pressed in the mice given mecamylamine, but not in those administered with anabasine (Fig. 4). These results indicated that the ACh/AChR system is associated with post-PH STAT3 activation in the liver. The finding that STAT3 plays a pivotal role in liver regeneration suggests that the vagus nerve is involved in liver regeneration as well as STAT3 activation after PH.

Experiment 2—Effect of Ach on IL-6 Production in Kupffer Cells

To assess the specificity of the IL-6 response to ACh, we evaluated IL-6 levels in the culture medium of Kupffer cells cultured for 6 h with or without ACh. Kupffer cells obviously released IL-6 in the presence of ACh (Fig. 5A). We also found that IL-6 mRNA levels were significantly increased in Kupffer cells incubated with ACh for 3 h (Fig. 5B). These data indicated that ACh stimulates Kupffer cells to produce and release IL-6.

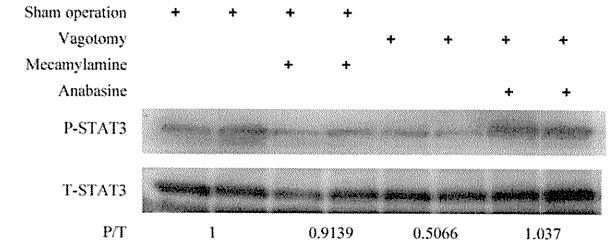

FIG. 4. Effect of AChR antagonist and AChR agonist on activation of STAT3. Western blot analysis of liver tissue in vagotomized, vagotomized + anabasine, control, and control + mecamylamine mice 2 h after PH. Vagotomized and control mice underwent PH 15 min after the administration of anabasine (AChR agonist) and mecamylamine (AChR antagonist), respectively.

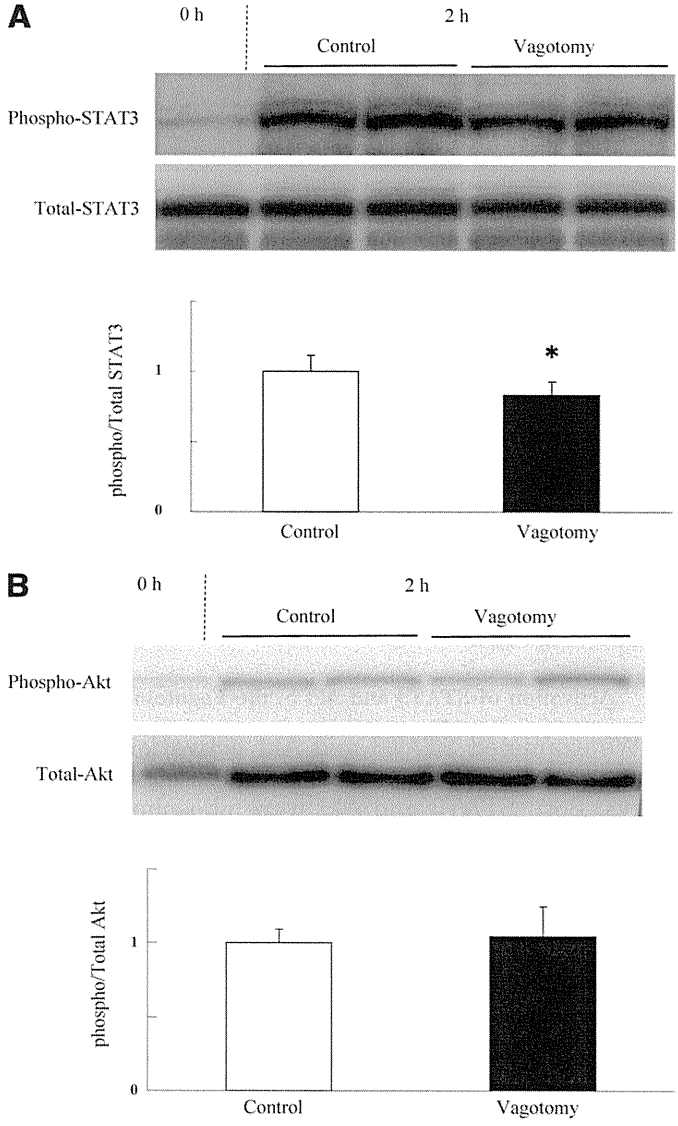

FIG. 3. Phosphorylation of STAT3 and Akt in control and vagotomy group. Western blot analysis of liver tissue 2 h after PH. (A) Upper panel: total and phosphor-STAT3. Lower panel: corresponding histograms from two separate analyses. (B) Upper panel: total and phosphor-Akt. Lower panel: corresponding histograms from two separate analyses. Data are presented as means ± S.D. (n = 5 per group). *$P < 0.05$ *versus* control group.

DISCUSSION

The autonomic nervous system innervates the liver and plays a role in metabolic control [29]. Subdiaphragmatic vagotomy causes more loss of body weight than sham vagotomy after PH, and this affects the restoration of liver mass [16, 30]. However, we created a mouse model of hepatic branch vagotomy that did not influence body weight after PH (data not shown). Hepatic branch vagotomy delays but does not suppress the increase in hepatic DNA synthesis activity of thy-

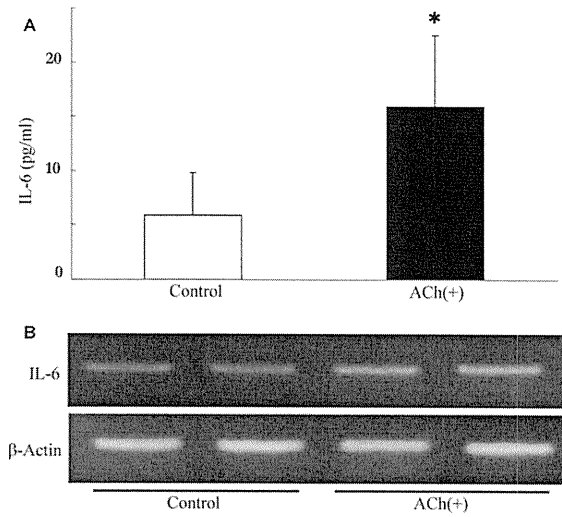

FIG. 5. Effect of ACh on IL-6 production in Kupffer cells. (A) IL-6 contents in medium of cultured Kupffer cells. Values are presented as means ± S.D. (n = 4 per group). *$P < 0.05$. (B) Expression of IL-6 mRNA by reverse transcriptase polymerase chain reaction in Kupffer cells incubated with saline or ACh (100 μM). Internal standard was β-actin.

midine kinase after PH [16, 30]. In our study, liver regeneration was also delayed by hepatic branch of vagotomy. In addition, the activity of STAT3 after PH was significantly suppressed by hepatic branch vagotomy. The effect of total autonomic denervation on liver regeneration is associated with regulation of the vascular system within the liver [21], but sectioning of the hepatic branch of vagus nerve did not change the blood flow of the hepatic artery or the portal vein [17]. Little is understood about the pathophysiological roles of autonomic nerves on the liver. We found that the vagus nerve is involved in liver regeneration through IL-6/STAT3 signaling via Kupffer cells/hepatocytes.

