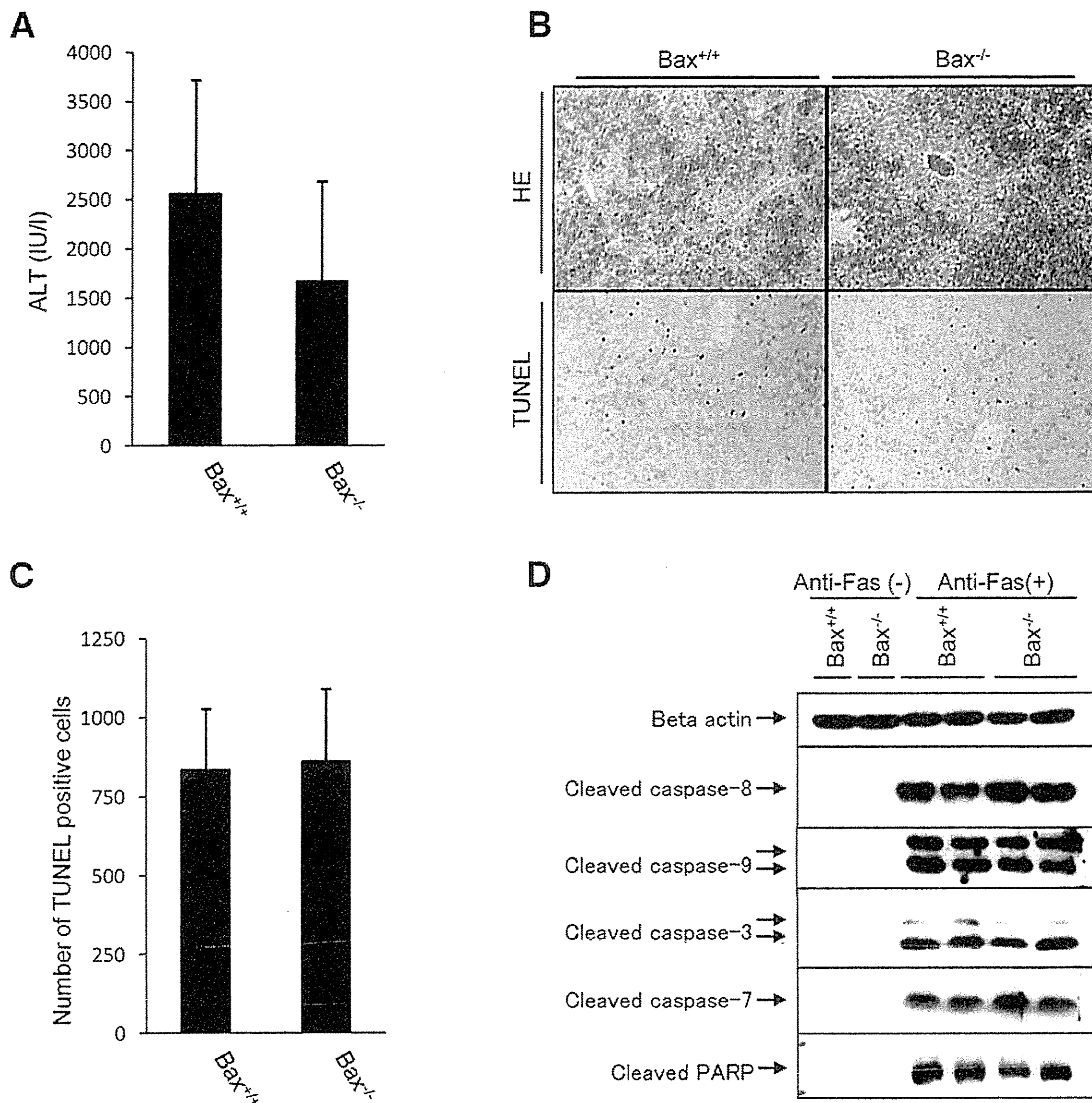

Fig. 2. Bax KO mice are not resistant to Fas-induced hepatocellular apoptosis. Bax KO mice ($Bax^{-/-}$) or control WT littermates ($Bax^{+/+}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (A) Serum ALT levels (n = 11 per group). (B) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (C) Number of TUNEL-positive cells (n = 8 per group). (D) Western blot analysis for the expressions of cleaved caspase-8, -9, -3, -7, and PARP.

PARP in Bax KO livers did not differ from those of WT livers (Fig. 2D). These findings demonstrate that, in contrast to Bak deficiency, Bax deficiency was not able to inhibit Fas-induced hepatocellular apoptosis.

Bax Deficiency Completely Blocks Fas-Induced Early-Onset Hepatocellular Apoptosis in a Bak-Deficient Background. To examine the impact of Bax in a Bak-deficient background, hepatocyte-specific Bak/Bax DKO mice (*bak*$^{-/-}$ *bax*$^{flox/flox}$ *Alb-Cre*) and Bak KO mice (*bak*$^{-/-}$ *bax*$^{flox/flox}$), which served as control littermates of this mating, were injected with Jo2 and analyzed 3 hours later. We confirmed the hepatocyte-specific defects of Bax protein in Bak/Bax DKO mice by way of western blot analysis (Fig. 3A). The serum ALT levels of Bak/Bax DKO mice were in the normal range and were significantly lower than those of Bak KO mice (Fig. 3B). Liver histology and TUNEL staining did not show evidence of hepatocyte apoptosis in Bak/Bax DKO livers, in contrast to Bak KO livers (Fig. 3C,D). Taken together, these results indicate that Bak and Bax are basically redundant molecules for execution of hepatocellular apoptosis induced by Fas

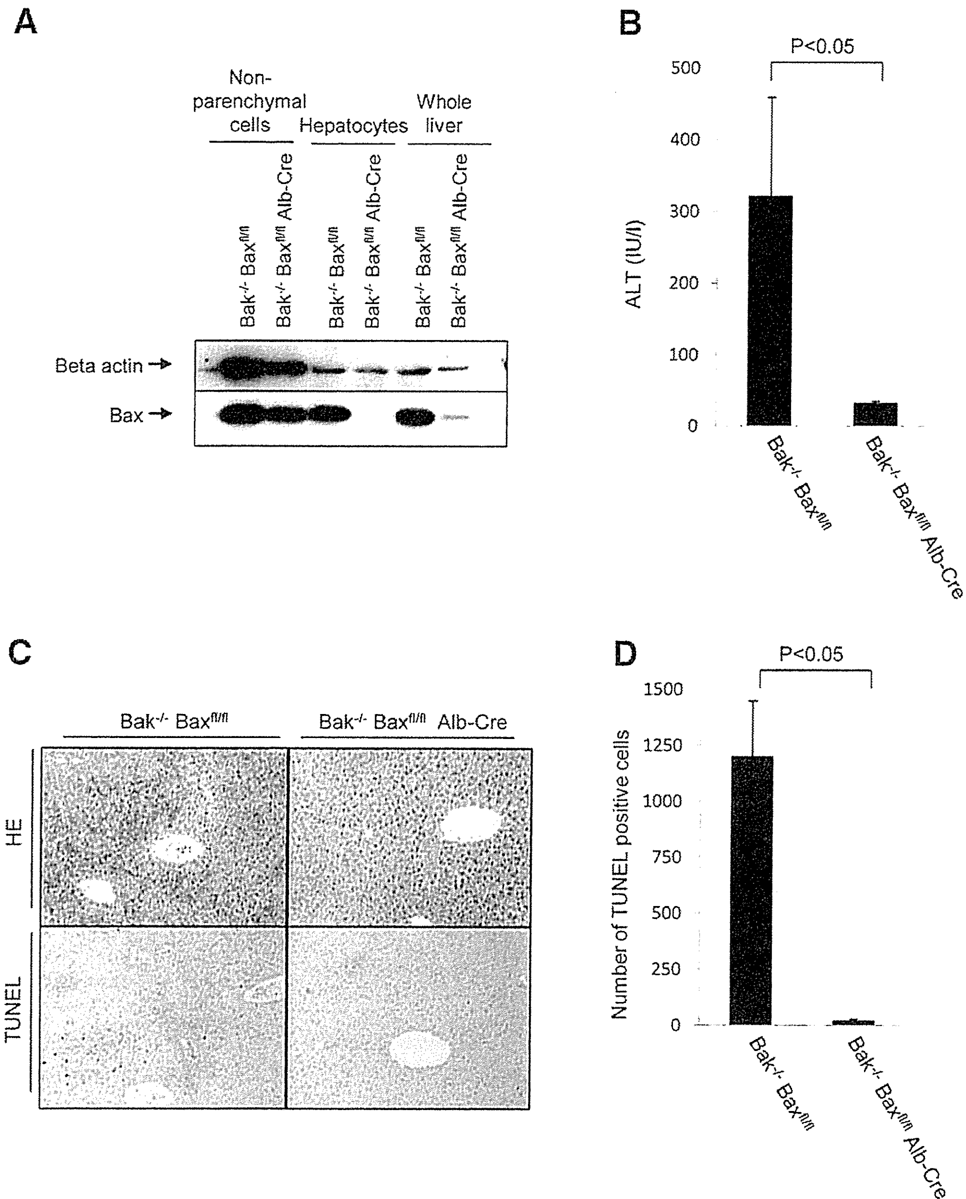

Fig. 3. Bak/Bax DKO mice are fully resistant to Fas-induced hepatocellular apoptosis in early phase. (A) Western blot analysis of the indicated fraction of the liver for the expressions of Bax. (B-D) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (B) Serum ALT levels (n = 10 per group). (C) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (D) Number of TUNEL-positive cells (n = 9 per group).

activation, although the former appears to be clearly required for full-blown apoptosis in vivo.

