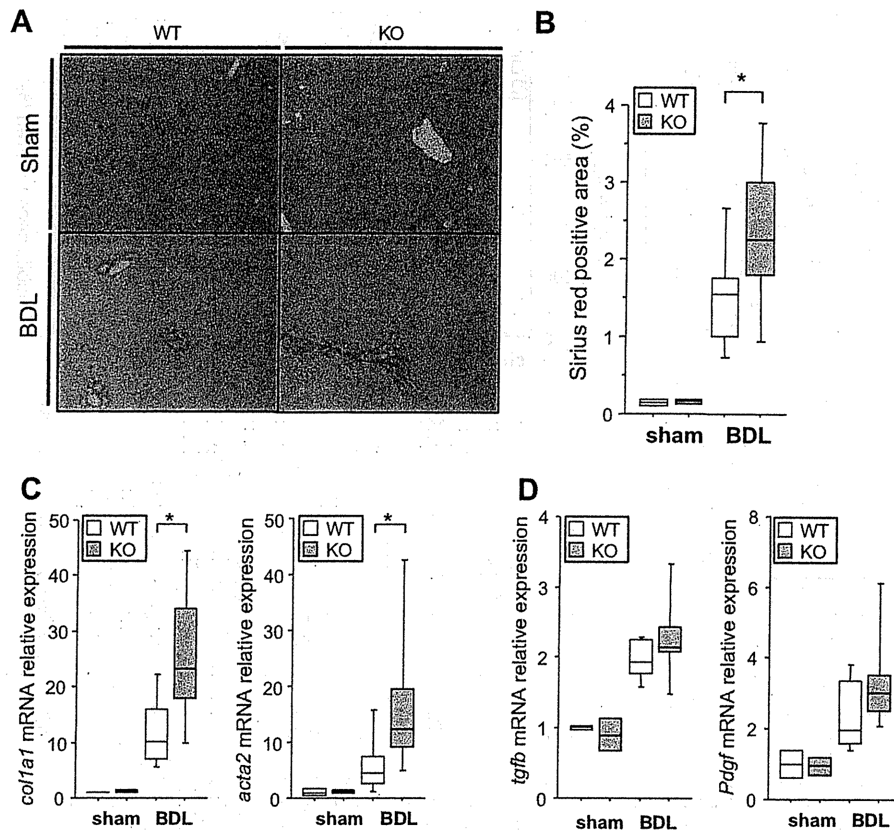

Fig. 2. Exacerbation of liver fibrosis in L-STAT3 KO mice after BDL. L-STAT3 KO mice (KO) and WT littermates (WT) were subjected to BDL or sham-operation (sham) and examined 10 days later. (A) Representative views of picrosirius red staining of the liver sections. (B) Morphometric analysis for picrosirius red staining, $n = 8$/group, $^*p < 0.05$. (C) Hepatic expression of αSMA and type I collagen α1 genes by real-time rtPCR analysis, $n = 8$/group, $^*p < 0.05$. (D) Hepatic expression of TGFβ and PDGF genes by real-time rtPCR analysis, $n = 8$/group.

factors released from hepatocytes repressed activated HSCs and their collagen synthesis. We isolated primary hepatocytes from L-STAT3 KO mice and WT controls, and stimulated them with or without IL-6. The cultured medium of stimulated hepatocytes was collected after 24 h. Whereas faint signals of STAT3 phosphorylation were observed under resting conditions in WT hepatocytes, administration of IL-6 clearly activated STAT3 phosphorylation in WT hepatocytes in contrast to STAT3 KO hepatocytes (Fig. 4A). Accordingly, IL-6 administration activated SAA and haptoglobin gene expression in WT hepatocytes, leading to production of higher levels of SAA and haptoglobin in culture supernatant compared with STAT3 KO hepatocytes (Fig. 4B, Supplementary Fig. 3).

Activated HSCs isolated from C57BL/6J were cultured with supernatant taken from IL-6-treated WT hepatocytes (sup-WT) or that from IL-6-treated STAT3 KO hepatocytes (sup-KO). Activated HSCs were also incubated with medium containing the same amount of IL-6 (sup-control) as a control. The mRNA expression of αSMA and type I collagen α1 in HSCs cultured with sup-WT significantly decreased compared with sup-control (Fig. 4C). On the other hand, the expression of these genes in HSCs cultured with sup-KO was similar to the levels of sup-control (Fig. 4C). In addition, activated HSCs were cultured with recombinant SAA. The expression of αSMA gene in HSCs decreased in dose-dependent manner, although the expression of type I collagen α1 gene did not change (Fig. 4D).

4. Discussion

Liver fibrosis is a consequence of chronic liver injury and inflammation. Accumulating evidence suggests that liver fibrosis is to some extent reversible by appropriate therapeutic intervention for chronic liver diseases [1]. Clarifying the cellular and molecular mechanisms involved in fibrogenesis and its progression has become very important for efficacious treatment. In the present study, we used L-STAT3 KO mice to examine the significance of this signaling pathway in liver fibrogenesis, because hepatocyte STAT3 is a crucial signaling transducer and transcription factor that regulates most, if not all, APPs which have been shown to possess a variety of biological properties during inflammation. We have demonstrated here that lack of STAT3 accelerates liver fibrosis during cholestasis and suggested that STAT3-dependent soluble factors collectively serve as a negative regulator for activation of HSCs.

Very recent research has shown that lack of gp130 or STAT3 in hepatocytes exacerbates liver fibrosis in murine sclerosis cholangitis models induced by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet or genetic deletion of multidrug resistance gene 2 (*mdr2*), respectively. In those studies, deletion of gp130 or STAT3 induced severer cholestasis compared with control mice, leading to enhanced inflammatory cell infiltration and injury in the liver [10,11]. Therefore, exacerbation of liver fibrosis observed in those models might be ascribed to exacerbated cholestasis and liver