After PH, TNF-α signals from TNFRI activate nuclear factor kappa B on Kupffer cells that translocates into the nucleus to induce IL-6 gene expression [31–33]. Released IL-6 binds to its receptor and sends signals to STAT3 in hepatocytes for immediate liver regeneration [34]. The activation of STAT3 is important for stimulating quiescent hepatocytes to re-enter the cell cycle in a proliferative response to PH [34, 35]. We found here that STAT3 activation and the immediate mitotic response of the vagotomized liver after hepatectomy was significantly suppressed, which might be a key cause of impaired liver regeneration. The following data also supported the notion that inactivated STAT3 was restored by AChR agonists, which we used instead of ACh because ACh is degraded in extremely short time *in vivo* whereas AChR agonists are stable, and that AChR antagonists inactivated STAT3 in normal mice.

Kupffer cells are activated by lipopolysaccharide and secrete TNF-α and IL-6 [36]. During systemic inflammation, TNF-α and IL-6 act as pro-inflammatory cytokines, which activate mainly neutrophils, lymphocytes, and vascular endothelium, and cause cell and tissue damage [38]. Under this condition, the vagus nerve suppresses acute inflammatory reactions by inhibiting the release of proinflammatory cytokines [24], while after PH, which is performed without damage to the residual liver and is not associated with inflammation [1], Kupffer cells also secrete IL-6, which acts on hepatocytes [14, 37], activates STAT3 [10–12], and promotes liver regeneration [13]. We found that STAT3 activation after PH was suppressed by vagotomy. And ACh stimulated Kupffer cells to produce IL-6 at the transcriptional level and to secrete IL-6 *in vitro*. Kupffer cells can be directly activated via AChR (via the vagus nerve) and produce/secrete IL-6 through nuclear factor kappa B activation immediately after PH.

Liver regeneration is very complicated. It consists of cell proliferation and apoptosis. IL-6 acts not only mitogenic but also antiapoptotic for hepatocytes [39]. There might be some effects of the vagus nerve on apoptosis. Various kinds of stimuli such as hepatocyte growth factor (HGF), epidermal growth factor, norepinephrine, and insulin besides IL-6 and TNFα participate in liver regeneration [1]. Especially, HGF is well known as a potent stimulator of DNA synthesis in hepatocytes. HGF activates a receptor tyrosine kinase c-Met, which stimulates diverse signaling pathways including Ras, mitogen-activated protein kinase, phosphatidylinositol 3′-kinase, and phospholipase C [40]. The effects of the vagus nerve on these signaling pathways remain to be unknown.

In conclusion, we investigated the mechanism of liver regeneration from the viewpoint of autonomic regulation, especially by the vagus nerve. We found that the vagus nerve stimulates Kupffer cells to produce/secrete IL-6 in response to PH, activates STAT3 in hepatocytes, and eventually promotes liver regeneration. The mechanism of up-stream regulation of the vagus nerve after PH remains to be determined. However, the present findings provided a clue to the mechanisms of immediate liver regeneration after PH. Under extreme clinical situations such as massive hepatectomy or liver transplantation, our findings might provide some hints to help overcome postoperative liver failure.

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The Survival Pathways Phosphatidylinositol-3 Kinase (PI3-K)/Phosphoinositide-Dependent Protein Kinase 1 (PDK1)/Akt Modulate Liver Regeneration Through Hepatocyte Size Rather Than Proliferation

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Liver regeneration comprises a series of complicated processes. The current study was designed to investigate the roles of phosphoinositide-dependent protein kinase 1 (PDK1)-associated pathways in liver regeneration after partial hepatectomy (PH) using liver-specific *Pdk1*-knockout (L-*Pdk1*KO) and *Pdk1/STAT3* double KO (L-DKO) mice. There was no liver regeneration, and 70% PH was lethal in L-*Pdk1*KO mice. Liver regeneration was severely impaired equally in L-*Pdk1*KO and L-DKO mice, even after nonlethal 30% PH. There was no cell growth (measured as increase of cell size) after hepatectomy in L-*Pdk1*KO mice, although the post-PH mitotic response was the same as in controls. As expected, hepatectomy did not induce hepatic Akt-phosphorylation (Thr308) in L-*Pdk1*KO mice, and post-PH phosphorylation of Akt, mammalian target of rapamycin (mTOR), p70 ribosomal S6 kinase ($p70^{S6K}$), and S6 were also reduced. To examine the specific role of PDK1-associated signals, a "pif-pocket" mutant of PDK1, which allows PDK1 only to phosphorylate Akt, was used. Liver regeneration was recovered in L-*Pdk1*KO mice with a "pif-pocket" mutant of PDK1. This re-activated Akt in L-*Pdk1*KO mice liver and induced post-PH cell growth, without affecting cell proliferation. Further deletion of STAT3 (L-DKO mice) did not further deteriorate liver regeneration, although this certainly reduced post-PH mitotic response. These findings indicate that PDK1/Akt contribute to liver regeneration by regulating cell size. Regarding phosphatidylinositol-3 kinase (PI3-K), immediate upstream signal of PDK1, activation of PI3-K induced cell proliferation via STAT3 activation in the liver of L-*Pdk1*KO mice but did not improve impaired liver regeneration. This confirmed the pivotal role of PDK1 in liver regeneration and cell growth. *Conclusion:* PDK1/Akt-mediated responsive cell growth is essential for normal liver regeneration after PH, especially when cell proliferation is impaired. (HEPATOLOGY 2009;49:204-214.)