Fas Stimulation Leads to Late-Onset Hepatocellular Death Even in Bak/Bax Deficiency with Moderate Caspase-3/7 Activation Without Mitochondrial Disruption. To examine whether the inhibition of Fas-induced rapid liver injury in Bak/Bax deficiency is a durable effect, we analyzed the survival rate after Jo2 injection. The survival rate of Bak/Bax DKO mice was significantly higher than that of Bak KO mice, but approximately half of the Bak/Bax DKO mice died within 12 hours (Fig. 4A). To examine the cause of this late-onset lethality, we analyzed the serum ALT levels and liver tissue 6 hours after Jo2 injection. Unexpectedly, the serum ALT levels were highly elevated in Bak/Bax DKO mice (Fig. 4B). Liver histology revealed many hepatocytes with cellular shrinkage and scattered regions of sinusoidal hemorrhage (Fig. 4C), indicating that Bak/Bax DKO mice still developed severe liver injury at this time point. TUNEL staining

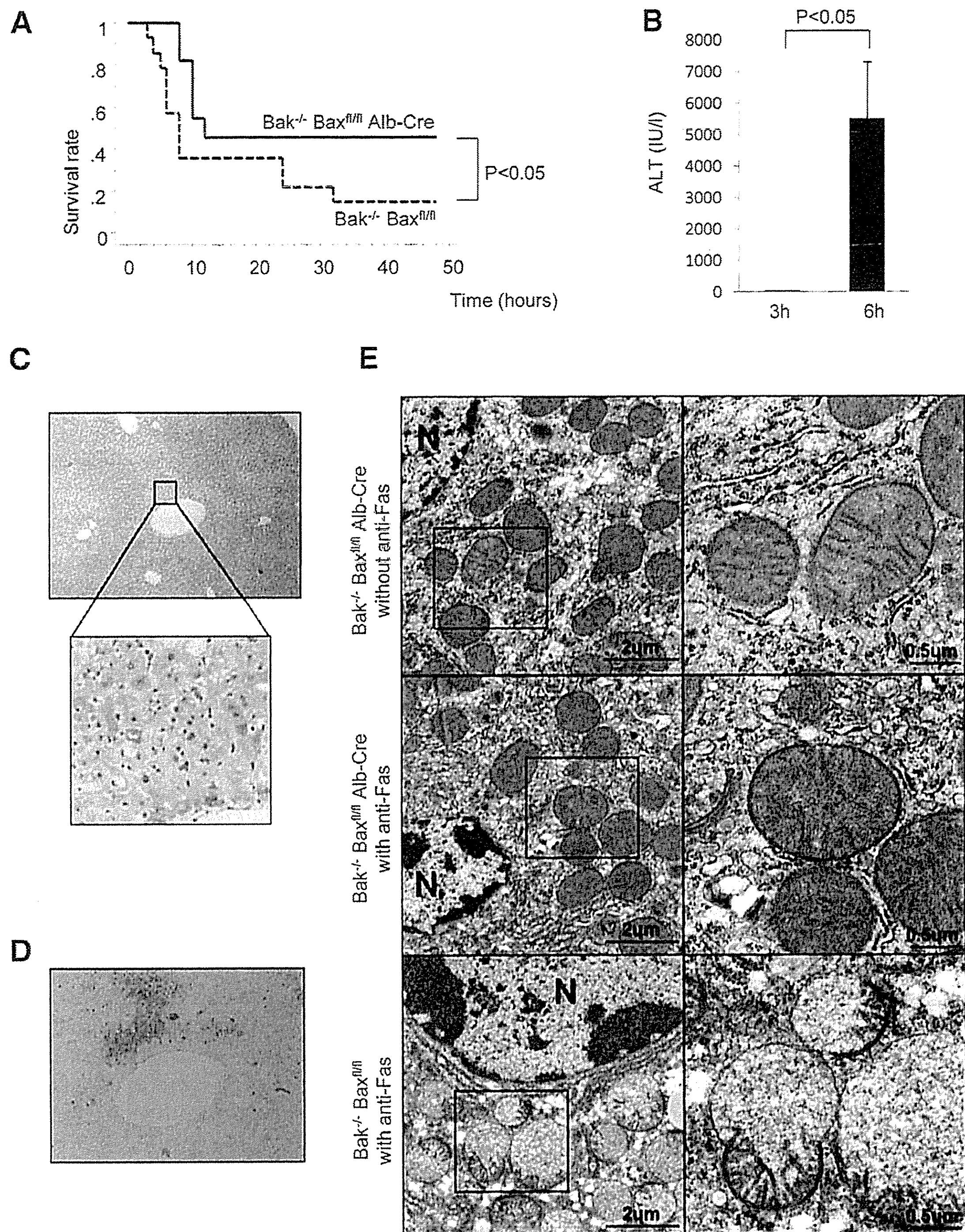

Fig. 4. Bak/Bax DKO mice develop late-onset severe liver injury upon Fas stimulation. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. (A) Survival rate after Jo2 injection (n = 11 per group). (B) Serum ALT levels of Bak/Bax DKO mice. (C, D) Hematoxylin and eosin (C) and TUNEL (D) staining of the liver sections of Bak/Bax DKO mice 6 hours after Jo2 injection. Representative photomicrographs are shown. (E) Representative electron microscopy photomicrographs of the livers of Bak/Bax DKO mice before and 6 hours after Jo2 anti-Fas injection (1.5 mg/kg) and control Bak KO mice 2 hours after Jo2 anti-Fas injection (1.5 mg/kg). Right panels are enlarged images of the square area of each left panel. N, nucleus.

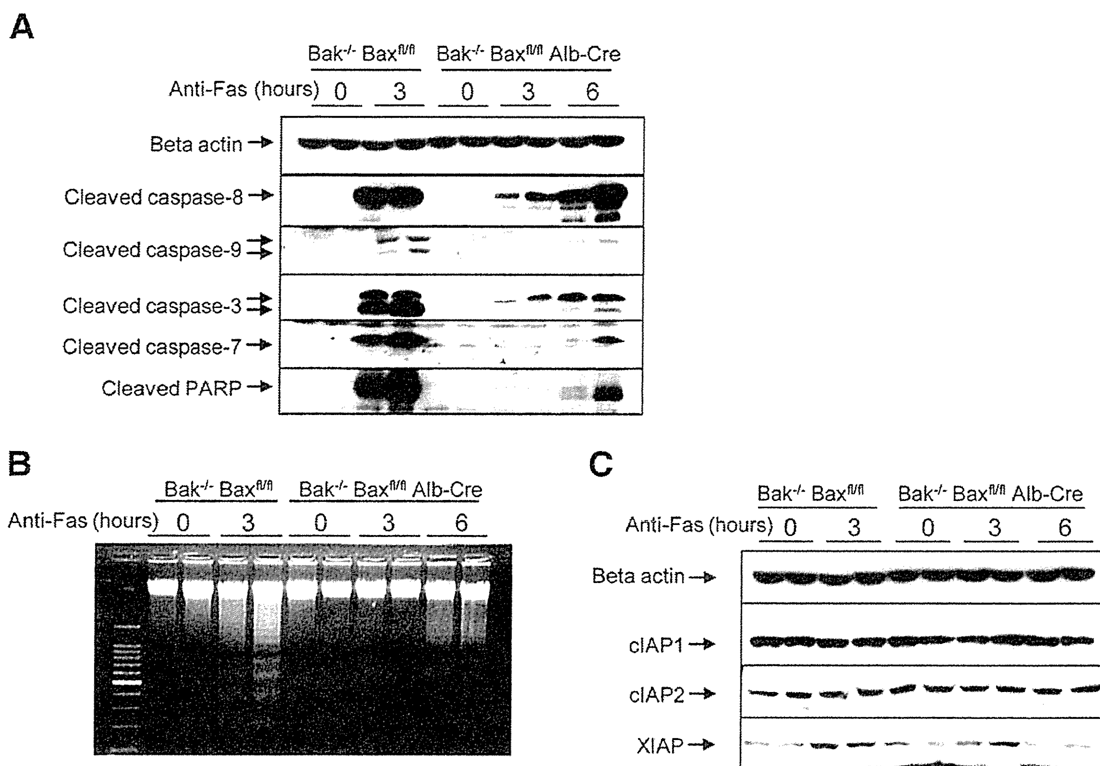

Fig. 5. Fas-mediated hepatocellular death in Bak/Bax DKO mice is associated with caspase-3/7 activation and oligonucleosomal DNA breaks. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with Jo2 anti-Fas antibody (1.5 mg/kg). (A) Western blot analysis for expression of cleaved caspase-8, -9, -3, -7, and PARP. (B) DNA laddering of the liver. (C) Western blot analysis for expression of IAP family proteins.

revealed many TUNEL-positive hepatocytes in the liver sections. Of importance, electron microscopic analysis revealed mitochondrial alterations (such as disruption of the membrane and herniation of the matrix) in hepatocytes of Bak KO mice but not in hepatocytes of Bak/Bax DKO mice with chromatin condensation (Fig. 4E). Because some reports showed that hepatocytes act like type I cells with a high dose of Jo2 anti-Fas antibody and that anti-Fas antibody does not always reliably mimic the action of the physiological Fas ligand,[23,24] we also injected 0.5 mg/kg Jo2 or recombinant Fas ligand into Bak/Bax DKO mice. Similarly, both injected mice showed severe elevation of serum ALT levels and severe hepatitis with many TUNEL-positive cells at 6 hours (Supporting Figs. 1 and 2).

To examine the kinetics of caspase activation and apoptosis in the liver after Jo2 administration, we performed western blot analysis for caspase activation and agarose gel electrophoresis for DNA laddering. All signals for cleaved forms of caspase-3, caspase-7, and PARP in the liver were clearly detected at 6 hours in Bak/Bax DKO mice, although they were weaker than those at 3 hours in control Bak KO littermates (Fig. 5A). Regarding the cleaved form of caspase-9, two bands were detected at 3 hours in Bak KO liver, but not in Bak/Bax DKO liver. Previous research established that procaspase-9 has two sites for cleavage upon activation: both Asp353 and Asp368 sites are autoprocessed by caspase-9 activation after cytochrome c release, whereas the Asp368 site is preferentially processed over the Asp358 site by caspase-3.[25] In our western blot analysis, the slow migrating species corresponding to the fragment cleaved at Asp368, but not the rapid migrating species corresponding to that at Asp353, was weakly detected at 6 hours in Bak/Bax DKO liver. This indicated that caspase-3–mediated cleavage of procaspase-9 takes place without evidence of cytochrome c–induced autoprocessing of procaspase-9. Agarose gel electrophoresis clearly detected oligonucleosomal DNA laddering at 6 hours in Bak/Bax DKO livers, similar to our observation at 3 hours in control Bak KO livers (Fig. 5B). Collectively, these morphological and biochemical data support the idea that hepatocellular death occurring at 6 hours in the Bak/Bax DKO liver seems to involve apoptosis.

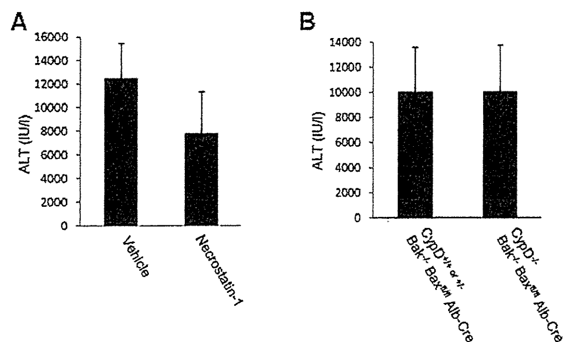

Fig. 6. Fas-induced hepatocellular death in Bak/Bax DKO mice is independent of RIP kinase and/or CypD. (A) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Cre) were intraperitoneally injected with 2 mg/kg necrostatin-1 in vehicle containing 0.2% dimethylsulfoxide or vehicle alone at 2 hours after injection of 1.5 mg/kg Jo2 anti-Fas antibody. Serum ALT levels at 6 hours after Jo2 injection are shown (n = 8 per group). (B) $CypD^{+/+\ or\ +/-}$ mice in a Bak/Bax-deficient background ($CypD^{+/+\ or\ +/-}$ $Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control $CypD^{-/-}$ littermates ($CypD^{-/-}$ $Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. Serum ALT levels at 6 hours after injection are shown (n = 7 per group or 8 per group, respectively).