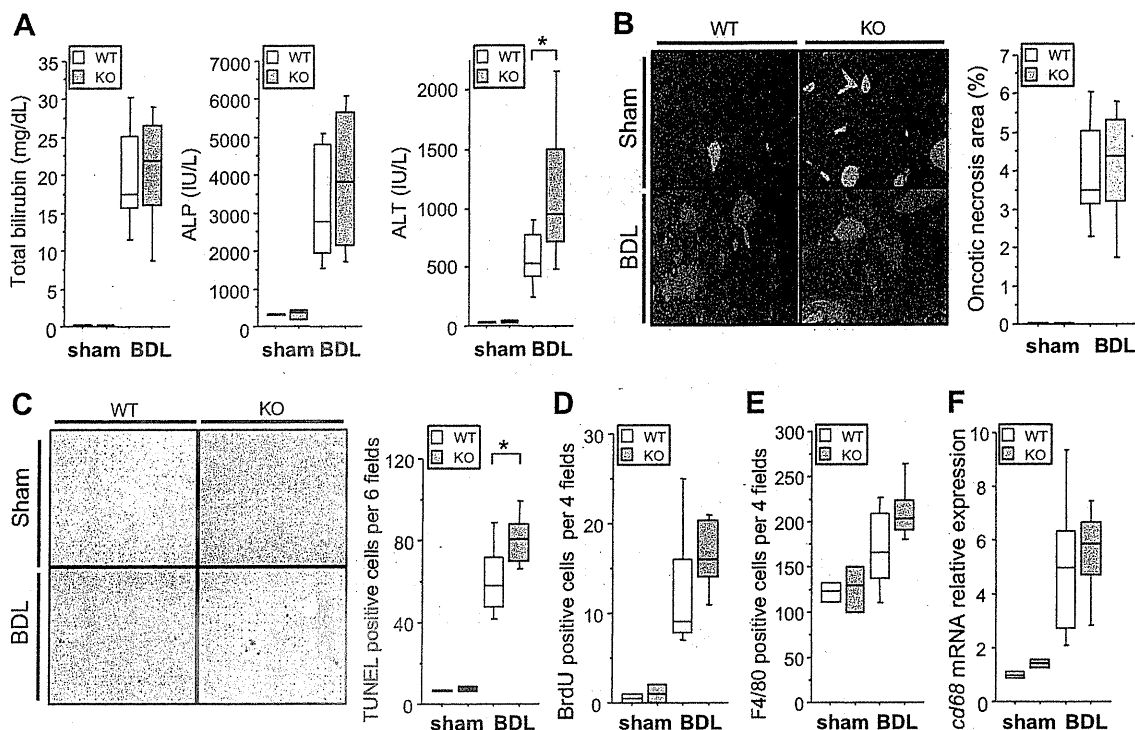

Fig. 3. Exacerbation of liver injury in L-STAT3 KO mice after BDL. L-STAT3 KO mice (KO) and WT littermates (WT) were subjected to BDL or sham-operation (sham) and examined 10 days later. (A) Serum levels of total bilirubin, alkaline phosphatase (ALP) and ALT, $n = 8$/group, $^*p > 0.05$. (B) Representative views of H&E staining of the liver sections and statistics for the area of oncotic necrosis determined by morphometric analysis of H&E staining, $n = 6$/group. (C) Representative views of TUNEL staining of the liver sections and statistics of TUNEL positive cells per 6 fields, $n = 8$/group. (D) The numbers of BrdU-positive cells of the liver sections, $n = 8$/group. 5-bromo-2-deoxyuridine (BrdU) was administered 2 h before sacrifice. (E) The numbers of F4/80-positive cells of the liver sections, $n = 8$/group. The liver sections were stained with anti-F4/80 antigen. (F) Hepatic mRNA expression of CD68 determined by real-time rtPCR analysis, $n = 8$/group.

inflammation. In the present study, we subjected our L-STAT3 KO mice to BDL, a well-established murine model of cholestasis, and examined them 10 days later. We found that hepatocyte-specific deletion of STAT3 promotes liver injury and fibrosis although the mice developed similar levels of cholestasis and inflammatory cell infiltration in the liver, compared with their control littermates. These results clearly indicated that hepatocyte STAT3 signaling negatively regulates liver fibrosis independently of cholestasis and inflammatory cell infiltration in our BDL model. An earlier study showed that deletion of the gp130 gene induced by Cre-mediated recombination under control of the Mx1 gene promoter exacerbated bacterial infection and increased mortality by 10 days after BDL, compared with control mice [17]. In our mice in which STAT3 had been deleted under control of the albumin gene promoter, no rise in mortality occurred after BDL. Since Mx1-Cre-mediated genetic recombination should occur not only in hepatocytes but also in other cell types including a variety of hematopoietic cells, the gp130/STAT3 signaling pathway of other cell types besides hepatocytes might have an impact on the infection control and survival after BDL.

L-STAT3 KO mice showed severer liver injury with increases in the levels of serum ALT and TUNEL-positive cells in the liver sections. This is consistent with the general concept that STAT3 promotes apoptosis resistance by regulating the expression of a variety of anti-apoptotic genes. Indeed previous research showed that the absence of hepatic STAT3 makes hepatocytes more vulnerable to Fas-mediated apoptosis [6]. In contrast to a previous finding that liver regeneration is suppressed in STAT3 KO mice after partial hepatectomy [5], compensatory regeneration after BDL did not differ between L-STAT3 KO mice and the control littermates. Accumulating evidence suggests that hepatocyte apoptosis promotes a liver fibrotic response via HSC activation. Kupffer cells and HSCs were reported to be able to engulf apoptotic bodies and to produce TNFα and TGFβ, respectively [18,19]. These cellular events may lead to the induction of profibrotic responses. Indeed, we previously reported that spontaneous apoptosis did induce mild fibrotic response with increased production of TGFβ *in vivo* [13]. In the present study, STAT3 KO mice displayed increased hepatic expression of αSMA and type I collagen α1 genes suggesting activation of HSCs after BDL, compared with WT controls. However, at the same time, there were no significant differences in the mRNA expression of proinflammatory cytokines such as TNFα (data not shown) and profibrogenic cytokines such as TGFβ and PDGF in the liver between the two groups. This suggested that other factors or cellular events except cytokines or apoptosis were involved in the difference of HSC activation between the two groups in our model.

APPs, defined as proteins whose serum levels change by >25% during inflammation, are mainly produced in the liver and regulated via gp130/STAT3 signaling [20]. Consistent with this, L-STAT3 KO mice showed impaired production of APPs in BDL. Recent research showed that APPs are regarded as important biological components of the immune response to infection and tissue injury [9,21]. The IL-6 family/gp130/STAT3 signaling pathway in hepatocytes regulates the acute phase response and the importance of this pathway during host defense has become evident recently. The present study demonstrated that the absence of STAT3 in hepatocytes during cholestasis led to progression of liver fibrosis as shown by collagen deposition and activation of HSCs. We investigated the direct influence of the soluble factors released from hepatocytes via STAT3 signaling on HSCs activation and collagen production *in vitro*. Interestingly, the mRNA expression of αSMA and type I collagen α1 significantly decreased in activated HSCs