Abbreviations: BrdU, bromodeoxyuridine; L-DKO, liver-specific Pdk1/Stat3 double knockout; L-Pdk1KO, liver-specific Pdk1 knockout; L-Stat3KO, liver-specific Stat3 knockout; mTOR, mammalian target of rapamycin; myr-p110, myristoylated form of p110 (catalytic subunit) of PI3-K; PCNA, proliferating cell nuclear antigen; PDK1, phosphoinositide-dependent protein kinase 1; PH, partial hepatectomy; pif, PDK1-interacting fragment; PI3-K, phosphatidylinositol-3 kinase; $p70^{S6K}$, p70 ribosomal S6 kinase; SEM, standard error of the mean; STAT3, signal transducer and activator of transcription protein 3; S6, ribosomal S6; WB, western blot

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Liver regeneration is a physiopathological phenomenon of quantitative recovery from loss of liver mass to compensate for decreased hepatic volume and impaired function. The liver has a unique ability to restore lost volume, rarely seen in other organs.[1,2] It is well established that normal adult hepatocytes are usually quiescent but have the potential ability to replicate. After surgical procedures that reduce liver mass, such as partial hepatectomy (PH) or live donor liver transplantation, rapid enlargement of the residual or grafted liver commonly takes place to restore liver mass and function. Clinically, liver regeneration has important implications because many therapeutic strategies for surgical treatment of liver diseases such as removal of liver tumors and liver transplantation depend on the ability of liver to regenerate physically and functionally. Poor or insufficient liver regeneration may be potentially fatal for these patients.[3-5] Therefore, better understanding of the physiopathological features of liver regeneration could lead to clinical benefits.

Numerous studies so far have sought to elucidate the mechanisms responsible for liver regeneration, investigating the regulation of cell proliferation in simple rodent hepatectomy models.[6-11] The importance of interleukin-6/signal transducer and activator of transcription-3 (STAT3) pathway in liver regeneration has been established, as reported by a number of researchers[12,13] using liver-specific *Interleukin-6* or *Stat3* knockout mice. Regarding STAT3, it was found that the defective mitotic response to hepatectomy in liver-specific *Stat3*-KO (L-*Stat3*KO) mice did not affect physical and functional liver regeneration at all.[6] Although the hepatocyte mitotic response was suppressed or greatly delayed in L-*Stat3*KO mice, Akt and its associated signaling molecules such as p70 ribosomal S6 kinase ($p70^{S6K}$), mammalian target of rapamycin (mTOR), and glycogen synthase kinase-3 were immediately phosphorylated after PH. These findings suggest that when cell proliferation is impaired, these molecules mediate a possible compensatory mechanism of liver regeneration.

The phosphatidylinositol-3 kinase (PI3-K)/phosphoinositide-dependent protein kinase 1 (PDK1)/Akt pathway, known as a survival pathway, targets various molecules involved in anti-apoptosis, anti-oxidation, and protein synthesis.[14-18] Recent reports have shown that PI3-K/PDK1/Akt pathway and its associated molecules are responsible for determining cell size and functions.[6,19-24] Many of these studies used animals with targeted gene disruption to demonstrate the crucial roles of these molecules (mTOR, $p70^{S6K}$, and glycogen synthase kinase-3) in determining the "inherent size of cells" in the specific organ. In contrast, liver-specific knockout of *phosphatase tensin homolog deleted on chromosome 10*, a negative regulator of PI3-K/Akt pathway, induced enlargement of the organ. Persistent stimulation of PI3-K/Akt pathway in hepatocytes resulted in an enlarged liver mass mainly by stimulating glycogen/fatty acid synthesis.[25] Because the PI3-K/Akt pathway is certainly involved in glucose/fat metabolism as well as protein metabolism in the liver,[26,27] these findings suggest a crucial role for these molecules in cell growth and liver regeneration during an acute response, such as occurs post-PH. However, the role of this pathway in acute responses to such stimuli has not yet been clearly determined.

We hypothesized that PI3-K/PDK1 plays a pivotal role in liver regeneration by positively regulating cell growth, especially in cases in which cell proliferation is severely impaired. The current study was therefore designed to further examine the roles of PI3-K/PDK1 and associated molecules in liver regeneration and to determine the molecule(s) critically responsible for post-PH liver regeneration in mice.

Materials and Methods

Generation of Liver-Specific Knockout Mice. We generated liver-specific *Pdk1*-knockout (L-*Pdk1*KO) mice and liver-specific *Pdk1/Stat3* double knockout (L-DKO) mice,[28] which harbor a transgene for Cre-recombinase under the control of albumin gene promoter and are homozygous for a floxed allele of *Pdk1* or both *Pdk1* and *Stat3*, respectively, by crossing Alb-*Cre* mice[29] with *Pdk1*-flox mice and *Stat3*-flox; *Pdk1*-flox mice, respectively. We used *Stat3*-flox/flox and *Pdk1*-flox/flox littermates as controls for L-*Pdk1*KO and L-DKO mice, respectively. Although mice with a liver-specific deficiency of PDK1 were previously shown to develop a severe condition characterized by prominent edema and premature death,[27] L-*Pdk1*KO mice generated in the current study appeared normal until at least 6 months of age. The metabolic phenotypes of L-*Pdk1*KO and L-DKO mice have been described elsewhere.[28]

Animal Experiments. L-*Pdk1*KO, L-DKO, and C57BL/6 mice (male, 8-10 weeks) were used for simple 70%/30% PH experiments. Anesthesia was induced with an intraperitoneal injection of Nembutal (pentobarbital sodium, 60 mg/100 g body weight). Mice were fasted overnight before the experiments. After laparotomy, the left and median liver lobes were surgically resected for 70% PH, and the left lobe for 30% PH. The mice were sacrificed for collection of liver specimens at the indicated times before or after hepatectomy, and the liver/body weight ratios were calculated to estimate the recovery of liver mass. The animals were maintained under standard

conditions and treated according to the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University School of Medicine.

Cell Proliferation Assay. To evaluate proliferation of hepatocytes after PH, proliferating cell nuclear antigen (PCNA)-positive, bromodeoxyuridine (BrdU)-labeled hepatocytes and mitotic hepatocytes were counted. Liver tissues were removed before and 48 hours or 72 hours after hepatectomy (for PCNA and BrdU or mitosis assessment, respectively), fixed in 10% buffered formalin, and paraffin embedded. Hematoxylin-eosin staining and immunohistochemical staining with anti-PCNA were performed. For BrdU labeling assay, BrdU labeling reagent was injected intravenously into mice at 1 mL/100 g body weight 1 hour before sacrifice. BrdU was immunostained with anti-BrdU antibody according the manufacturer's recommendations (Roche, Basel, Switzerland). At least 500 hepatocytes were counted for mitotic or PCNA/BrdU positivity at least three times in different sections in each group.