To examine the underlying mechanisms by which caspase-3/7 was increasingly activated from 3 to 6 hours in Bak/Bax DKO mice, we analyzed the expression of inhibition of apoptosis proteins (IAPs), which can block cleavage of procaspase-3, -7, and -9.[26] The expression levels of cIAP1 and cIAP2 were not changed in the liver after Jo2 injection (Fig. 5C, Supporting Fig. 3). In contrast, the expression levels of XIAP were up-regulated in the livers of both Bak KO and Bak/Bax DKO mice at 3 hours after Jo2 injection, as in WT mice (Fig. 5C, Supporting Fig. 3), which is consistent with previous reports.[27] However, this up-regulation disappeared from the livers of Bak/Bax DKO mice at 6 hours. Repression of XIAP overexpression might explain why weak activation of capsase-3/7 gradually increased from 3 to 6 hours in the Bak/Bax DKO liver.

Cell Death with Bak/Bax Deficiency Is Not Dependent on a Necrotic Pathway. Fas activation was reported to induce not only caspase-dependent apoptosis but also caspase-independent necrosis, which is required for receptor-interacting protein (RIP) kinase.[28] To exclude the possibility of this necrotic cell death in the Bak/Bax DKO liver, we first examined the effect of necrostatin-1, which specifically inhibits RIP kinase to protect against necrotic cell death caused by death-domain receptor stimulation.[2,29] Bak/Bax DKO mice were injected with 2 mg/kg necrostatin-1 at 2 hours after or 1 hour before Jo2 injection. The ALT levels at 6 hours after Fas stimulation were clearly elevated without a significant difference between the necrostatin-1 injection group and the vehicle injection group (Fig. 6A and Supporting Fig. 4). We next examined the effect of CypD, which is a key molecule of mitochondrial permeability transition generated by Ca^{2+} overload and/or oxidative stress leading to necrotic cell death.[14,30] We injected Jo2 into $CypD^{-/-}$ mice with a Bak/Bax-deficient background (*$cypd^{-/-}$ $bak^{-/-}$ $bax^{flox/flox}$ Alb-Cre*) or control $CypD^{+/+\ or\ +/-}$ littermates (*$cypd^{+/+\ or\ +/-}$ $bak^{-/-}$ $bax^{flox/flox}$ Alb-Cre*). The ALT levels of CypD/Bak/Bax triple KO mice upon Fas stimulation were the same as those of control mice (Fig. 6B). These results indicate that liver injury in Bak/Bax deficiency induced by Fas stimulation was not dependent on the necrotic pathway, at least that mediated by RIP kinase and/or CypD.

Late-Onset Cell Death in Bak/Bax Deficiency Is Completely Dependent on Caspase. Although cell death observed in Bak/Bax DKO mice appears to be apoptosis, the question arose of whether relatively weak caspase-3/7 activity compared with that observed in Bak KO mice is sufficient for inducing liver injury 6 hours after Fas simulation. To this end, Bak/Bax DKO mice were given 40 mg/kg Q-VD-Oph, a potent broad spectrum caspase inhibitor,[31] 2 hours after injection of Jo2. Western blot analysis revealed the existence of truncated Bid and cleaved caspase-8 in the liver 2 hours after Jo2 injection, demonstrating that caspase-8 had already been activated by this point (Fig. 7A). Administration of the caspase inhibitor at 2 hours completely blocked the elevation of serum ALT levels and hepatocellular apoptosis, as evidenced by liver histology and TUNEL staining 6 hours after Jo2 injection (Fig. 7B-D). Finally, we tried to analyze the survival rate of Bak/Bax DKO mice and control Bak KO mice when therapeutically injected with the caspase inhibitor 2 hours after Jo2 injection. None of the Bak/Bax DKO mice showed lethal liver injury upon Jo2 injection, whereas half of the Bak KO mice died from severe liver injury (Fig. 7E). These findings suggest that Fas-induced liver injury in Bak/Bax deficiency was dependent on caspase activity, which could be fully negated by the caspase inhibitor. On the other hand, caspase activation in Bak KO mice was too high to be negated by the same dose of the caspase inhibitor.

Discussion

In the present study, we demonstrate that Bak KO, but not Bax KO, provides partial resistance to Fas-induced hepatocellular apoptosis in vivo. We demonstrated previously that Bak KO mice, but not Bax KO

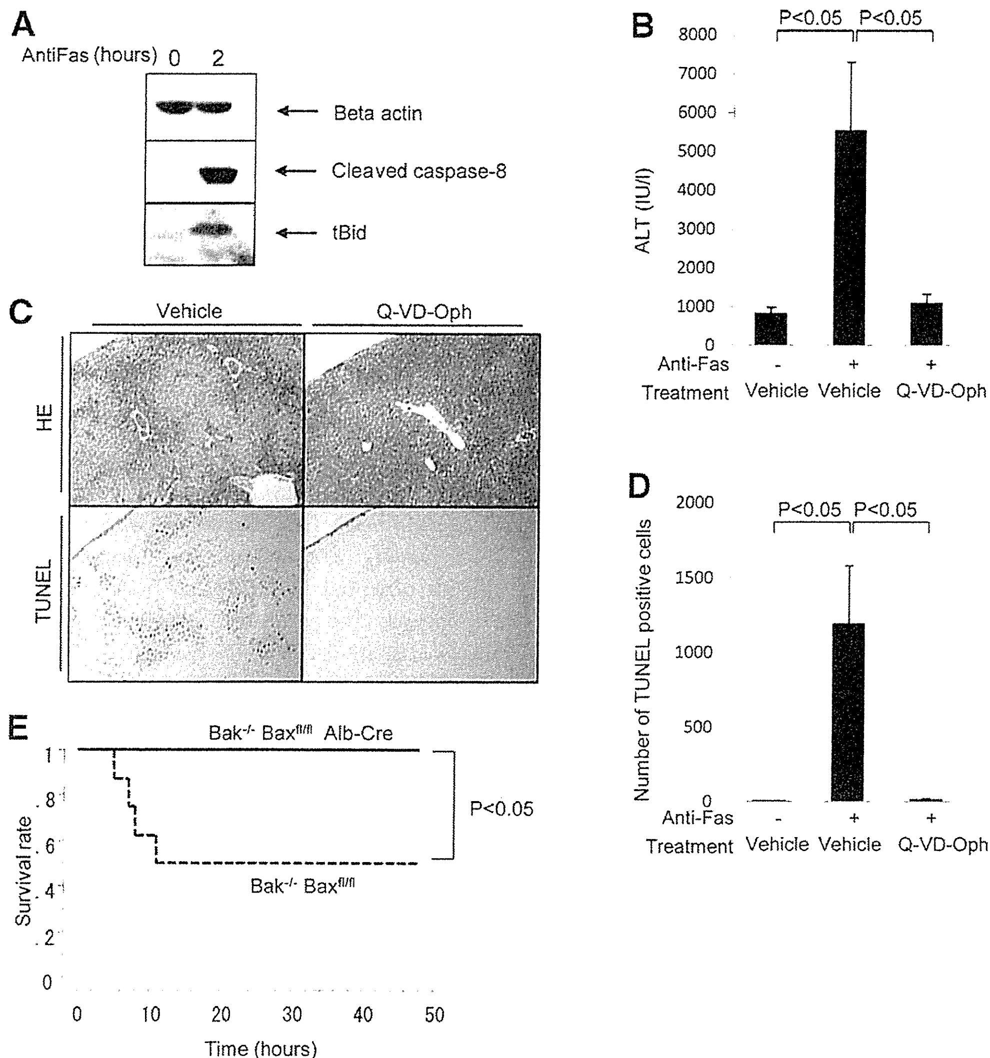

Fig. 7. Hepatocellular death in Bak/Bax DKO mice is dependent on caspase activation. (A) Bak/Bax DKO mice were analyzed before and 2 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). Western blot analysis of the liver for the expression of cleaved caspase-8 and truncated Bid (tBid). (B-D) Bak/Bax DKO mice were intraperitoneally administered 40 mg/kg Q-VD-Oph in 10 mL/kg dimethylsulfoxide (DMSO) or DMSO alone, as a vehicle, 2 hours after injection of 1.5 mg/kg Jo2 anti-Fas antibody and analyzed at 6 hours. (B) Serum ALT levels ($n = 6$ or 7 per group, respectively). (C) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (D) Number of TUNEL-positive cells ($n = 6$ or 7 per group, respectively). Because intraperitoneal injection of DMSO leads to injury at the surface layer of the liver, TUNEL positivity close to the surface layer was excluded from the cell count. (E) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were given 40 mg/kg Q-VD-Oph intraperitoneally in 10 mL/kg DMSO or DMSO alone at 2 hours after injection of 1.5 mg/kg anti-Fas antibody. The disease-free survival rate of lethal liver injury after Jo2 injection is shown ($n = 8$ per group).

mice, showed resistance to apoptosis induced by Bcl-xL deficiency, which depended mainly on Bid activation.[16] Research has shown that Fas induces apoptosis in hepatocytes through the Bid pathway,[10,11] and the present study also demonstrates that Bid becomes truncated in the liver upon anti-Fas injection. Therefore, truncated Bid may preferentially activate Bak rather than Bax in the liver. However, the present study also reveals that, in the absence of Bak, Bax plays an essential role in mediating the early onset of hepatocellular apoptosis. The most important finding of this study is that Bak/Bax deficiency failed to protect against the late onset of liver injury after Jo2 anti-Fas injection as well as Fas agonist injection. Wei et al.,[32] in their historical paper establishing the importance of Bak and Bax in the mitochondrial pathway of apoptosis, reported that hepatocytes were protected from Jo2-induced apoptosis in traditional Bak/Bax DKO mice (*bak*$^{-/-}$ *bax*$^{-/-}$). Because perinatal lethality occurs with most traditional Bak/Bax DKO mice,

they could only analyze three animals, which did not enable detailed analysis of cell death due to Jo2 stimulation. The present study is the first to (1) thoroughly examine the impact of Bak and Bax in the liver using conditional KO mice and (2) demonstrate that Bak/Bax deficiency can protect against Fas-induced severe injury in the early phase but not in the late phase.

The late onset of liver injury observed in Bak/Bax DKO appeared to be apoptosis based on biochemical and morphological observations, including caspase activation, oligonucleosomal DNA breaks and, most importantly, identification of cell death with caspase dependency. In addition, the well-established necrotic pathway mediated by RIP kinase and/or CypD was not involved. However, the difference from apoptosis observed in Bak KO mice was the absence of mitochondrial alteration or cytochrome c–dependent caspase-9 processing in Bak/Bax DKO mice. We also confirmed that Bak/Bax-deficient mitochondria were not capable of releasing cytochrome c in the presence of truncated Bid (Supporting Fig. 5). These data support the idea that activation of the mitochondrial pathway of apoptosis is fully dependent on either Bak or Bax even in the late phase, indicating at the same time that late onset of apoptosis takes place through an extrinsic pathway rather than the mitochondrial pathway.