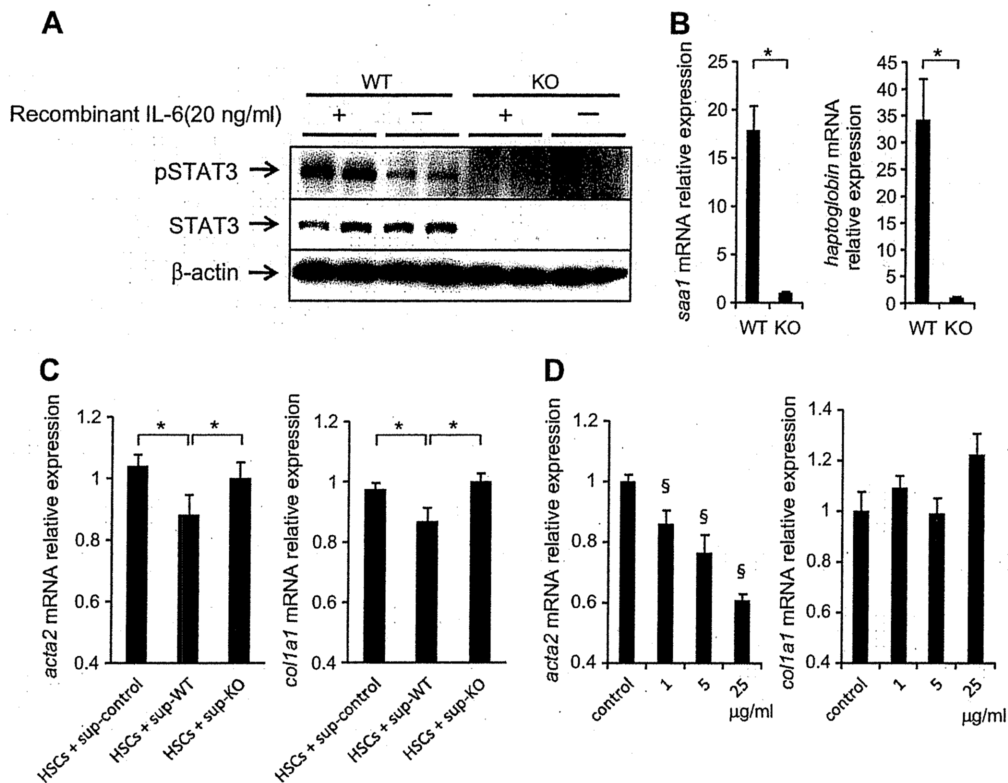

Fig. 4. Involvement of STAT3-dependent hepatic soluble factors in the suppression of activated HSCs. Primary hepatocytes isolated from L-STAT3 KO mice (KO) and WT mice (WT) were stimulated with or without 20 ng/ml of IL-6 for 24 h. (A) Expressions of STAT3 and pSTAT3 in hepatocytes by western blot analysis. β-actin is included as a control. (B) Real-time rtPCR analysis of mRNA expression of SAA and haptoglobin in IL-6-treated hepatocytes, $n = 4$/group, $^{*}p < 0.05$. (C) Real-time rtPCR analysis of αSMA and type I collagen α1 mRNA expression in HSCs, $n = 4$/group, $^{*}p < 0.05$. Activated HSCs were cultured for 24 h with control medium (sup-control), the cultured supernatant from WT hepatocytes (sup-WT) or that from STAT3 KO hepatocytes (sup-KO), stimulated with 20 ng/ml IL-6, respectively. (D) Real-time rtPCR analysis of αSMA and type I collagen α1 mRNA expression in HSCs, $n = 4$/group, $^{\S}p < 0.05$ vs the other three groups. Activated HSCs were cultured with recombinant SAA (0, 1, 5, 25 μg/ml) for 24 h.

cultured with sup-WT compared with control medium. In contrast, the expression levels of these genes in activated HSCs remained unchanged when cultured with sup-KO. Since Sup-WT was abundant with SAA and haptoglobin, these findings imply that the hepatocyte STAT3-dependent soluble factors, such as APPs, directly repressed activated HSCs and their collagen production. Indeed, SAA negatively regulated HSC activation, although the mRNA expression of type I collagen α1 was unchanged, providing an example among APPs being able to downregulate activation marker of HSCs. Although further study is needed, the present study suggested that APPs could collectively inhibit HSC activity and collagen production.

In conclusion, the present study demonstrated that the absence of STAT3 in hepatocytes exacerbated liver injury and fibrosis during cholestasis. We speculated that both increase in hepatocyte apoptosis and lack of an acute phase response may contribute to accelerated liver fibrosis in this model. APPs had been individually analyzed to have pro- and anti-inflammatory properties during inflammation. The current study unveiled a previously unrecognized role of STAT3-dependent hepatic APPs in collectively serving as a negative regulator for HSC activation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.105.

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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.

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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 *loxP* 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 × *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 × *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 × *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 × *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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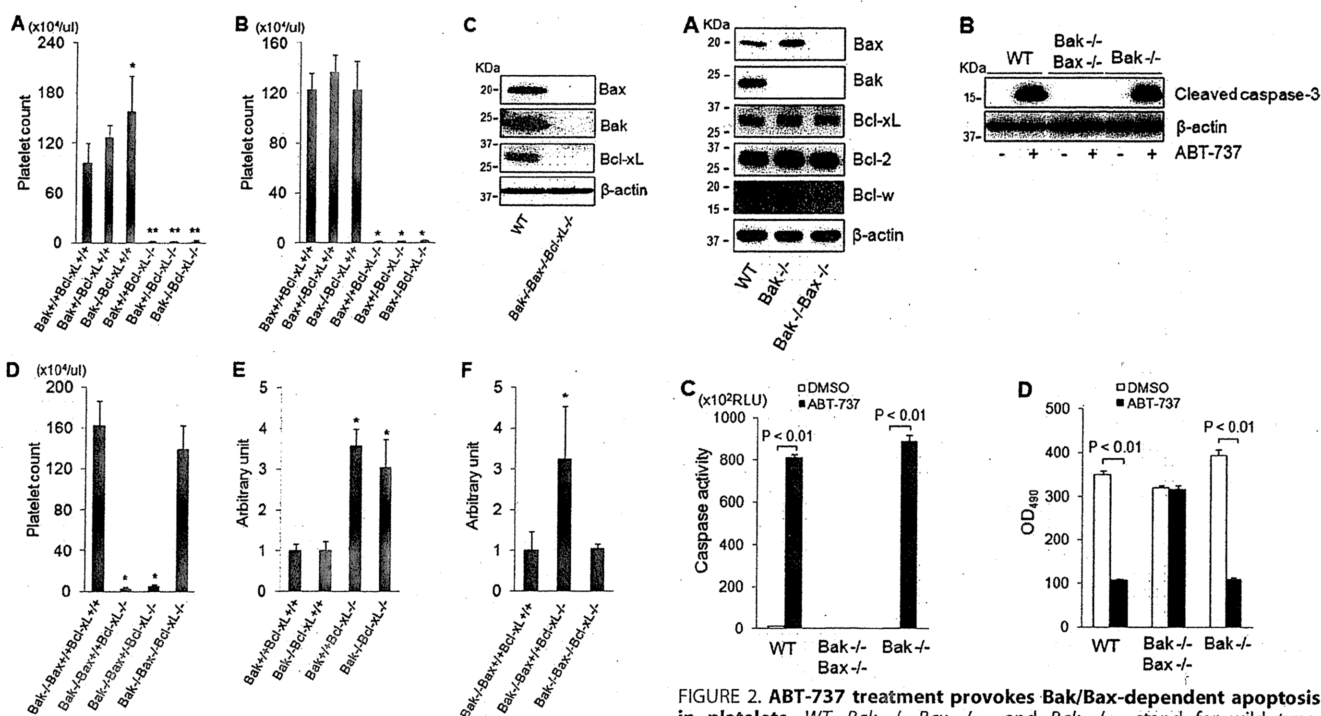

FIGURE 1. **Thrombocytopenia induced by Bcl-xL deficiency is dependent on Bcl-2 effector proteins Bak and Bax.** *Bcl-xL+/+* and *Bcl-xL−/−* stand for *bcl-x$^{flox/flox}$* without *pf4-Cre* and *bcl-x$^{flox/flox}$* with *pf4-Cre*, respectively. *Bak+/+*, *Bak+/−*, and *Bak−/−* stand for *bak$^{+/+}$*, *bak$^{+/-}$*, and *bak$^{-/-}$*, respectively. *WT* stands for wild type. *A*, platelet counts of the offspring from mating of *bak$^{+/-}$bcl-x$^{flox/flox}$ pf4-Cre* mice and *bak$^{+/-}$bcl-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-x$^{flox/flox}$ pf4-Cre* mice and *bax$^{+/-}$bcl-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-x$^{flox/flox}$ pf4-Cre* mice and *bak$^{-/-}$bax$^{flox/+}$bcl-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-x$^{flox/flox}$ pf4-Cre* mice and *bak$^{+/-}$bcl-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-x$^{flox/flox}$ pf4-Cre* mice and *bak$^{-/-}$bax$^{flox/+}$bcl-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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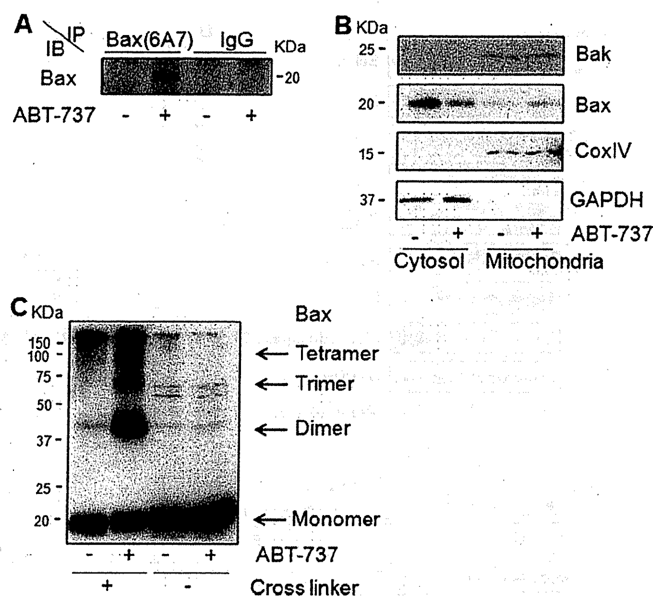

FIGURE 3. **ABT-737 treatment causes Bax activation in platelets.** *A–C*, platelets (1.0 × 10^8) isolated from C57BL/6J mice were incubated with 10 μM ABT-737 or vehicle for 2 h at room temperature. *A*, Western blot of platelet lysates for the expression of Bax after immunoprecipitation (*IP*) using 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.