Electrophoretic Mobility Shift Assay. STAT3 DNA-binding activity was assayed using mutant 67 of serum inducible element of c-fos gene promoter (SIE-m67) oligonucleotide as a probe (5′-actgGGATTTTTC-CCGTAAATGGTC-3′). The reaction mixture contained nuclear protein extract (5 μg), dithiothreitol (2 mM), poly(deoxyinosinic-deoxycytidylic) acid sodium salt (dI-dC) (2 μg), single-stranded DNA (10 μg/mL), and ^{32}P-labeled SIE-m67 probe (5×10^5 cpm). The specimens were electrophoresed on 5% polyacrylamide native gels at 4°C in 0.25× Tris-Borate/ethylenediaminetetra-acetic acid buffer.

Western Blot Analysis. Thirty micrograms whole liver protein extract was separated by 10% sodiumk dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following antibodies were used as primary antibodies: PDK1, STAT3/phospho-STAT3 (Santa Cruz, CA), Akt/phospho-Akt (Thr and Ser), p70^{S6K}/phospho-p70^{S6K}, mTOR/phospho-mTOR (Cell Signaling, MA), and PCNA (Santa Cruz, CA).

Measurement of Cell Size. The method to measure the size of hepatocytes in liver sections was described previously.[6] Briefly, individual hepatocytes were outlined and cross-sectional area was determined with a computer-assisted image analysing system (LSM Image Browser, Carl Zeiss GmBH, Jena, Germany). Cell areas of at least 500 hepatocytes were randomly selected in zone 2 and calculated in triplicate using different sections in each group.

Adenoviral Vectors (LacZ, L155E, and myr-p110). The replication-deficient adenovirus encoding *β-galactosidase* was used as a control vector (LacZ). An adenovirus vector encoding PDK1-interacting fragment (pif)-pocket mutant of *Pdk1*(L155E) was generated, where Leu155 was replaced by Glu, allowing PDK1 signaling exclusively to Akt, but not to p70^{S6K} or any others.[30] An adenovirus vector encoding a hemagglutinin-tagged myristoylated form of p110 (catalytic subunit) of PI3-K (myr-p110) was generated as described previously.[31] All viruses were produced in human embryonic kidney 293 cells, purified on double cesium chloride gradients, and plaque-titered. All adenoviruses were injected intravenously via tail vein 72 hours before the experiments.

Statistical Analysis. Results are expressed as means ± standard error of the mean (SEM). Statistical analyses were performed with Fishers' test, and *P* values less than 0.05 were considered significant.

Results

L-Pdk1KO Mice and L-DKO Mice. Western blot (WB) analyses showed the expression of PDK1 and PDK1/STAT3 in the livers of L-*Pdk1*KO and L-DKO mice, respectively (Fig. 1A). Only a trace amount of PDK1 was detected in L-*Pdk1* KO liver, but no PDK1 and STAT3 were detected in L-DKO liver. These mice, however, showed normal liver structure and morphology (Fig. 1B), as reported previously.[27] Liver and body weights of these knockout mice were no different from control mice at 8 to 10 weeks of age (data not shown).

Survival and Liver Regeneration After Hepatectomy in L-Pdk1KO Mice and L-DKO Mice. Six of 10 L-*Pdk1*KO mice with conventional 70% PH died within 12 hours. Liver regeneration of the remaining four L-*Pdk1*KO mice was severely impaired (Fig. 2A). However, 30% PH allowed all mice to survive until at least 14 days post-PH (Fig. 2B). Liver/body weight ratios showed that estimated liver regeneration was significantly and persistently suppressed at this time in L-*Pdk1*KO mice. Because albumin is produced exclusively by hepatocytes, serum levels of albumin were measured to evaluate functional liver regeneration after PH in L-*Pdk1*KO mice (Fig. 2C). It was found that serum albumin levels were maintained in the normal range even after PH in control mice, but were still reduced 2 weeks post-PH in L-*Pdk1*KO mice. These data thus suggest that PDK1 is essential for the recovery of liver mass and function after hepatectomy. Regarding the other markers of liver function/injury, serum levels of glutamic oxaloacetic transaminase/glutamate pyruvate transaminase/lactate dehydrogenase and bilirubin did not differ between control and L-*Pdk1*KO mice before PH, and were moderately increased after PH, showing somewhat higher levels in the latter mice (Fig. 2D). Blood glucose levels were decreased

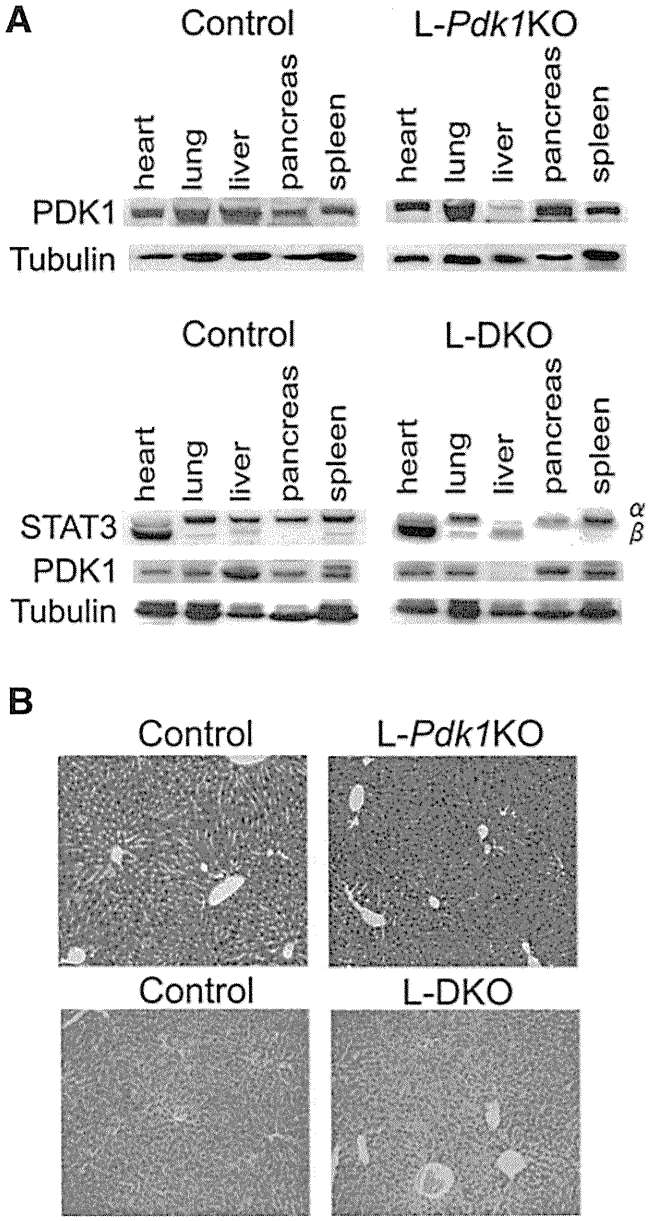

Fig. 1. Expression of PDK1 and STAT3 in liver tissues (A) and liver histology (B) in L-*Pdk1*KO and L-DKO mice. (A) WB showing liver-specific deletion of PDK1 and PDK1/STAT3 in L-*Pdk1*KO and L-DKO mice, respectively. (B) Hematoxylin-eosin staining showing normal liver structure of both L-*Pdk1*KO and L-DKO mice (original magnification ×200)

after PH but did not show any difference between the two groups.