Although hepatocytes are generally considered to be type II cells, recent work has shown that the requirement of the mitochondrial pathway may be overcome through changes induced by in vitro culture conditions[33,34] or the strength of Fas stimulation.[23] Schüngel et al.[23] demonstrated that hepatocytes act as type II cells with a low-dose Jo2 injection (0.5 mg/kg) and act as type I cells with an extremely high-dose Jo2 injection (5 mg/kg). This agrees with the generally accepted idea that type I cells exhibit strong activation of DISC and caspase-8, which itself is sufficient to induce apoptosis, whereas type II cells exhibit weak activation and therefore require amplification of the apoptosis signal through the mitochondrial loop. In the present study, we used 1.5 mg/kg or 0.5 mg/kg Jo2 antibody, which could be considered relatively low doses, and found that hepatocytes act like type II cells in WT mice or Bak/Bax single KO mice but act like type I cells in Bak/Bax DKO mice. The present study therefore demonstrates that hepatocytes can act as type I cells in the absence of Bak and Bax independent of the strength of DISC formation or signals from microenvironments.

The question arises of why hepatocytes can act as type I cells where the levels of DISC formation or caspase-8 activation may be insufficient to induce activation of downstream caspases. Recently, Jost et al.[27] reported a discriminating role of XIAP between type I and type II cells; in type II cells, the levels of XIAP expression increased after Fas stimulation but decreased in type I cells. In agreement with this report, XIAP expression was up-regulated at 3 hours in both Bak KO and Bak/Bax DKO livers. Interestingly, this XIAP up-regulation disappeared at 6 hours after Jo2 injection in Bak/Bax DKO mice. Because XIAP is a potent inactivator of caspase-3, -7, and -9 processing, repression of XIAP may be one reason why hepatocytes can act as type I cells at this time point.

Previous studies have reported that liver endothelial cells express Fas receptor and have suggested that apoptosis of these cells may participate in the liver damage in mice receiving Jo2 antibody, especially in the case of high-dose administration.[35] However, we did not find liver injury in the sinusoidal hemorrhage in Bak/Bax DKO mice at 3 hours after Jo2 injection, which is the time point when Bak KO mice developed it (Fig. 3C). Together with the fact that Bax, but not Bak, was active in liver nonparenchymal cells in our Bak/Bax DKO mice, as was the case in Bak KO mice (Fig. 3A), we speculate that Bak-deficient sinusoidal cells could not contribute much to liver injury at 3 hours after Jo2 injection (1.5 or 0.5 mg/kg).

Recently, a pan-caspase inhibitor was reported to reduce hepatic damage in liver transplant recipients and patients with chronic hepatitis C in clinical trials.[36,37] For treatment of fulminant liver injury, caspase inhibitors seem to be attractive drugs. However, the present study demonstrates that Fas-induced apoptotic signals could be efficiently amplified through the mitochondrial pathway, leading to high lethality even if caspase inhibitor was administered 2 hours after Jo2 injection. In contrast, administration of the same dose of the caspase inhibitor was able to fully block hepatocyte apoptosis and lethality in Bak/Bax DKO mice. From a clinical point of view, when using caspase inhibitors to prevent fulminant liver failure, concomitant inactivation of the mitochondrial amplification loop may be required.

In conclusion, the extrinsic pathway of apoptosis exists in hepatocytes and causes late onset of lethal liver failure in the absence of Bak and Bax independent of the strength of Fas ligation. This pathway could be therapeutically intervened through the use of caspase inhibitors, presumably due to low levels of DISC formation and subsequent weak activation of effector caspases in hepatocytes. The present study unveils the entire framework of the Fas-mediated signaling

pathway in hepatocytes, placing the mitochondrial pathway of apoptosis as a potent loop for amplifying activation of the caspase cascade to execute complete and rapid cell death in hepatocytes.

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BH3-only Activator Proteins Bid and Bim Are Dispensable for Bak/Bax-dependent Thrombocyte Apoptosis Induced by Bcl-xL Deficiency

MOLECULAR REQUISITES FOR THE MITOCHONDRIAL PATHWAY TO APOPTOSIS IN PLATELETS[S]

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A pivotal step in the mitochondrial pathway of apoptosis is activation of Bak and Bax, although the molecular mechanism remains controversial. To examine whether mitochondrial apoptosis can be induced by just a lack of antiapoptotic Bcl-2-like proteins or requires direct activators of the BH3-only proteins including Bid and Bim, we studied the molecular requisites for platelet apoptosis induced by Bcl-xL deficiency. Severe thrombocytopenia induced by thrombocyte-specific Bcl-xL knock-out was fully rescued in a Bak and Bax double knock-out background but not with single knock-out of either one. In sharp contrast, deficiency of either Bid, Bim, or both did not alleviate thrombocytopenia in Bcl-xL knock-out mice. An *in vitro* study revealed that ABT-737, a Bad mimetic, induced platelet apoptosis in association with a conformational change of the amino terminus, translocation from the cytosol to mitochondria, and homo-oligomerization of Bax. ABT-737-induced Bax activation and apoptosis were also observed in Bid/Bim-deficient platelets. Human platelets, upon storage, underwent spontaneous apoptosis with a gradual decline of Bcl-xL expression despite a decrease in Bid and Bim expression. Apoptosis was attenuated in Bak/Bax-deficient or Bcl-xL-overexpressing platelets but not in Bid/Bim-deficient platelets upon storage. In conclusion, platelet lifespan is regulated by a fine balance between anti- and proapoptotic multidomain Bcl-2 family proteins. Despite residing in platelets, BH3-only activator proteins Bid and Bim are dispensable for Bax activation and mitochondrial apoptosis.

Platelets are unique blood cells that do not have a nucleus but contain mitochondria and have the daily job of handling hemostasis and thrombosis (1). They are produced from megakaryocytes and once released into circulation can function for about 10 days in humans and 4–5 days in mice (2). They are then thought to be destroyed by the reticuloendothelial system. Regarding the mechanism that controls their lifespan, several studies have observed a decrease in mitochondrial membrane potential, caspase activation, and phosphatidylserine exposure in platelets, leading to the conclusion that platelets undergo apoptotic cell death (3–5). It has been demonstrated that platelets contain several apoptosis-related proteins such as Bcl-2 family proteins and a variety of caspase family proteins (3–7). Recently, Mason *et al.* (8) reported that knock-out of a single allele of the *bcl-x* gene results in mild thrombocytopenia, which is ameliorated in a Bak knock-out background. We have also reported previously that thrombocyte-specific homozygous Bcl-xL knock-out mice show marked thrombocytopenia (9). These findings established the critical role of Bcl-2 family proteins in regulating platelet apoptosis and lifespan. Platelets may be the simplest model for the study of Bcl-2 biology with physiological relevance because they neither perform *de novo* protein synthesis nor undergo proliferation.

The proapoptotic multidomain Bcl-2 family proteins Bak and Bax serve as effector molecules for the mitochondrial pathway of apoptosis. Upon activation, they form pores by homo-oligomerization at the mitochondrial outer membrane through which apoptogenic factors such as cytochrome *c* are released into the cytosol (10). Currently, three models for regulation of Bak/Bax-dependent mitochondrial apoptosis by Bcl-2 family proteins have been proposed (11–15). One, referred to as the indirect model or displacement model, argues that Bak and Bax are constitutively active and are neutralized by binding to at least one or more antiapoptotic members of the multidomain Bcl-2 family proteins including Bcl-2, Bcl-xL, Mcl-1, Bcl-w, and Bfl-1/A1. BH3[3]-only proteins such as Bad, Bid, Bim, Noxa, and Puma bind to the antiapoptotic Bcl-2 proteins to unleash Bak and Bax (15). The second model, referred to as the direct model, argues that Bak and Bax are inactive by default and require activator proteins to function. Among BH3-only proteins, Bid and Bim are classified as activator proteins with the others

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[3] The abbreviations used are: BH3, Bcl-2 homology domain 3; Pf4, platelet factor 4; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium.

classified as sensitizer proteins because only Bid and Bim have been demonstrated to directly activate Bak and Bax (16, 17). In this model, Bid and Bim are sequestered by the antiapoptotic Bcl-2 family proteins, and the sensitizer BH3-only proteins bind to the antiapoptotic Bcl-2 proteins to liberate Bid and Bim so they can directly engage Bak and Bax (14). The third model, referred to as the embedded together model, argues that BH3-only activator proteins can recruit not only Bax but also antiapoptotic Bcl-2 proteins to mitochondrial membranes. Although membrane-bound Bax can form oligomers, membrane-bound antiapoptotic Bcl-2 proteins function as a dominant-negative Bax by competitively binding to Bax (12, 18).

In the physiological setting, genetic studies have revealed a functional relationship between BH3-only activator proteins and multidomain Bcl-2 family proteins. For instance, fatal polycystic kidney disease and lymphopenia caused by loss of Bcl-2 are ameliorated in a Bim knock-out background (19). Similarly, we reported previously that spontaneous hepatocyte apoptosis caused by hepatocyte-specific deficiency of Bcl-xL or Mcl-1 is alleviated by Bid deficiency (20, 21). These studies indicated that Bid or Bim is apparently involved in apoptotic phenotypes induced by lack of an antiapoptotic Bcl-2 family protein. However, it had not been established whether or not these direct activators are required for Bak/Bax activation, leading to mitochondrial apoptosis.

In the present study, we explored the molecular requisites for platelet apoptosis induced by Bcl-xL deficiency. We observed complete recovery from severe thrombocytopenia in Bcl-xL knock-out mice with a Bak and Bax double knock-out background, confirming that Bcl-xL deficiency causes apoptotic cell death through a Bak/Bax-dependent mitochondrial apoptosis machinery. Deficiency of either Bid, Bim, or both did not alleviate thrombocytopenia in Bcl-xL knock-out mice. An *in vitro* study revealed that pharmacological inhibition of antiapoptotic Bcl-2 family proteins sufficiently activated Bax protein to cause apoptosis even in Bid/Bim-deficient platelets. Our current study indicates that Bak/Bax can be activated by neutralization of antiapoptotic Bcl-2 family proteins for the execution of apoptotic cell death without involvement of the BH3-only direct activator proteins Bid and Bim in specific cellular contexts.