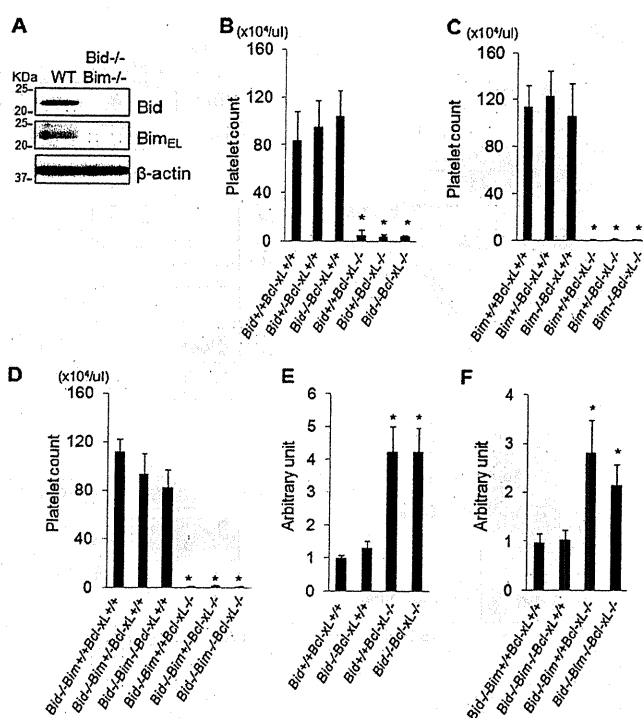

FIGURE 4. **Thrombocytopenia induced by Bcl-xL deficiency does not require BH3-only activator proteins Bid and Bim.** *Bcl-xL+/+* and *Bcl-xL−/−* stand for *bcl-x$^{flox/flox}$* without *pf4-Cre* and *bcl-x$^{flox/flox}$* with *pf4-Cre*, respectively. *Bid+/+*, *Bid+/−*, and *Bid−/−* stand for *bid$^{+/+}$*, *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-x$^{flox/flox}$ pf4-Cre* mice and *bid$^{+/-}$bcl-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-x$^{flox/flox}$ pf4-Cre* mice and *bim$^{+/-}$bcl-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-x$^{flox/flox}$ pf4-Cre* mice and *bid$^{-/-}$bim$^{+/-}$bcl-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-x$^{flox/flox}$ pf4-Cre* mice and *bid$^{+/-}$bcl-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-x$^{flox/flox}$ pf4-Cre* mice and *bid$^{-/-}$bim$^{+/-}$bcl-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 antiapoptotic 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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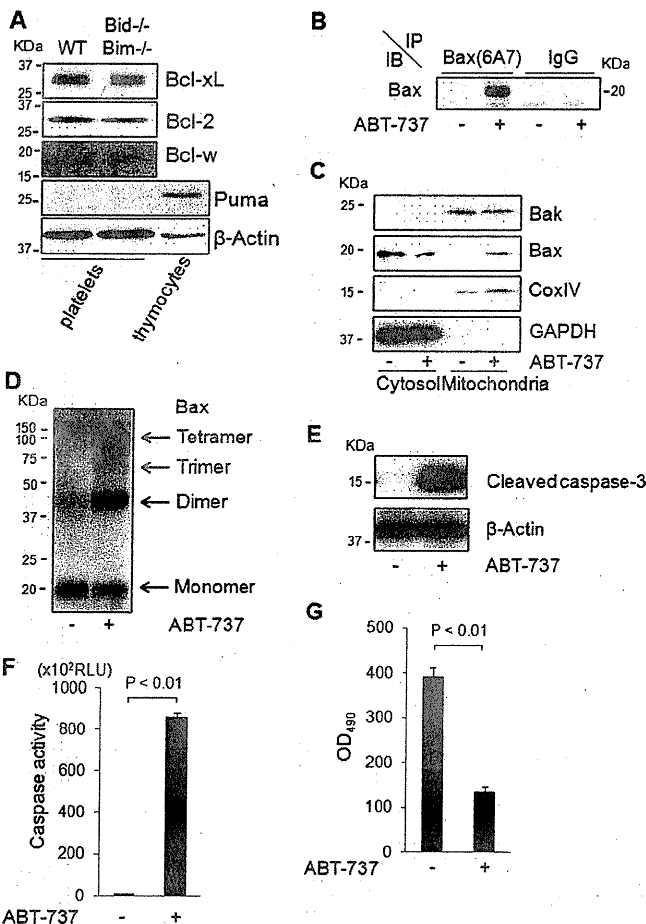

FIGURE 5. **Bax activation and subsequent apoptotic cell death provoked by Bcl-xL deficiency can proceed in absence of Bid and Bim.** *A*, Western blot of platelet lysates for the expression of Puma, Bcl-2, Bcl-w, and Bcl-xL. *WT* and *Bid−/−Bim−/−* stand for wild type and $bid^{-/-}bim^{-/-}$, respectively. *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.

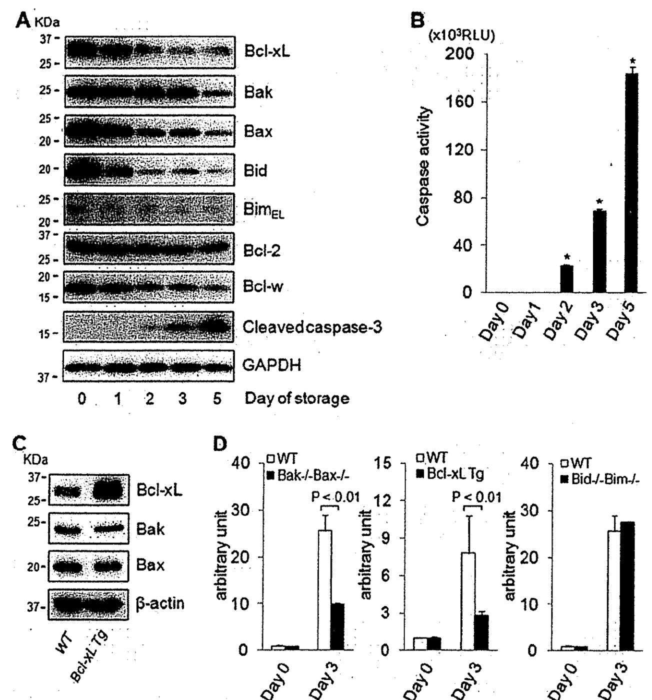

FIGURE 6. **Spontaneous apoptotic cell death in stored platelets occurs with decline of Bcl-xL despite decrease in Bid and Bim.** *A* and *B*, platelet-rich plasma derived from a healthy volunteer was stored for the indicated time course. *A*, Western blot of stored platelet lysates for the expression 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.