Apoptotic cell death was very mildly induced by PH equally in control and L-*Pdk1* KO liver, but did not differ between the groups (Fig. 2E). This fact indicates that apoptotic cell death did not contribute to the reduced liver mass recovery in L-*Pdk1* KO mice.

Mitotic Responses in L-Pdk1KO Mice After Hepatectomy. Mitotic responses began immediately after PH in L-*Pdk1*KO mice even though liver regeneration was severely impaired (Fig. 3). Mitotic hepatocytes 3 days post-PH (Fig. 3A) and BrdU/PCNA-positive hepatocytes 2 days post-PH (Fig. 3B and C) were equally apparent in control and L-*Pdk1*KO mice. The numbers of mitotic cells, BrdU-positive cells, and PCNA-positive cells observed at each time (48 hours, 72 hours) in the post-PH liver tissues were not statistically different between the groups. BrdU incorporation into nuclei, as well as PCNA expression, also remained identical. These data suggest that deletion of PDK1 in liver did not affect the initial mitotic response of hepatocytes after PH.

In support of these observations, STAT3, which is a pivotal transcription factor in the post-PH mitotic response, showed similar activity in both control and L-*Pdk1*KO mice (Fig. 3D). Phosphorylation of STAT3 at Tyr705 and Ser727 and its binding to DNA occurred immediately after PH, and recovered to basal levels by 72 hours in both knockout and control mice. STAT3 activation after PH also did not differ in its intensity and timing in control and L-*Pdk1*KO mice.

Cell Size (Cell Growth) After Hepatectomy and Activation of PDK1-Associated Signals. Hepatocyte cell size in L-*Pdk1*KO mice without PH was slightly smaller than in controls, but the difference was not statistically significant (Fig. 4A). Cell size in normal livers increased significantly after PH, but not in L-*Pdk1*KO mice; rather, there was a tendency to decrease in the latter.

PDK1 specifically phosphorylates Akt at Thr308, and its activity can be assessed in this way (Fig. 4B). We found that Akt was phosphorylated at Thr308 immediately but transiently after PH in controls, but not in L-*Pdk1*KO livers up to 14 days post-PH. Akt phosphorylation at Thr308 was completely abolished in L-Pdk1 KO liver. This means that the liver of L-*Pdk1* KO mice is enough to study the function of PDK1, although PDK1 expression is slightly detected in L-*Pdk1* KO liver (Fig. 1A). Interestingly, Akt was strongly and persistently phosphorylated at Ser473 in L-*Pdk1*KO liver even in the quiescent state, as well as after PH, although this is normally carried out by PI3-K–regulated kinases other than PDK1. Also, the increased amounts of total Akt were observed in L-*Pdk1* KO liver, which may be the result of increased production of Akt in response to the reduced signal from PDK1.

Among the molecules downstream of PDK1, Akt, mTOR, $p70^{S6K}$, and S6 may potentially contribute to the maintenance of normal liver regeneration when cell proliferation is suppressed.[6] To confirm the involvement of these molecules in the impairment of liver regeneration in L-*Pdk1*KO mice, phosphorylation (activation) of mTOR, $p70^{S6K}$, and S6, downstream targets of $p70^{S6K}$, as well as Akt, was also examined (Fig. 4B). Mtor and $p70^{S6K}$ were weakly phosphorylated even in quiescent liver. Al-