EXPERIMENTAL PROCEDURES

Mice—Mice carrying a *bcl-x* gene with two *lox*P sequences at the promoter region and a second intron (*bcl-x$^{flox/flox}$*) (22) and heterozygous *pf4-Cre* transgenic mice expressing the Cre recombinase gene under the regulation of the promoter of the platelet factor 4 gene (23) have been described previously. Thrombocyte-specific Bcl-xL knock-out mice (*bcl-x$^{flox/flox}$ pf4-Cre*) (9) and systemic Bid knock-out mice (24) also have been described previously. We purchased C57BL/6J mice from Charles River (Osaka, Japan) and systemic Bim knock-out mice, systemic Bak knock-out mice, systemic Bax knock-out mice, and conditional Bak/Bax double knock-out mice (*bak$^{-/-}$ bax$^{flox/flox}$*) from The Jackson Laboratory (Bar Harbor, ME). We generated thrombocyte-specific Bcl-xL/Bid double knock-out mice (*bid$^{-/-}$ bcl-x$^{flox/flox}$ pf4-Cre*), Bcl-xL/Bim double knock-out mice (*bim$^{-/-}$ bcl-x$^{flox/flox}$ pf4-Cre*), Bcl-xL/Bid/Bim triple knock-out mice (*bid$^{-/-}$ bim$^{-/-}$ bcl-x$^{flox/flox}$ pf4-Cre*), Bcl-xL/Bak double knock-out mice (*bak$^{-/-}$ bcl-x$^{flox/flox}$ pf4-Cre*), Bcl-xL/Bax double knock-out mice (*bax$^{-/-}$ bcl-x$^{flox/flox}$ pf4-Cre*), Bcl-xL/Bak/Bax triple knock-out mice (*bak$^{-/-}$ bax$^{flox/flox}$ bcl-x$^{flox/flox}$ pf4-Cre*), and Bak/Bax double knock-out mice (*bak$^{-/-}$ bax$^{flox/flox}$ pf4-Cre*) by mating the strains. We also generated systemic Bid/Bim double knock-out mice (*bid$^{-/-}$ bim$^{-/-}$*) by mating the strains. Heterozygous *HA-hBcl-xL* transgenic mice expressing human Bcl-xL gene under the regulation of the CAG promoter were generated according to a procedure described previously (25) using a hemagglutinin-tagged human bcl-xL expression plasmid, pcDNA$_3$HAbcl-xL (26). Mice were maintained in a specific pathogen-free facility and treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Hematological Analyses—Blood was collected from the inferior vena cava of mice. Complete blood cell counts were determined using an automated cell counter (Sysmex, Kobe, Japan).

Platelet Isolation, Storage, and Preparation of Lysates—Platelets were isolated as described previously (9). Briefly, whole blood collected from mice or healthy donors was mixed with ¼ volume of citrate-phosphate-dextrose (Sigma-Aldrich). Platelet-rich plasma was obtained by centrifugation at $100 \times g$ for 15 min at room temperature without braking. To avoid mechanical aggregation of platelets by centrifugation, platelet-rich plasma was incubated with 1 μM prostaglandin E_1 (Sigma-Aldrich) and 1 unit/ml apyrase (Sigma-Aldrich) (27). Next, platelets were isolated by centrifugation at $200 \times g$ at room temperature for 15 min. Washed platelets were resuspended in modified Tyrode's buffer (5 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4 \cdot 2H_2O$, 2.8 mM dextrose, pH 7.4) and left standing for 30 min before use. In some experiments, platelet-rich plasma or platelet suspension was stored under continuous gentle agitation in an incubator at 25 °C for the indicated time. For preparation of cell lysates, the platelet pellet was obtained by centrifugation at $200 \times g$ at room temperature for 10 min after incubation with 1 μM prostaglandin E_1 (Sigma-Aldrich) for 10 min and lysed in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1× protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan), 1× phosphatase inhibitor mixture (Nacalai Tesque), phosphate-buffered saline, pH 7.4) unless otherwise indicated. The platelet lysates were cleared by centrifugation at $10,000 \times g$ at 4 °C for 15 min. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). We confirmed that incubation with prostaglandin E_1 did not affect the caspase-3/7 activity of isolated platelet supernatant (data not shown).

In Vitro ABT-737 Experiment—ABT-737, provided by Abbott Laboratories (Abbott Park, IL), was dissolved with DMSO. Platelets were treated with 10 μM ABT-737 or DMSO for the indicated times.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Assay—The MTS assay is a colorimetric assay for measuring the ability of living cells to reduce the uncolored MTS substrate to purple formazan. In platelets, this activity is directly related to cellular viability (4, 5). The MTS assay was performed with a cell proliferation kit (CellTiter 96 AQ$_{ueous}$, Promega, Tokyo, Japan) according to the manufacturer's protocol. Upon addition of

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MTS solution, the reaction plate was incubated at 37 °C for 4 h, and then the absorbance was read at 490 nm with a plate reader (Bio-Rad).

Caspase-3/7 Activity—Serum or platelet supernatant caspase-3/7 activity was measured with a luminescent substrate assay for caspase-3 and caspase-7 (Caspase-Glo assay, Promega) according to the manufacturer's protocol.

Western Blot Analysis—Equal amounts of protein lysates were electrophoretically separated using SDS-PAGE and transferred onto PVDF membrane unless otherwise indicated. For immunodetection, the following antibodies were used: rabbit polyclonal antibody to Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody to Bid, rabbit polyclonal antibody to Bax, rabbit polyclonal antibody to cleaved caspase-3, rabbit polyclonal antibody to Bim, rabbit polyclonal antibody to Puma, rabbit polyclonal antibody to Bcl-2, rabbit polyclonal antibody to Bcl-w, rabbit polyclonal antibody to cytochrome c oxidase IV (Cell Signaling Technology, Beverly, MA), rabbit polyclonal antibody to Bak, rabbit polyclonal antibody to Bax (Millipore, Billerica, MA), rabbit polyclonal antibody to GAPDH (Trevigen, Gaithersburg, MD), rabbit polyclonal antibody to Bim (Assay Designs, Ann Arbor, MI), and mouse monoclonal antibody to β-actin (Sigma-Aldrich).

Isolation of Mitochondria-rich and Cytosolic Fractions—Platelet homogenates were prepared by repeated freeze-and-thaw methods (28). Briefly, platelets in isolation buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 1 mg/ml fatty acid-free BSA, 10 mM HEPES-KOH, 1× proteinase inhibitor mixture, 1× phosphatase inhibitor mixture, pH 7.4) were frozen in liquid nitrogen for 1 min and then thawed at 37 °C for 3 min. This freeze-and-thaw sequence was repeated for two more cycles, and then the samples were centrifuged at 700 × g for 10 min at 4 °C. The supernatant was further centrifuged at 15,000 × g for 10 min at 4 °C. The pellet was regarded as the mitochondria-rich fraction, and the supernatant was the cytosolic fraction.

Immunoprecipitation—Platelets (1.0×10^8) were lysed in HNC buffer (25 mM HEPES/Na, 300 mM NaCl, 2% CHAPS, 1× protease inhibitor mixture, 1× phosphatase inhibitor mixture, pH 7.5) and immunoprecipitated using mouse monoclonal antibody to Bax (clone 6A7) (Abcam, Cambridge, MA) with an immunoprecipitation kit (Dynabeads Protein G, Invitrogen). Control immunoprecipitations were performed using mouse control IgG (Abcam).

Detection of Bax Oligomerization—Bax oligomerization was detected as described previously (29). Briefly, 5.0×10^7 platelets were lysed with HNC buffer. Next, ~50 mg of platelet lysates was incubated with 5 mM bismaleimidohexane (Pierce) and 5 mM bis(sulfosuccinimidyl) suberate (Pierce) for 30 min at room temperature. To quench cross-linkers, the lysates were incubated with 100 mM Tris-HCl, pH 7.5 for 15 min at room temperature. Bax oligomers were detected by Western blot using rabbit polyclonal antibody to Bax (Cell Signaling Technology).

Statistical Analysis—All data are expressed as mean ± S.D. Statistical analyses were performed by unpaired Student's t test or by one-way analysis of variance. When analyses of variance were applied, differences in the mean values among the groups were examined by Scheffe post hoc correction. $p < 0.01$ was considered statistically significant.

RESULTS

Thrombocytopenia Induced by Bcl-xL Deficiency Is Dependent on Proapoptotic Effector Proteins Bax and Bak—Previous research has reported that the mild thrombocytopenia caused by heterozygous Bcl-xL knock-out is prevented in a Bak knock-out background (8). We therefore first examined whether the severe thrombocytopenia seen in the thrombocyte-specific homozygous Bcl-xL knock-out mice (9) could also be prevented by loss of Bak. Bcl-xL and Bak double knock-out mice were generated by mating thrombocyte-specific Bcl-xL knock-out mice and systemic Bak knock-out mice. Bcl-xL and Bak double knock-out mice were born at the expected Mendelian frequency, but unexpectedly, their platelet count did not show any difference from that of the thrombocyte-specific Bcl-xL knock-out mice (Fig. 1*A*). Among Bcl-2 family proteins, not only Bak but Bax is also a well recognized proapoptotic effector protein. Therefore, we next generated Bcl-xL and Bax double knock-out mice by mating thrombocyte-specific Bcl-xL knock-out mice and systemic Bax knock-out mice. Bcl-xL and Bax double knock-out mice were also born at the expected Mendelian frequency, and their platelet count also was not different from that of the thrombocyte-specific Bcl-xL knock-out mice (Fig. 1*B*). To investigate whether the Bak/Bax-dependent mitochondrial apoptotic pathway is actually involved in thrombocytopenia caused by Bcl-xL deficiency, we generated Bcl-xL, Bak, and Bax triple knock-out mice by mating Bcl-xL and Bak double knock-out mice with thrombocyte-specific Bax knock-out mice because systemic Bak and Bax double knock-out mice usually die as neonates (30). Triple knock-out mice were born at the expected Mendelian frequency and did not show any protein expression of Bcl-xL, Bak, and Bax in their platelets on examination by Western blotting (Fig. 1*C*). The platelet count of the triple knock-out mice was almost normal and not significantly different from that of systemic Bak knock-out mice, which served as a control for this mating (Fig. 1*D*). These findings clearly demonstrated that the severe thrombocytopenia induced by thrombocyte-specific Bcl-xL knock-out was fully dependent on Bak/Bax. Serum caspase-3/7 activity, monitored by specific cleavage of the Ac-DEVD-*p*-nitroanilide substrate, was significantly higher in thrombocyte-specific Bcl-xL knock-out mice than control littermates (Fig. 1*E*), suggesting platelet apoptosis in the knock-out mice. Caspase activation in the Bcl-xL knock-out mice was not alleviated in a Bak knock-out background (Fig. 1*E*) but was diminished with a Bak and Bax double knock-out background (Fig. 1*F*), suggesting that Bcl-xL deficiency caused platelet apoptosis through a Bak/Bax-dependent mitochondrial pathway. These results also implied that either Bak or Bax was sufficient to induce apoptosis in Bcl-xL-deficient platelets.