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

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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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Delayed-Onset Caspase-Dependent Massive Hepatocyte Apoptosis upon Fas Activation in Bak/Bax-Deficient Mice

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The proapoptotic Bcl-2 family proteins Bak and Bax serve as an essential gateway to the mitochondrial pathway of apoptosis. When activated by BH3-only proteins, Bak/Bax triggers mitochondrial outer membrane permeabilization leading to release of cytochrome c followed by activation of initiator and then effector caspases to dismantle the cells. Hepatocytes are generally considered to be type II cells because, upon Fas stimulation, they are reported to require the BH3-only protein Bid to undergo apoptosis. However, the significance of Bak and Bax in the liver is unclear. To address this issue, we generated hepatocyte-specific Bak/Bax double knockout mice and administered Jo2 agonistic anti-Fas antibody or recombinant Fas ligand to them. Fas-induced rapid fulminant hepatocyte apoptosis was partially ameliorated in Bak knockout mice but not in Bax knockout mice, and was completely abolished in double knockout mice 3 hours after Jo2 injection. Importantly, at 6 hours, double knockout mice displayed severe liver injury associated with repression of XIAP, activation of caspase-3/7 and oligonucleosomal DNA breaks in the liver, without evidence of mitochondrial disruption or cytochrome c–dependent caspase-9 activation. This liver injury was not ameliorated in a cyclophilin D knockout background nor by administration of necrostatin-1, but was completely inhibited by administration of a caspase inhibitor after Bid cleavage. Conclusion: Whereas either Bak or Bax is critically required for rapid execution of Fas-mediated massive apoptosis in the liver, delayed onset of mitochondria-independent, caspase-dependent apoptosis develops even in the absence of both. The present study unveils an extrinsic pathway of apoptosis, like that in type I cells, which serves as a backup system even in type II cells.
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Fas, also called APO-1 and CD95, is one of the death receptors that are potent inducers of apoptosis and constitutively expressed by every cell type in the liver.[1] Dysregulation of Fas-mediated apoptosis is involved in several liver diseases.[2] In the liver of patients with chronic hepatitis C, Fas is overexpressed in correlation with the degree of hepatitis, and Fas ligand can be detected in liver-infiltrating mononuclear cells.[3,4] Fas is also strongly expressed in the livers of patients with chronic hepatitis B, autoimmune hepatitis, and nonalcoholic steatohepatitis.[4,5] Moreover, in the liver of patients with fulminant hepatitis, Fas is up-regulated with strong detection of Fas ligand.[6] In mice, injection of Jo2 agonistic anti-Fas antibody leads

Abbreviations: ALT, alanine aminotransferase; CypD, cyclophilin D; DISC, death-inducing signaling complex; DKO, double knockout; DMSO, dimethylsulfoxide; IAP, inhibition of apoptosis protein; KO, knockout; PARP, poly(adenosine diphosphate ribose) polymerase; RIP, receptor-interacting protein; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; WT, wild-type.

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to massive hepatocyte apoptosis and lethality, suggesting that the hepatocyte is one of the most sensitive cell types to Fas stimulation.[7] This model is considered to at least partly mimic human fulminant liver failure.

Fas, upon ligation by Fas ligand, activates caspase-8 through the recruitment of Fas-associated protein with a death domain and formation of the death-inducing signaling complex (DISC).[1,2] Whereas activated caspase-8 directly activates effector caspases such as caspase-3 and caspase-7 through the so-called extrinsic pathway, leading to apoptosis in type I cells, it activates caspase-3/7 through the mitochondrial pathway in type II cells. In type II cells, activated caspase-8 cleaves the BH3-only protein Bid into its truncated form, which in turn directly or indirectly activates and homo-oligomerizes Bak and/or Bax to form pores at the mitochondrial outer membrane, leading to the release of cytochrome c. After being released, cytochrome c assembles with Apaf-1 to form apoptosomes which promote self-cleavage of procaspase-9 followed by activation of caspase-3/7 to cleave a variety of cellular substrates such as poly(adenosine diphosphate ribose) polymerase (PARP) and finally to execute apoptosis.[8,9] Hepatocytes are considered to be typical type II cells, because Bid knockout (KO) mice were reported to be resistant to hepatocyte apoptosis upon Fas activation.[10,11] Although Bak and Bax are crucial gateways to apoptosis of the mitochondrial pathway, little information is available about their significance in hepatocyte apoptosis because most traditional Bak/Bax double knockout (DKO) mice (*bak*$^{-/-}$ *bax*$^{-/-}$) die perinatally.[12]

In the present study, we tried to address this issue by generating hepatocyte-specific Bak/Bax DKO mice. We demonstrate that either Bak or Bax is required and sufficient to induce Fas-mediated early-onset hepatocyte apoptosis and lethal liver injury. Importantly, even if deficient in both Bak and Bax, Bak/Bax DKO mice still develop delayed-onset caspase-dependent massive hepatocyte apoptosis, suggesting that the mitochondria-independent pathway of apoptosis, as observed in type I cells, works as a backup system when the mitochondrial pathway of apoptosis in the liver is absent. This study is the first to demonstrate the significant but limited role of Bak and Bax in executing Fas-induced apoptosis in the liver.

Materials and Methods

Mice. Heterozygous Alb-Cre transgenic mice expressing Cre recombinase gene under the promoter of the albumin gene were described.[13] We purchased Bak KO mice (*bak*$^{-/-}$), Bax KO mice (*bax*$^{-/-}$), and Bak KO mice carrying the *bax* gene flanked by 2 *lox*P sites (*bak*$^{-/-}$ *bax*$^{flox/flox}$) from the Jackson Laboratory (Bar Harbor, ME). Traditional cyclophilin D (CypD) KO mice have been described.[14] All mice strains that we used were created from a mixed background (C57BL/6 and 129). We generated hepatocyte-specific Bak/Bax DKO mice (*bak*$^{-/-}$ *bax*$^{flox/flox}$ *Alb-Cre*) or hepatocyte-specific CypD/Bak/Bax triple KO mice (*cypd*$^{-/-}$ *bak*$^{-/-}$ *bax*$^{flox/flox}$ *Alb-Cre*) by mating the strains. Mice were injected intraperitoneally with 1.5 or 0.5 mg/kg Jo2 anti-Fas antibody (BD Bioscience, Franklin Lakes, NJ) or intravenously with 0.25 mg/kg recombinant Fas ligand (Alexis Biochemicals, San Diego, CA) cross-linked with 0.5 mg/kg anti-Flag M2 antibody (Sigma-Aldrich, St. Louis, MO) to induce apoptosis. In some experiments, mice were intraperitoneally injected with 2 mg/kg necrostatin-1 (Sigma-Aldrich) or 40 mg/kg Q-VD-Oph (R&D Systems, Minneapolis, MN). They were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School.

Apoptosis Assay. Measurement of serum alanine aminotransferase (ALT) levels, hematoxylin and eosin staining, and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) of liver sections have been described.[15] Analysis of cytochrome c release from isolated mitochondria have also been described.[16] To detect DNA fragmentation, 1.5 μg DNA extracted from 30 mg liver tissue by Maxwell16 (Promega, Madison, WI) was incubated with 0.5 μg RNase A (Qiagen, Tokyo, Japan) and separated by way of electrophoresis on a 1.5% agarose gel.