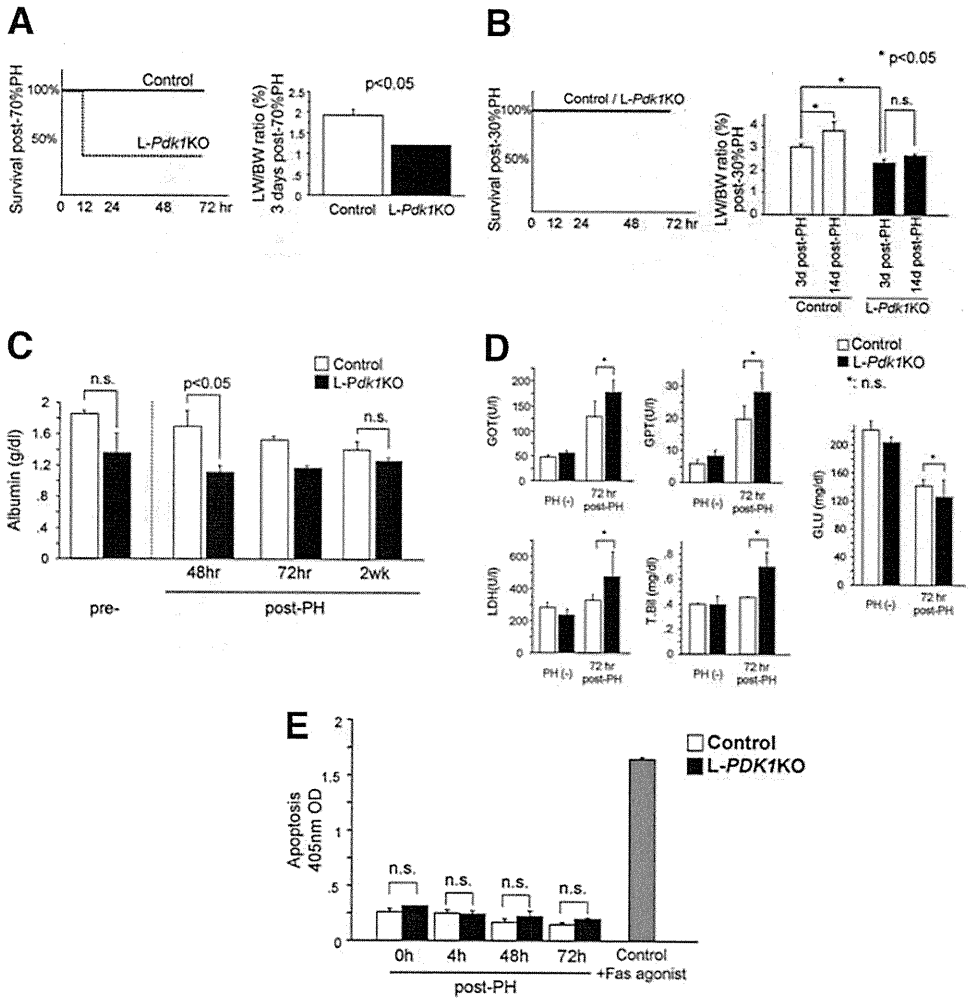

Fig. 2. Impaired liver regeneration after PH in L-*Pdk1*KO mice. (A) Seventy percent PH was lethal within 12 hours with no liver regeneration. (B) Thirty percent PH allowed the L-*Pdk1*KO mice to survive at least until 14 days post-PH with no liver regeneration. (C) Serum albumin levels were reduced in L-*Pdk1*KO mice transiently after PH. (D) Serum levels of glutamic oxaloacetic transaminase/glutamate pyruvate transaminase/lactate dehydrogenase and bilirubin and blood glucose level were measured pre-PH and post-PH. No significant differences were observed between control and L-*Pdk1*KO mice. Data are from at least three independent experiments, expressed as mean ± SEM.

though mTOR, $p70^{S6K}$, and S6 were all phosphorylated markedly from 4 hours after PH up until 72 hours in controls, they were not or were only very weakly phosphorylated in L-*Pdk1*KO liver after PH. S6 was markedly phosphorylated even at quiescence, which was slightly and transiently increased after PH in control livers. Deletion of PDK1 suppressed post-PH phosphorylation of S6, which quickly returned to the pre-PH level up to 72 hours post-PH in L-*Pdk1*KO liver regardless of liver mass recovery. $p70^{S6K}$ responded immediately to PH and possibly phosphorylated S6, but the latter is presumably also phosphorylated by kinases other than $p70^{S6K}$. Taken together, these findings may suggest the possibility that PDK1-Akt(/mTOR) pathway plays a more important role in post-PH liver regeneration by cell growth, even though $p70^{S6K}$/S6 pathway is certainly involved in cell growth in other types of cells.[32,33]

PDK1/Akt-Mediated Liver Regeneration by Cell Growth Rather than Cell Proliferation. To confirm the role of PDK1-Akt/mTOR pathway in liver regeneration, we employed the "pif-pocket" mutant of PDK1, which allows PDK1 to signal exclusively to Akt but not to $p70^{S6K}$ or others.[30,34-36] Adenovirus-mediated introduction of the pif-pocket mutant (L155E) did re-phosphorylate Akt (Thr308), but not $p70^{S6K}$ (Thr389), 4 hours after PH in L-*Pdk1*KO mice (Fig. 5A). Re-activation of Akt, but not $p70^{S6K}$, in L-*Pdk1*KO mice allowed responsive cell growth again in the post-PH liver without affecting cell proliferation, and eventually improved liver regeneration in L-*Pdk1*KO mice (Fig. 5A, B).

We also performed the experiment of 70% PH with hepatic introduction of the pif-pocket mutant (L155E), to confirm the effect of Akt on liver cell growth or proliferation after PH (Fig. 5C). Hepatic introduction of pif-pocket mutant let the 70% PH mice survive at least until 72 hours post-PH and recovered liver sufficiently. Post-PH mitosis and cell growth in L-*Pdk1* KO liver were observed to the same degree as the post-PH liver of control mice.

Furthermore, we performed an additional experiment to ask whether $p70^{S6K}$ indeed has no influence on hepatocyte cell size. Sodium salicylate, a known inhibitor of $p70^{S6K}$,[37,38] clearly suppressed phorbol myristate acetate–