ABT-737 Treatment Provokes Bak/Bax-dependent Apoptosis in Platelets—To investigate the molecular mechanisms of Bak/Bax-dependent platelet apoptosis provoked by a lack of antiapoptotic Bcl-2 proteins, we conducted an *in vitro* study using ABT-737, a Bad mimetic, which antagonizes the antiapoptotic function of Bcl-xL, Bcl-2, and Bcl-w by binding to the hydro-

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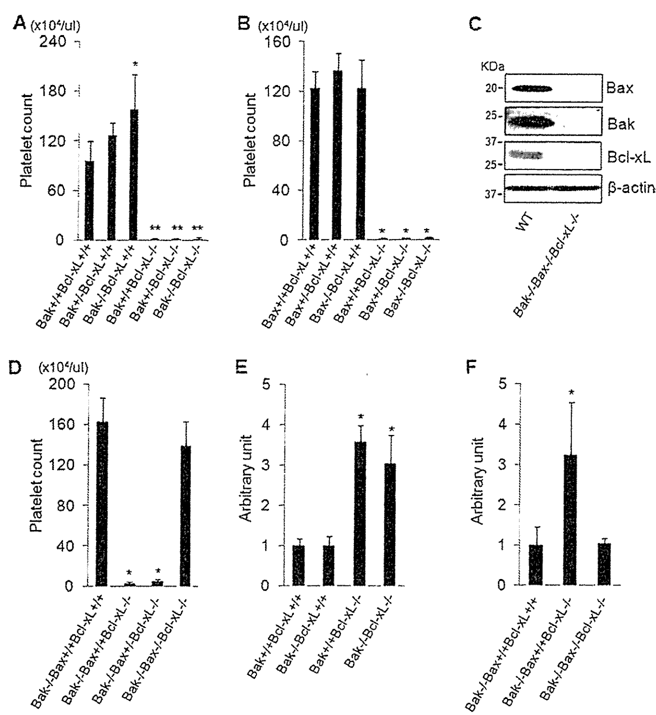

FIGURE 1. **Thrombocytopenia induced by Bcl-xL deficiency is dependent on Bcl-2 effector proteins Bak and Bax.** *Bcl-xL+/+* and *Bcl-xL−/−* stand for *bcl-x*$^{flox/flox}$ without *pf4-Cre* and *bcl-x*$^{flox/flox}$ with *pf4-Cre*, respectively. *Bak+/+*, *Bak+/−*, and *Bak−/−* stand for $bak^{+/+}$, $bak^{+/-}$, and $bak^{-/-}$, respectively. *WT* stands for wild type. *A*, platelet counts of the offspring from mating of $bak^{+/-}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bak^{+/-}bcl\text{-}x^{flox/flox}$ mice (more than four mice per group; *, $p < 0.01$ *versus* all other groups; **, $p < 0.01$ *versus* Bcl-xL+/+ groups). *B*, platelet counts of the offspring from mating of $bax^{+/-}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bax^{+/-}bcl\text{-}x^{flox/flox}$ mice (more than five mice per group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *Bax+/+*, *Bax+/−*, and *Bax−/−* stand for $bax^{+/+}$, $bax^{+/-}$, and $bax^{-/-}$, respectively. *C*, Western blot of platelet lysates for the expression of Bcl-xL, Bak, and Bax. *D*, platelet counts of the offspring from mating of $bak^{-/-}bax^{flox/+}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bak^{-/-}bax^{flox/+}bcl\text{-}x^{flox/flox}$ mice (more than eight mice per group; *, $p < 0.01$ *versus* Bak−/−Bax+/+Bcl-xL+/+ group and Bak−/−Bax−/−Bcl-xL−/− group. *Bax+/+*, *Bax+/−*, and *Bax−/−* stand for $bax^{+/+}$, $bax^{flox/+}$ with *pf4-Cre*, and $bax^{flox/flox}$ with *pf4-Cre*, respectively. *E*, serum caspase-3/7 activity of the offspring from mating of $bak^{+/-}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bak^{+/-}bcl\text{-}x^{flox/flox}$ mice (n = 5 or 6/group; *, $p < 0.01$ *versus* Bcl-xL+/+ group). *F*, serum caspase-3/7 activity of the offspring from mating of $bak^{-/-}bax^{flox/+}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bak^{-/-}bax^{flox/+}bcl\text{-}x^{flox/flox}$ mice (n = 8/group; *, $p < 0.01$ *versus* all). *Bax+/+*, *Bax+/−*, and *Bax−/−* stand for $bax^{+/+}$, $bax^{flox/+}$ with *pf4-Cre*, and $bax^{flox/flox}$ with *pf4-Cre*, respectively.

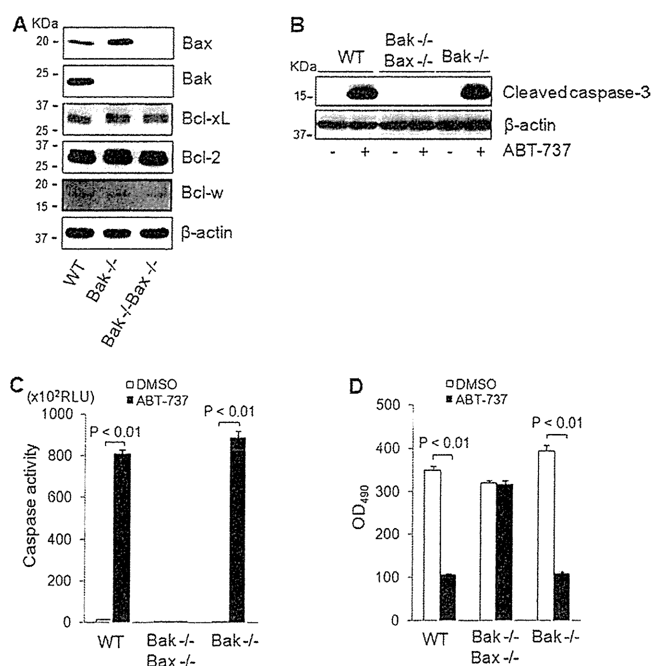

FIGURE 2. **ABT-737 treatment provokes Bak/Bax-dependent apoptosis in platelets.** *WT*, *Bak−/−Bax−/−*, and *Bak−/−* stand for wild type, *bak−/−bax*$^{flox/flox}$ with *pf4-Cre*, and *bak−/−*, respectively. *A*, Western blot of platelet lysates for the expression of Bak, Bax, Bcl-xL, Bcl-2, and Bcl-w. *B*, platelets (3.0×10^7) were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. A Western blot of platelet lysates for the expression of cleaved caspase-3 is shown. *C* and *D*, platelets (2.0×10^6) were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *C*, caspase-3/7 activity of platelet supernatant (n = 4/group). *D*, MTS assay (n = 5/group). *RLU*, relative light units.

phobic groove of these proteins (31). Western blot revealed that these antiapoptotic Bcl-2 proteins existed in platelets (Fig. 2*A*), and ABT-737 has already been reported to cause apoptosis in platelets in both *in vivo* and *in vitro* settings (7, 8). We first examined whether ABT-737-induced platelet apoptosis was executed via the Bak/Bax-dependent mitochondrial pathway. In platelets isolated from wild-type mice, administration of ABT-737 caused cleavage of caspase-3 (Fig. 2*B*). Supernatants of ABT-737-treated platelets showed marked elevation of caspase-3/7 activity (Fig. 2*C*). In addition, platelet cellular viability, which can be assessed by MTS assay (3, 4), decreased upon ABT-737 treatment (Fig. 2*D*). On the other hand, although expression of targeted antiapoptotic Bcl-2 proteins was not different between platelets from wild-type mice and Bak/Bax double knock-out mice (Fig. 2*A*), ABT-737 treatment neither caused caspase activation nor impaired cellular integrity in Bak/Bax-deficient platelets (Fig. 2, *B–D*). These findings demonstrated that ABT-737 caused platelet apoptosis via the Bak/Bax-dependent mitochondrial pathway. Interestingly, unlike what was reported previously (8), Bak deficiency could alleviate neither caspase activation nor loss of cellular viability in ABT-737-treated platelets (Fig. 2, *B–D*), offering evidence of the redundancy of Bak and Bax proteins in executing apoptosis in platelets under inhibition of these antiapoptotic Bcl-2 proteins.

ABT-737 Treatment Causes Bax Activation in Platelets—After ABT-737 treatment of the platelets, we next examined the activation status of the Bax protein in these platelets. In general, Bax activation is divided into sequential steps. When subjected to a variety of apoptotic stimuli, the Bax protein first undergoes a conformational change such as exposure of the amino terminus. This active form is translocated from the cytosol to the mitochondria. Finally, mitochondrial Bax undergoes self-oligomerization, leading to permeabilization of the outer mitochondrial membrane (32). We found that upon addition of ABT-737 to platelets the Bax protein underwent a conformational change as demonstrated by Western blotting upon immunoprecipitation with an antibody that specifically recognizes the amino terminus of the Bax protein (33) (Fig. 3*A*). In addition, upon ABT-737 treatment, the Bax protein was translocated from the cytosol to the mitochondria (Fig. 3*B*) and then underwent homo-oligomerization (Fig. 3*C*). These findings indicated that inhibition of antiapoptotic Bcl-2 proteins in

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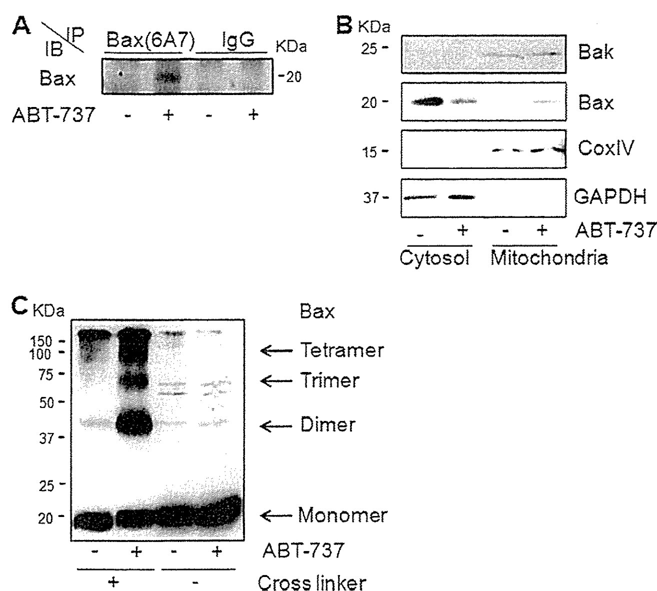

FIGURE 3. **ABT-737 treatment causes Bax activation in platelets.** *A–C*, platelets (1.0×10^8) isolated from C57BL/6J mice were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *A*, Western blot of platelet lysates for the expression of Bax after immunoprecipitation (*IP*) using mouse antibody that specifically recognizes activated Bax (6A7) or mouse control IgG (active Bax exposes an amino-terminal epitope (amino acids 12–24) that is recognized by 6A7). *B*, Western blot for the expression of Bak, Bax, *CoxIV* (cytochrome c oxidase IV), and GAPDH after cellular fractionation of the platelet lysates. *C*, Western blot for the expression of Bax after incubation of the platelet lysates with or without protein cross-linkers (5 mM bismaleimidohexane and 5 mM bis(sulfosuccinimidyl) suberate). *IB*, immunoblot.