Western Blot Analysis. For western immunoblotting, the following antibodies were used: anti–full-length Bid, anti–Cox IV, anti–cleaved caspase-3, anti–caspase-7, anti–caspase-8, anti–caspase-9, anti-PARP, anti-Bax, anti-cIAP1, and anti-XIAP antibodies were

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obtained from Cell Signaling Technology (Beverly, MA); anti-Bax and anti-cIAP2 antibodies were obtained from Millipore (Billerica, MA); anti-Bid antibody, which detects truncated Bid, was generously provided by Xiao-Ming Yin (Indiana University School of Medicine, Indianapolis, IN)[17]; and anti–β-actin antibody was obtained from Sigma-Aldrich. For isolation of the mitochondria-rich fraction, a Mitochondrial Isolation Kit (Thermo Scientific, Rockford, IL) was used. The isolation of hepatocytes from whole liver has been described.[13]

Detection of Bax Oligomerization. Liver tissue was lysed with HCN buffer (25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 300 mM NaCl, 2% CHAPS, protease inhibitor cocktail, phosphatase inhibitor cocktail, 100 μM BOC-Asp(OMe)CH2F [MP Biomedicals, Solon, OH]; pH 7.5). After the liver lysate was sonicated and centrifuged, the supernatant was collected and the concentration was adjusted. For cross-linking, 100 μL of the lysate was incubated with 5 μL 100 mM bis(maleimido)hexane (Thermo Scientific) and 5 μL 100 mM BS^3 (Thermo Scientific) for 30 minutes at room temperature as described.[18] After quenching the cross-linkers by way of incubation with 12 μL 1 M Tris-HCl (pH 7.5) for 15 minutes at room temperature, the lysate was boiled with sample buffer followed by western blot analysis for Bax.

Electron Microscopy. Livers were fixed by perfusion of phosphate-buffered saline with 2.5% glutaraldehyde solution buffered at pH 7.4 with 0.1 M Millonig's phosphate, postfixed in 1% osmium tetroxide solution at 4°C for 1 hour, dehydrated in graded concentrations of ethanol, and embedded in Quetol 812 epoxy resin (Nisshin EM, Tokyo, Japan). Ultrathin sections (80 nm) cut on ultramicrotome were stained with uranyl acetate and lead citrate and examined with an H-7650 electron microscope (Hitachi Ltd., Tokyo, Japan) at 80 kV.

Statistical Analysis. Data are presented as the mean ± SE. Differences between two groups were determined using the Mann-Whitney U test for unpaired observations. The survival curves were estimated using the Kaplan-Meier method and were tested by way of log-rank test. $P < 0.05$ was considered statistically significant.

Results

Bak Deficiency Partially Ameliorates Fas-Induced Hepatocellular Apoptosis but Fails to Prevent Animal Death. First, to examine the significance of Bak in hepatocellular apoptosis induced by Fas stimulation, Bak KO mice ($bak^{-/-}$) and wild-type (WT) littermates ($bak^{+/+}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody and analyzed 3 hours later. Consistent with previous reports,[10,19] WT mice showed severe elevation of serum ALT levels with massive hepatocellular apoptosis (Fig. 1A,B). Bak KO mice also developed liver injury, but the levels of serum ALT and the number of TUNEL-positive hepatocytes were significantly lower in Bak KO mice than in WT mice (Fig. 1A-C). Western blotting for cleaved caspase-3, caspase-7, and PARP revealed that activation of effector caspases were partially inhibited in KO livers compared with WT livers (Fig. 1D). Cleavage of procaspase-9, which is initiated by mitochondrial release of cytochrome c, was also suppressed in Bak KO livers compared with WT liver (Fig. 1D). The cleaved form of caspase-8, a direct downstream target of Fas activation, was detected in both mice, but its levels were reduced in Bak KO mice compared with WT mice (Fig. 1D). This reduction may be explained by the lesser activation of caspase-3/7, because it has been reported that caspase-3/7 could activate caspase-8 through an amplification loop during apoptosis.[20] Collectively, these findings demonstrated that Bak deficiency partially ameliorated Fas-induced hepatocellular apoptosis associated with reduced cleavage of caspase-9, caspase-3/7, and PARP. We then compared survival of mice after Jo2 injection but found that Bak KO mice also rapidly died with kinetics similar to those of WT mice, suggesting that partial amelioration of hepatocellular apoptosis induced by Bak deficiency did not lead to survival benefit under our experimental conditions (Fig. 1E). Because Bax residing in the cytosol moves to the mitochondria upon activation, where it undergoes oligomerization,[21] we analyzed its translocation and oligomerization in the liver at 3 hours after Jo2 injection. Western blot analysis revealed that the levels of Bax expression clearly increased in the mitochondrial fraction in both WT livers and Bak KO livers (Fig. 1F). Signals for the Bax dimer were also detected in both livers (Fig. 1F). These findings indicate that Bax is also activated after Fas stimulation, raising the possibility of its involvement in hepatocellular apoptosis.

Bax Deficiency Fails to Ameliorate Fas-Induced Hepatocellular Apoptosis. Next, to examine the significance of Bax in hepatocellular apoptosis induced by Fas stimulation, Bax KO mice ($bax^{-/-}$) and WT littermates ($bax^{+/+}$) were injected with Jo2 and examined 3 hours later. There was no significant difference in the levels of serum ALT or the number of TUNEL-positive hepatocytes between the two groups (Fig. 2A-C), which is consistent with a previous report.[22] The levels of the cleaved forms of caspase-8, -9, -3, -7, and