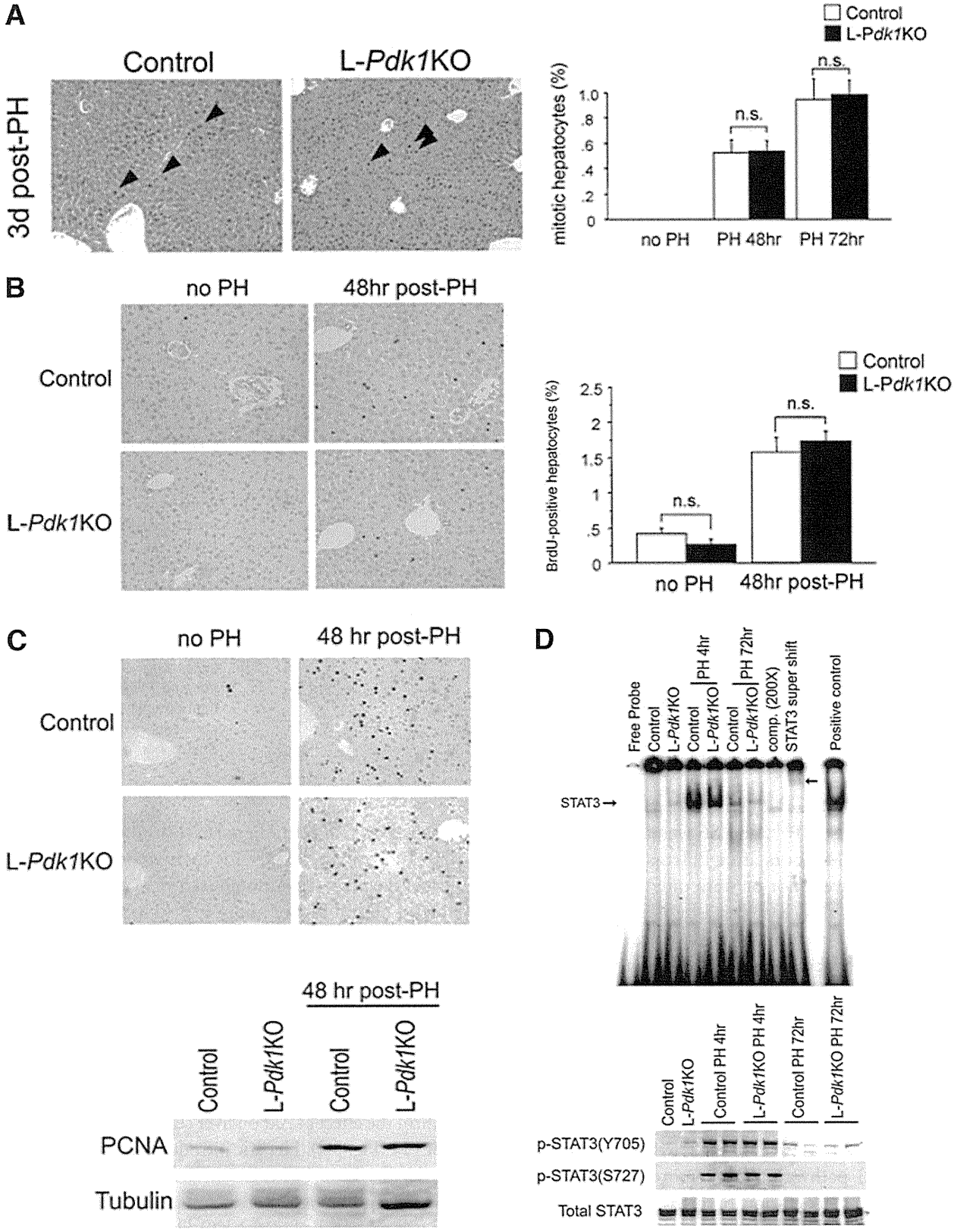

Fig. 3. Normal mitotic response after PH in L-*Pdk1*KO mice. (A) Hematoxylin-eosin staining showing mitotic hepatocytes 48 and 72 hours post-PH in control and L-*Pdk1*KO liver. (Arrowheads indicate mitotic hepatocytes. Original magnification ×200) Counts of mitotic, BrdU-positive, and PCNA-positive hepatocytes (immunohistochemistry and WB) in the regenerating liver document equivalent mitotic responses in control and L-*Pdk1*KO liver (A, B, C). Each photo is representative of at least three independent experiments. Data are expressed as mean ± SEM. (D) WB and electrophoretic mobility shift assays showing STAT3 phosphorylated (Tyr705 and Ser727) and activated to the same extent in control and L-*Pdk1*KO liver. Each blot is representative of at least three independent experiments.

induced phosphorylation of $p70^{S6K}$ in hepatocytes (Fig. 5D) but had no effect on Akt (data not shown). Inhibition of $p70^{S6K}$ did not affect hepatocyte cell size but slightly suppressed cell proliferation (Fig. 5D). This may suggest that $p70^{S6K}$ is involved mainly in transmitting mitotic signals, not cell growth signals. These data are consistent with our hypothesis that the PDK1–Akt pathway, but not PDK1–$p70^{S6K}$, plays a pivotal role in liver regeneration mainly by regulating cell size.

Activation of PI3-K Does Not Improve Post-PH Liver Regeneration in L-Pdk1KO Mice. To confirm the pivotal role of PDK1 in liver regeneration, we performed additional experiments to investigate the effects of PI3-K, a signaling molecule immediately upstream of PDK1, on liver regeneration in L-*Pdk1*KO mice.

In controls, activation of PI3-K by transducing myr-p110 leads to phosphorylation of Akt (Thr308 and Ser473) and STAT3 (Tyr705) in a dose-dependent manner and results in enlarged livers without PH (Fig. 6A). This may indicate that PI3-K signals to STAT3 for cell proliferation as well as Akt for cell growth, and contributes cooperatively to liver regeneration. An adenovirus vector of myr-p110 was injected to L-*Pdk1*KO mice at the time of PH (1×10^8 pfu/body). It was found that myr-p110 phosphorylated Akt at Thr308 and Ser473 in control mice, but only at Ser473 in L-*Pdk1*KO mice, because Akt Thr308 is phosphorylated only by PDK1 whereas Ser473 can be phosphorylated by PI3-K–regulated kinases other than PDK1 (Fig. 6B). Hepatic activation of PI3-K clearly induced more PCNA-positive and mitotic hepatocytes in the liver of L-*Pdk1*KO mice after PH than in controls (Fig. 6B). However, this was not sufficient to improve impaired liver regeneration and albumin synthesis in L-*Pdk1*KO mice (Fig. 6C). In contrast, additional deletion of STAT3 in the liver of L-*Pdk1*KO mice (L-DKO mice) did not affect its regeneration (Fig. 6D). These data indicate that PI3-K is important for the increase of liver mass by signaling STAT3 for cell proliferation and PDK1/Akt for cell growth, but cannot mediate liver regeneration if PDK1/Akt pathway is disturbed. PDK1-mediated signals for cell growth are thus crucial for liver regeneration.

To confirm the contribution of PI3-K/STAT3 to post-PH cell growth, we performed 30% PH in DKO mice with hepatic introduction of myr-p110. Constitutive activation of PI3-K failed to increase post-PH cell proliferation and growth, or to improve the impaired liver regeneration in DKO mice (Fig. 6E).

Discussion

Important roles for interleukin-6/STAT3 in liver regeneration have been reported previously.[7,8,10,12,13,39]