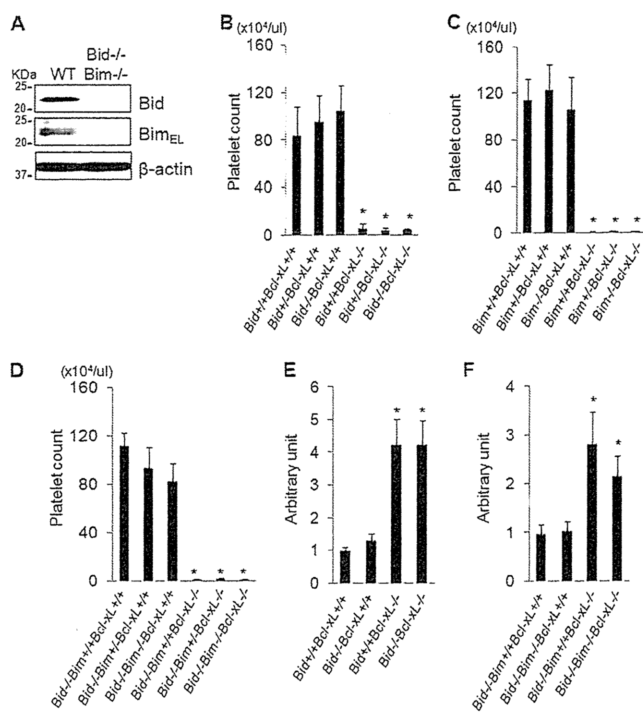

FIGURE 4. **Thrombocytopenia induced by Bcl-xL deficiency does not require BH3-only activator proteins Bid and Bim.** *Bcl-xL+/+* and *Bcl-xL−/−* stand for *bcl-x*$^{flox/flox}$ without *pf4-Cre* and *bcl-x*$^{flox/flox}$ with *pf4-Cre*, respectively. *Bid+/+*, *Bid+/−*, and *Bid−/−* stand for $bid^{+/+}$, $bid^{+/-}$, and $bid^{-/-}$, respectively. *Bim+/+*, *Bim+/−*, and *Bim−/−* stand for $bim^{+/+}$, $bim^{+/-}$, and $bim^{-/-}$, respectively. *WT* and *Bid−/−Bim−/−* stand for wild type and $bid^{-/-}bim^{-/-}$, respectively. *A*, Western blot of platelet lysates for the expression of Bid and Bim$_{EL}$. *B*, platelet counts of the offspring from mating of $bid^{+/-}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bid^{+/-}bcl\text{-}x^{flox/flox}$ mice (more than five mice per group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *C*, platelet counts of the offspring from mating of $bim^{+/-}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bim^{+/-}bcl\text{-}x^{flox/flox}$ mice (more than seven mice per group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *D*, platelet counts of the offspring from mating of $bid^{-/-}bim^{+/-}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bid^{-/-}bim^{+/-}bcl\text{-}x^{flox/flox}$ mice (more than five mice per group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *E*, serum caspase-3/7 activity of the offspring from mating of $bid^{+/-}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bid^{+/-}bcl\text{-}x^{flox/flox}$ mice (n = 4–6/group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups). *F*, serum caspase-3/7 activity of the offspring from mating of $bid^{-/-}bim^{+/-}bcl\text{-}x^{flox/flox}$ *pf4-Cre* mice and $bid^{-/-}bim^{+/-}bcl\text{-}x^{flox/flox}$ mice (n = 4–6/group; *, $p < 0.01$ *versus* Bcl-xL+/+ groups).

platelets caused Bax activation, promoting Bak/Bax-dependent mitochondrial apoptosis followed by caspase activation.

Thrombocytopenia Induced by Bcl-xL Deficiency Does Not Require BH3-only Activator Proteins Bid and Bim—We explored whether Bak/Bax-dependent platelet apoptosis induced by Bcl-xL deficiency requires the direct activator proteins Bid and Bim. Western blot revealed that Bid and Bim were both present in platelets (Fig. 4*A*). We generated Bcl-xL/Bid double knock-out mice and Bcl-xL/Bim double knock-out mice by mating thrombocyte-specific Bcl-xL knock-out mice with systemic Bid knock-out mice or Bim knock-out mice, respectively. These double knock-out mice showed massive thrombocytopenia that was not alleviated at all compared with that of thrombocyte-specific Bcl-xL knock-out mice (Fig. 4, *B* and *C*). It was possible that, in Bcl-xL-deficient platelets, the existence of either Bid or Bim was sufficient to activate Bak/Bax directly, leading to platelet apoptosis in these double knock-out mice. We then generated Bcl-xL, Bid, and Bim triple knock-out mice by mating Bcl-xL/Bid double knock-out mice with Bcl-xL/Bim double knock-out mice. These triple knock-out mice still showed massive thrombocytopenia without any difference of platelet count compared with that of Bcl-xL/Bid double knock-out mice (Fig. 4*D*). These findings clearly demonstrated that BH3-only activator proteins Bid and Bim were dispensable for the severe thrombocytopenia induced by thrombocyte-specific Bcl-xL deletion *in vivo*. In addition, caspase activation in thrombocyte-specific Bcl-xL knock-out mice was not alleviated even in the Bid and Bim double knock-out background (Fig. 4, *E* and *F*), suggesting that the lack of Bcl-xL required neither Bid nor Bim to trigger Bak/Bax-dependent platelet apoptosis.

Bax Activation and Subsequent Apoptotic Cell Death Provoked by ABT-737 Can Proceed in Absence of Bid and Bim—To investigate whether Bax can be activated by inhibition of anti-apoptotic Bcl-2 proteins even in the absence of Bid and Bim, we isolated platelets from Bid and Bim double knock-out mice. A Western blot study confirmed that neither Bid nor Bim existed in platelets of the double knock-out mice (Fig. 4*A*) and showed that Puma protein, another putative direct activator (13), was not detected in platelets of either wild-type mice or Bid/Bim double knock-out mice (Fig. 5*A*). The expression of antiapoptotic Bcl-2 proteins including Bcl-xL, Bcl-2, and Bcl-w was unchanged between these mice (Fig. 5*A*). Upon ABT-737 treatment, the Bax protein in Bid/Bim-deficient platelets could undergo conformational change (Fig. 5*B*), translocation from the cytosol to the mitochondria (Fig. 5*C*), and homo-oligomerization (Fig. 5*D*). These results clearly demonstrated that ABT-

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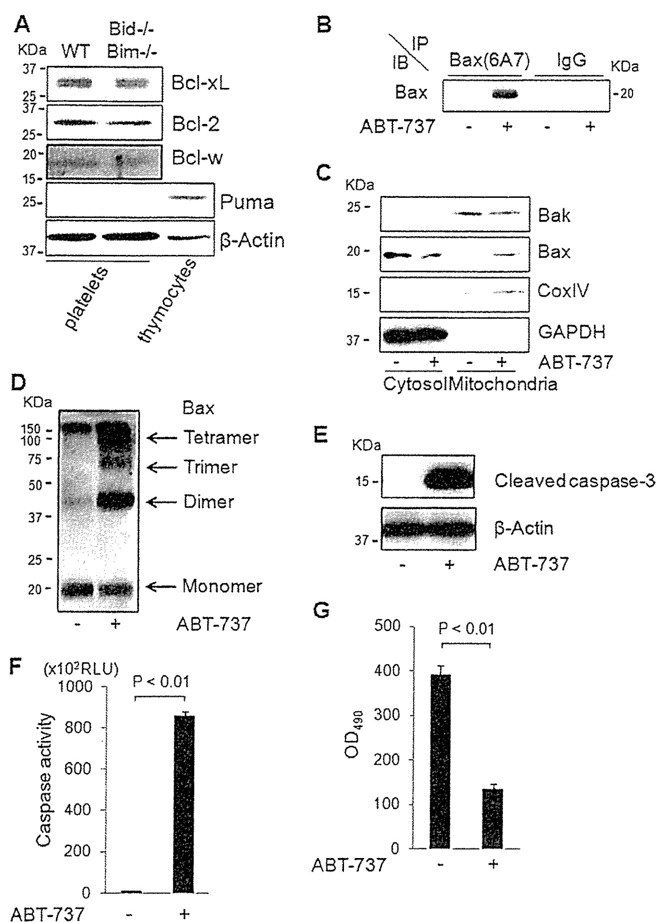

FIGURE 5. **Bax activation and subsequent apoptotic cell death provoked by Bcl-xL deficiency can proceed in absence of Bid and Bim.** *A*, Western blot of platelet lysates for the expression of Puma, Bcl-2, Bcl-w, and Bcl-xL. *WT* and *Bid−/−Bim−/−* stand for wild type and $bid^{-/-}bim^{-/-}$, respectively. *B–E*, platelets (1.0×10^8) isolated from Bid/Bim double knock-out mice were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *B*, Western blot for the expression of Bax after immunoprecipitation (*IP*) using mouse antibody that specifically recognizes activated Bax (6A7) or mouse control IgG. *C*, Western blot for the expression of Bak, Bax, *CoxIV* (cytochrome c oxidase IV), and GAPDH after cellular fractionation of the platelet lysates. *D*, Western blot for the expression of Bax after incubation of the platelet lysates with protein cross-linkers (5 mM bismaleimidohexane and 5 mM bis(sulfosuccinimidyl) suberate). *E*, Western blot of platelet lysates for the expression of cleaved caspase-3. *F* and *G*, platelets (2.0×10^6) isolated from Bid/Bim double knock-out mice were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *F*, caspase-3/7 activity of platelet supernatant (n = 4/group). *G*, MTS assay (n = 5/group). *IB*, immunoblot; *RLU*, relative light units.

737-induced Bax activation did not require the direct activator proteins Bid and Bim. Upon ABT-737 treatment of Bid/Bim-deficient platelets, cleavage of caspase-3 and elevation of caspase-3/7 activity were both observed (Fig. 5, *E* and *F*), and the MTS assay demonstrated that platelet cellular viability was also impaired (Fig. 5*G*). These findings indicated that Bid and Bim were dispensable for Bak/Bax-dependent platelet apoptosis provoked by inhibition of antiapoptotic Bcl-2 proteins.

Spontaneous Apoptotic Cell Death in Stored Human Platelets Occurs with Decline of Bcl-xL Despite Decrease in Bid and Bim— In stored human platelets, phosphatidylserine exposure increases with caspase-3 activation (4, 5), which leads to spontaneous platelet apoptosis, but the exact molecular mechanism of this process remains elusive. This led us to examine the profile of Bcl-2 family proteins in human platelets during the course of storage. In stored platelets, cleaved caspase-3 gradually increased (Fig. 6*A*) and caspase-3/7 activity rose simultaneously (Fig. 6*B*), indicating that the platelets steadily underwent apoptotic cell death with storage time. Regarding the Bcl-2 family protein profile, although expression of Bcl-xL and Bax proteins gradually decreased with time, the decrease in Bak expression occurred at a later time point (Fig. 6*A*). As for BH3-only direct activator proteins, Bid and Bim expression also decreased with time (Fig. 6*A*). To examine the involvement of Bcl-2 family proteins in spontaneous apoptosis in stored platelets, caspase-3/7 activity was measured in platelets from wild-type mice, Bak/Bax double knock-out mice, Bcl-xL transgenic mice, and Bid/Bim double knock-out mice upon storage. A Western blot revealed that Bcl-xL protein increased in platelets isolated from Bcl-xL transgenic mice compared with wild-type mice, whereas expression of effector proteins Bak and Bax did not differ between them (Fig. 6*C*). Although wild-type platelets showed elevation of the caspase-3/7 activity upon storage, it was significantly lower in Bak/Bax-deficient platelets than in