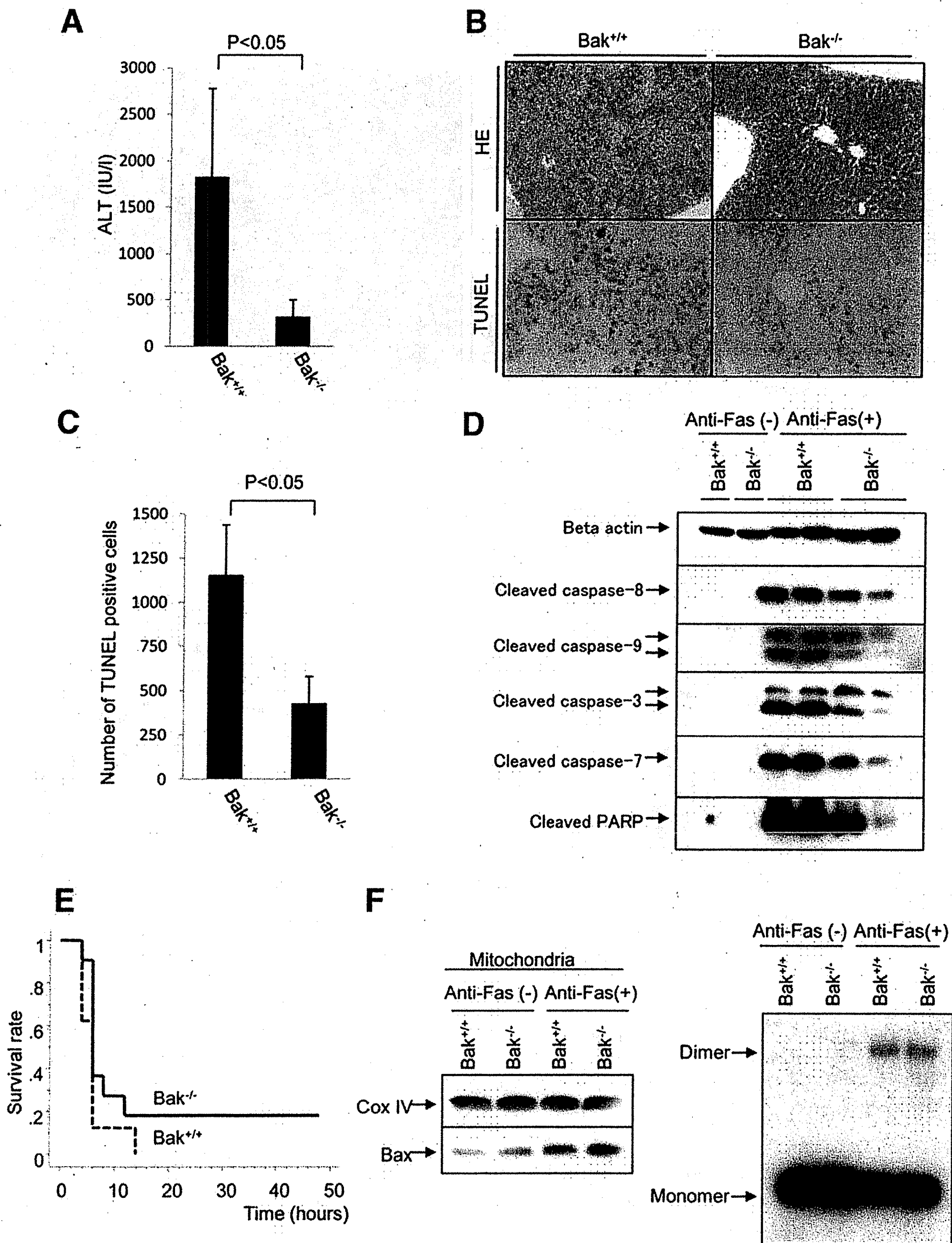

Fig. 1. Bak KO mice are partially resistant to Fas-induced hepatocellular apoptosis. Bak KO mice ($Bak^{-/-}$) or control WT littermates ($Bak^{+/+}$) were analyzed at 3 hours after intraperitoneal injection of 1.5 mg/kg Jo2 anti-Fas antibody. (A) Serum ALT levels (n = 10 or 11, respectively). (B) Hematoxylin and eosin (HE) and TUNEL staining of the liver sections. (C) Number of TUNEL-positive cells (n = 8 or 9, respectively). (D) Western blot analysis for the expressions of cleaved caspase-8, 9, -3, -7 and PARP. (E) Bak KO mice or control WT littermates were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody (n = 8 or 11, respectively). Survival rates after Jo2 injection are shown. (F) Bak KO mice or control WT littermates were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg) or vehicle. Left: Western blot analysis of the mitochondrial fraction of the liver for the expression of Bax. Right: Western blot analysis for the expression of Bax monomer and dimer in the liver.

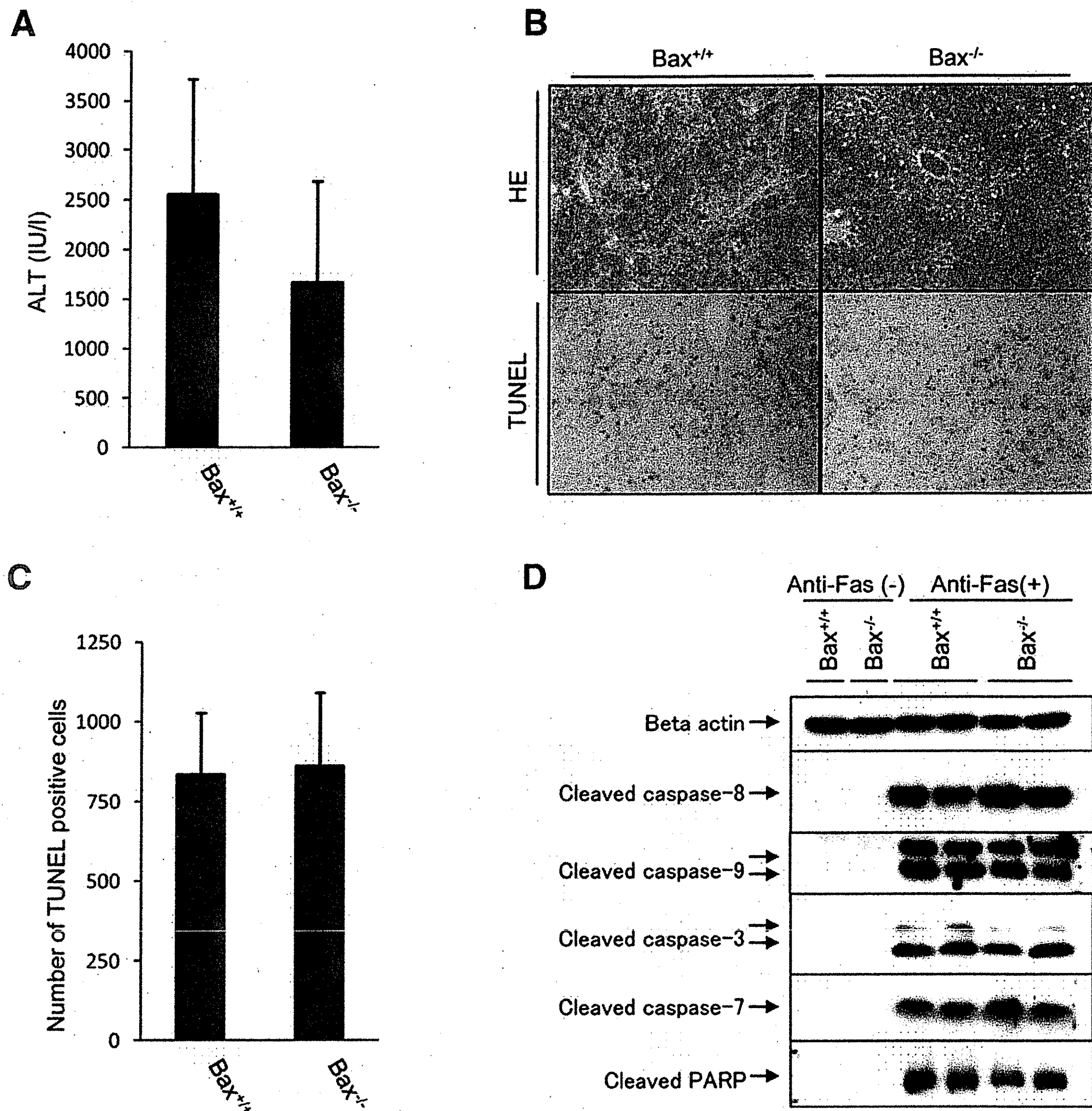

Fig. 2. Bax KO mice are not resistant to Fas-induced hepatocellular apoptosis. Bax KO mice (Bax$^{-/-}$) or control WT littermates (Bax$^{+/+}$) were analyzed 3 hours after intraperitoneal injection of Jo2 anti-Fas antibody (1.5 mg/kg). (A) Serum ALT levels (n = 11 per group). (B) Hematoxylin and eosin (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