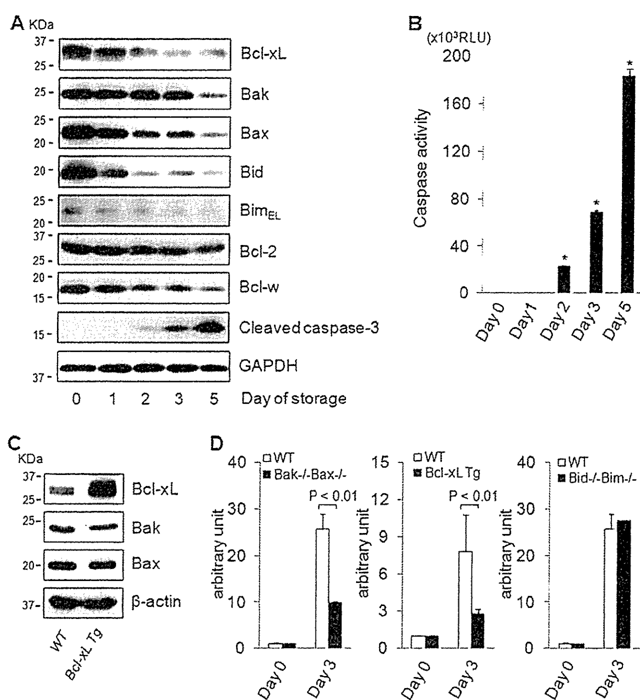

FIGURE 6. **Spontaneous apoptotic cell death in stored platelets occurs with decline of Bcl-xL despite decrease in Bid and Bim.** *A* and *B*, platelet-rich plasma derived from a healthy volunteer was stored for the indicated time course. *A*, Western blot of stored platelet lysates for the expression of Bcl-xL, Bak, Bax, Bid, Bim_{EL}, Bcl-w, Bcl-2, cleaved caspase-3, and GAPDH. Equal numbers of platelets were loaded per sample. *B*, caspase-3/7 activity of supernatant derived from platelet-rich plasma (n = 4/group; *, $p < 0.01$ *versus* all other groups). *C*, Western blot of platelet lysates derived from Bcl-xL transgenic mice for the expression of Bcl-xL, Bak, and Bax. *WT* and *Bcl-xL Tg* stand for wild-type mice and Bcl-xL transgenic mice, respectively. *D*, platelets derived from C57BL/6J mice, Bak/Bax double knock-out mice, Bcl-xL transgenic mice, and Bid/Bim double knock-out mice were stored for the indicated time course. Caspase-3/7 activity of stored platelet supernatant was assessed and is presented as the -fold induction compared with freshly isolated platelet supernatant (n = 4/group). *WT*, *Bak−/−Bax−/−*, and *Bid−/−Bim−/−* stand for wild-type, $bak^{-/-}bax^{flox/flox}$ with *pf4-Cre*, and $bid^{-/-}bim^{-/-}$ mice, respectively. *Bcl-xL Tg* stands for Bcl-xL transgenic mice. *RLU*, relative light units.

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wild-type platelets (Fig. 6*D*). These findings indicated that Bak/Bax-dependent mitochondrial apoptosis played an important role in the execution of spontaneous apoptosis in stored platelets. Furthermore, caspase activation was alleviated in Bcl-xL-overexpressing platelets compared with wild-type platelets upon storage (Fig. 6*D*), suggesting an antiapoptotic function of Bcl-xL in stored platelets. On the other hand, caspase-3/7 activity increased in Bid/Bim-deficient platelets and was not different from that in wild-type platelets (Fig. 6*D*), suggesting that direct activator proteins Bid and Bim are dispensable for the spontaneous platelet apoptosis upon storage.

DISCUSSION

In the mitochondrial pathway, apoptotic cell death is dependent on activation of the proapoptotic effector proteins Bak and Bax. Cells lacking both Bak and Bax are resistant to multiple apoptotic stimuli (34). Genetic studies have revealed that Bax or Bak single knock-out mice have less pronounced phenotypes compared with Bak/Bax double knock-out mice, which display various severe defects during development, indicating the redundancy of their involvement in apoptosis (30, 35). With regard to the mitochondrial apoptosis machinery in platelets, the involvement of Bax seemed to be less critical because platelet numbers in Bax knock-out mice were normal in contrast to the thrombocytosis displayed in Bak knock-out mice (30, 35). However, our *in vitro* study revealed that ABT-737 could provoke apoptosis even in Bak-deficient platelets. Moreover, our *in vivo* studies have clearly demonstrated that either Bax or Bak was sufficient to cause platelet apoptosis in the absence of Bcl-xL, indicating that Bax and Bak are redundant and equivalently important for the mitochondrial apoptosis in platelets.

In support of the displacement model, co-immunoprecipitation studies revealed complexes of Bak with a variety of antiapoptotic proteins (36). However, the major concern with this model is that Bax is presumed to exist mainly in a cytosolic fraction as a monomer (37). Thus, Bax activation might not be controlled by displacement (38). Unlike Bak activation, sequential steps are necessary for Bax activation such as a conformational change, mitochondrial translocation, and homo-oligomerization. Recent reports have revealed the mechanism of how activator proteins Bid and Bim are directly involved in these steps and initiate Bax activation (39, 40). In the present study, we showed that all the serial steps of Bax activation can adequately proceed without the involvement of the activator proteins Bid and Bim *in vitro*. Moreover, Bak/Bax-dependent mitochondrial apoptosis could be fully executed by inhibition of antiapoptotic Bcl-2-like proteins even if the direct activator proteins Bid and Bim did not exist. Similar results have been presented by Willis *et al.* (15), who showed that embryonic fibroblasts from Bid and Bim double knock-out, when infected with retrovirus expressing BH3 sensitizer proteins, could undergo apoptosis *in vitro*. Based on their results, they claimed that the Bax protein may be constitutively active and inhibited through binding to antiapoptotic Bcl-2-like proteins for cells to survive. However, in our *in vitro* study, we could not detect physiological interaction between Bax and Bcl-xL in platelets. Therefore, it is difficult to evaluate whether Bak and/or Bax is active or inactive at the default state in platelets. On the other hand, genetically modified mice clearly showed that retrieval of direct activator proteins could not prevent caspase activation and thrombocytopenia induced by the lack of Bcl-xL. These findings demonstrated, for the first time, *in vivo* evidence that direct activator proteins Bid and Bim were dispensable for apoptosis execution provoked by the loss of antiapoptotic Bcl-2-family proteins.

Because ABT-737 can bind to and neutralize Bcl-2, Bcl-w, and Bcl-xL, all of which are present in platelets (Figs. 2*A* and 6*A*), it is difficult to directly conclude that the *in vitro* results from our ABT-737 study exactly reflect our *in vivo* results obtained from Bcl-xL deletion. However, in addition to reports that neither systemic Bcl-w knock-out nor Bcl-2 knock-out mice exhibit any phenotypes with respect to platelet counts (41–43), our *in vivo* results of massive thrombocytopenia seen in thrombocyte-specific Bcl-xL knock-out mice indicated that the antiapoptotic role of Bcl-2 and Bcl-w in platelets was apparently less important than that of Bcl-xL. Even if Bcl-2 and Bcl-w were involved in our *in vitro* results, our present results clearly demonstrated that neither Bid nor Bim is required for Bax activation and following mitochondrial apoptosis by inhibition of antiapoptotic Bcl-2 family proteins. Regarding the other antiapoptotic members of the Bcl-2 family, systemic A1a knock-out mice were not reported with any phenotype with respect to platelet counts (44). Mcl-1 is a rapid turnover protein and could not be detected in platelets (supplemental Fig. 1). Therefore, Bcl-xL may be the main antiapoptotic Bcl-2 family protein with functional significance in platelets. This simplicity may explain why Bid and Bim deficiency failed to ameliorate the phenotype of Bcl-xL knock-out in platelets in contrast to other scenarios in which Bid or Bim is apparently indispensable (19–21). Fatal polycystic kidney disease and lymphopenia observed in Bcl-2 knock-out mice are ameliorated in a Bim knock-out background (19). In this case, lymphocytes and other cell lineages may possess Bcl-2 and other antiapoptotic Bcl-2 proteins such as Mcl-1 (45). Hepatocyte apoptosis observed in hepatocyte-specific knock-out of Mcl-1 or Bcl-xL is ameliorated in a Bid knock-out background (20, 21). In this case, hepatocytes clearly have two critical antiapoptotic Bcl-2 family proteins, Bcl-xL and Mcl-1, and Bid may switch binding partners from one to the other in the case of deficiency of either protein. Bid and Bim could regulate the rheostat balance between antiapoptotic and proapoptotic Bcl-2 family proteins, which may become irrelevant if none of the antiapoptotic Bcl-2 family proteins are present.

Although among the BH3-only proteins Bid and Bim are recognized as the putative direct activators, Puma, one of the other BH3-only proteins, has been reported to have the ability to interact directly with effector proteins (13). However, a recent report has pointed out that Puma is a sensitizer protein, which indirectly activates Bak or Bax (46). Hence, its actual mechanism of action in apoptosis remains obscure and disputed. Importantly, in contrast to thymocyte tissue, a Western blot did not show a detectable amount of Puma protein in platelets (Fig. 5*A*), indicating that it might not be involved in the platelet apoptosis machinery. However, we could not exclude the possibility that other proteins may function as alternative direct acti-

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vators in the absence of Bid and Bim, leading to Bax activation and mitochondrial apoptosis in platelets upon inactivation of antiapoptotic Bcl-2 family proteins.

In stored platelets, because of the lack of *de novo* protein synthesis, each protein may gradually decrease in relation to its half-life. Our current results showed that the decline of Bcl-xL and Bax protein was much faster than that of Bak protein, and the disruption of the balance between anti- and proapoptotic multidomain Bcl-2 proteins seemed to be associated with apoptosis in stored human platelets. In fact, upon storage, caspase activation was weakened in Bak/Bax-deficient or Bcl-xL-overexpressing platelets compared with wild-type platelets. Taken together with these findings, the balance between anti- and proapoptotic multidomain Bcl-2 family proteins seems to dictate the cellular fate of the life and death of stored platelets. Similar degradation of the Bcl-2 family proteins should occur in circulation, which may explain why Bak knock-out mice displayed mild thrombocytosis *in vivo* (Fig. 1*A*). On the other hand, spontaneous apoptosis occurred in stored platelets despite the absence of activator proteins Bid and Bim. Although in most physiological contexts cellular death is an active decision made by regulating BH3-only proteins, our present findings suggest that activator proteins Bid and Bim were dispensable for Bak/Bax-dependent spontaneous apoptosis in stored platelets.

How anti- and proapoptotic Bcl-2 family proteins interact to maintain cellular integrity and to command cellular survival and death is one of the most important issues that remain to be clearly determined. Although their networks seem to vary depending on the cellular context, our present findings provide an *in vivo* example indicating that the absence of antiapoptotic Bcl-2-like proteins can induce activation of the effector protein Bax, leading to apoptosis without the involvement of the activator proteins Bid and Bim.

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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

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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$^+$/TCRβ$^-$ lymphocytes, NKT cells as DX5$^+$/TCRβ$^+$ lymphocytes and T cells as DX5$^-$/TCRβ$^+$ 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 $12.6 \pm 2.4\%$ in IgG-injected liver, whereas it decreased to $0.76 \pm 0.04\%$ 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