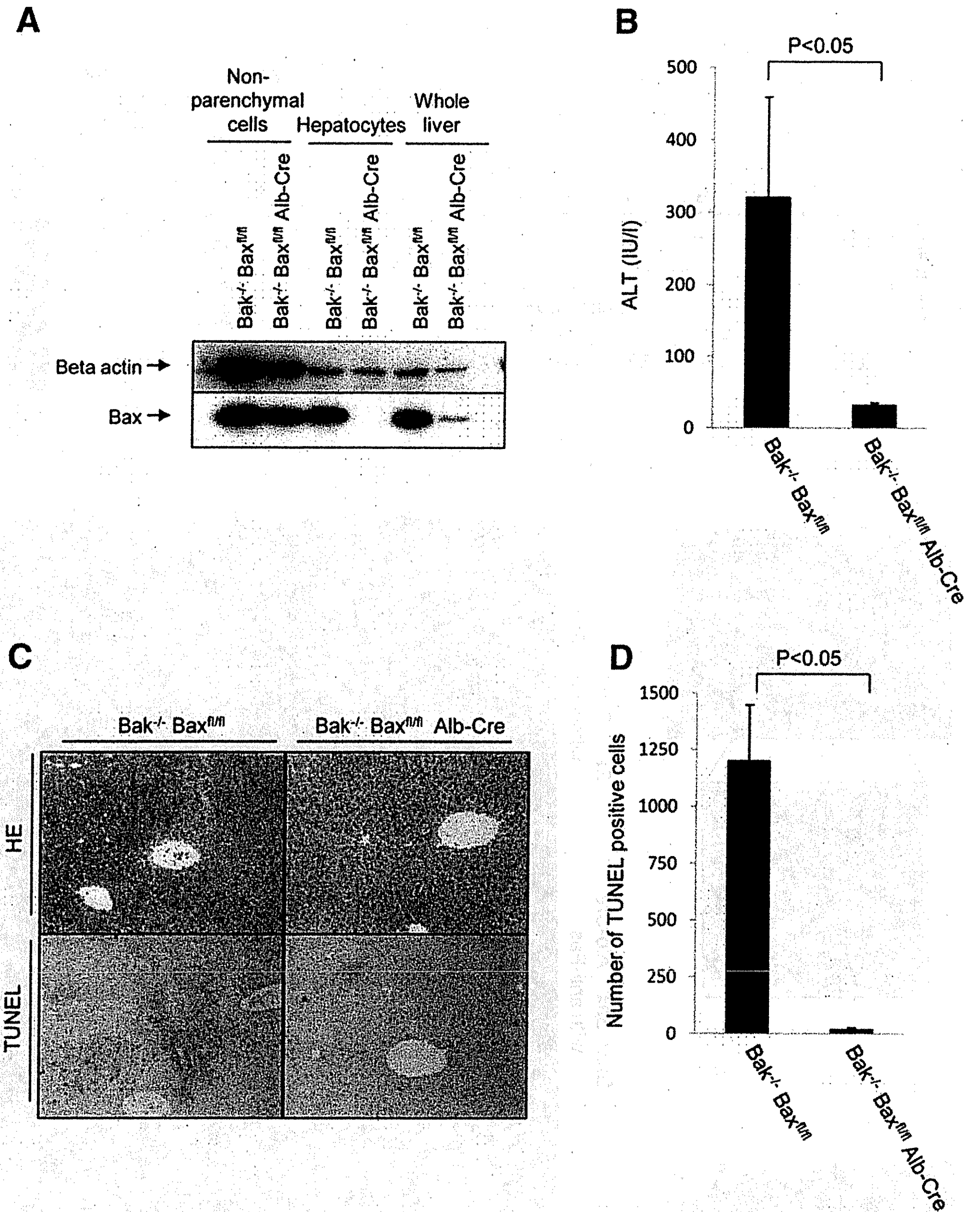

Fig. 3. Bak/Bax DKO mice are fully resistant to Fas-induced hepatocellular apoptosis in early phase. (A) Western blot analysis of the indicated fraction of the liver for the expressions of Bax. (B-D) Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) 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

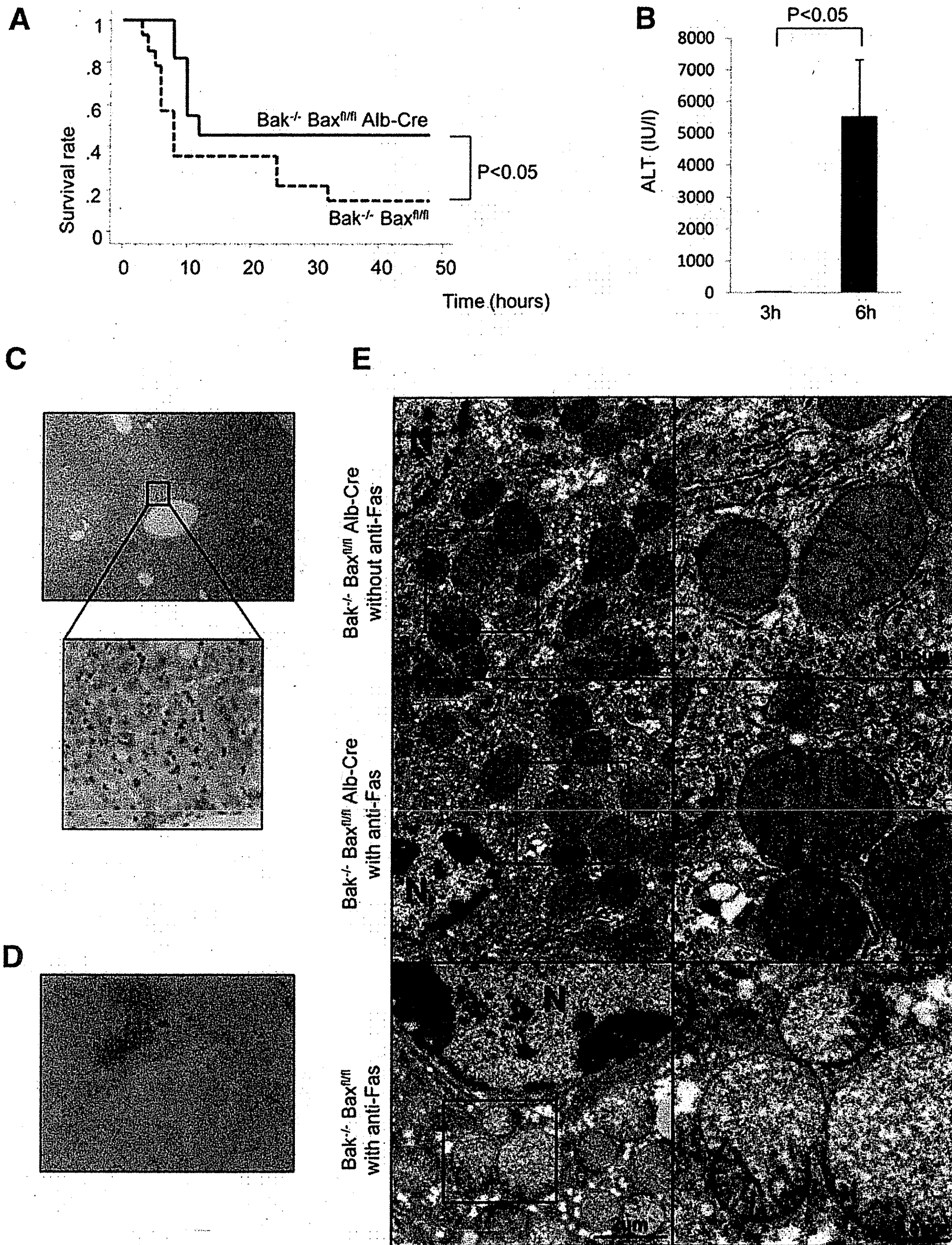

Fig. 4. Bak/Bax DKO mice develop late-onset severe liver injury upon Fas stimulation. Bak/Bax DKO mice ($Bak^{-/-}$ $Bax^{fl/fl}$ Alb-Cre) or control Bak KO littermates ($Bak^{-/-}$ $Bax^{fl/fl}$) were intraperitoneally injected with 1.5 mg/kg Jo2 anti-Fas antibody. (A) Survival rate after Jo2 injection (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.