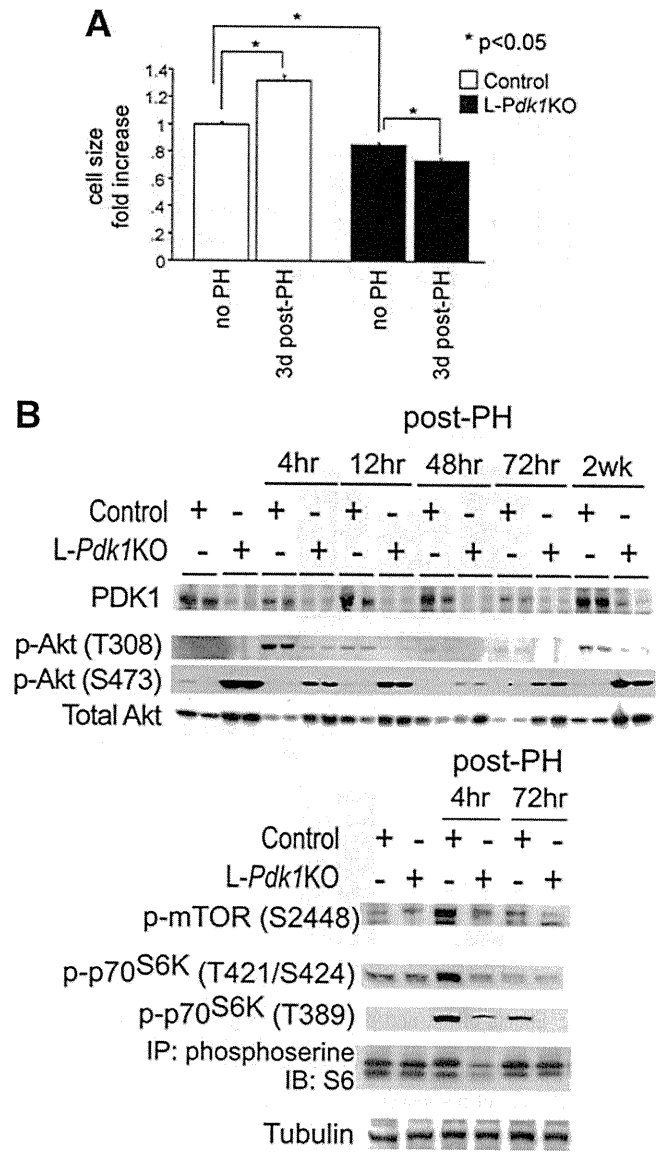

Fig. 4. Post-PH cell growth and activation of Akt, mTOR, $p70^{S6K}$, and S6 proteins in control and L-*Pdk1*KO liver (A, B). Responsive cell growth did not occur after PH in L-*Pdk1*KO mice. Akt, mTOR, $p70^{S6K}$, and S6 were all activated by PH in control mice, but hardly at all in L-*Pdk1*KO mice. The data at each time point consist of two representative blots from at least three independent experiments.

However, deletion of STAT3 in liver fails to affect liver regeneration despite almost completely suppressing the mitotic response, where Akt, $p70^{S6K}$, and mTOR signals are markedly activated.[6] It has therefore been suggested that "survival signals" such as Akt, $p70^{S6K}$, and mTOR may be responsible for the acute response in post-PH liver regeneration, possibly by regulating cell growth and thus may contribute to the initiation and maintenance of liver regeneration when the mitotic response is impaired.[6] Conversely, $p70^{S6K}$/S6, for example, is suggested to be involved in cell proliferation[40-42] and post-PH liver regeneration.[42] So far, the involvement and the roles of these

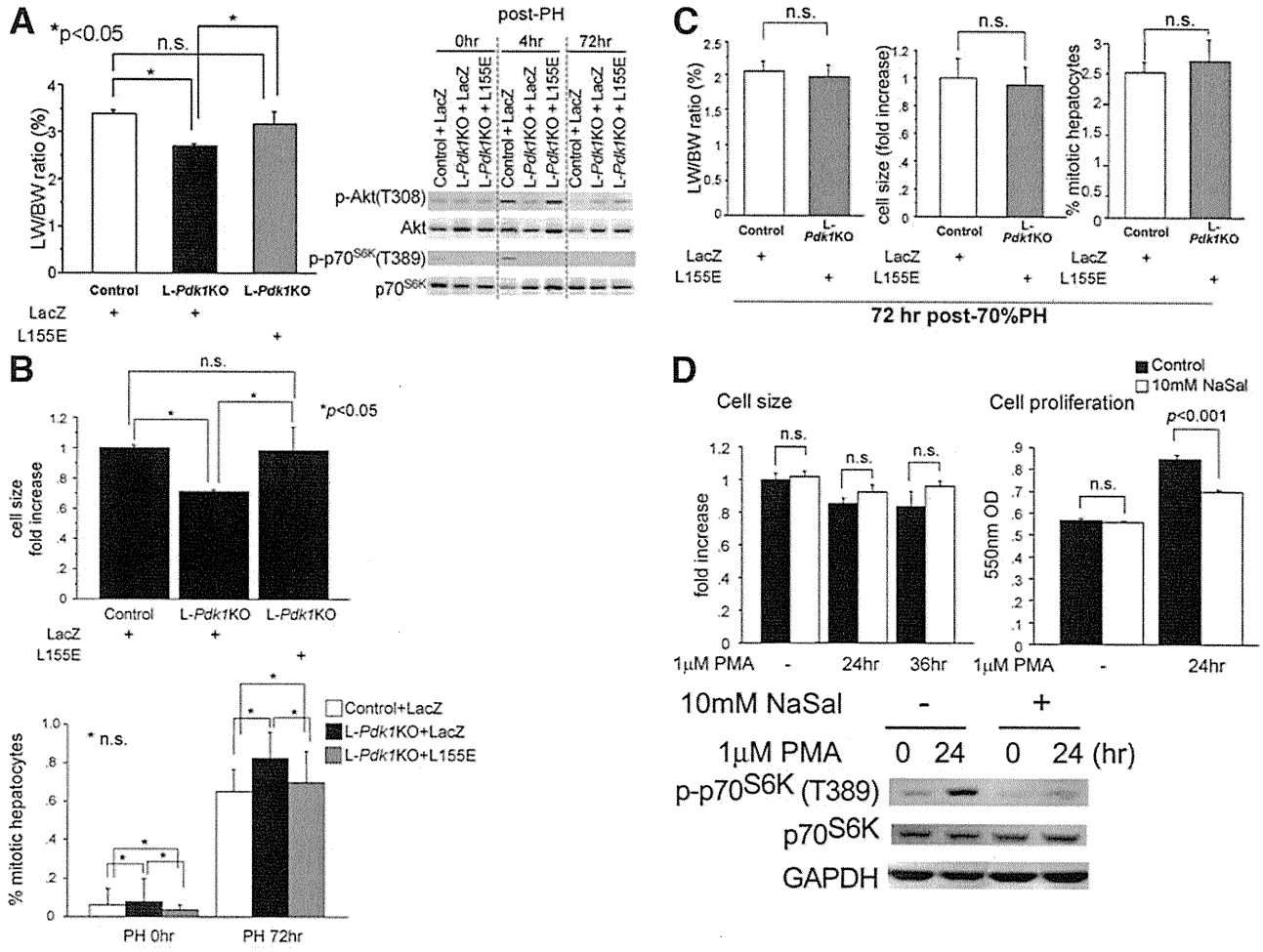

Fig. 5. Effects of Akt and p70^{S6K} on cell growth or proliferation in liver regeneration. (A) Introduction of pif-pocket mutant of PDK1 (L155E) to L-*Pdk1*KO liver allowed re-phosphorylation of Akt (Thr308), but not p70^{S6K} (Thr389), and normal regeneration of L-*Pdk1*KO liver 72 hours post-PH. (B) By introducing pif-pocket of PDK1 (L155E) into PDK1-deficient livers, hepatocytes re-grow in size, but post-PH mitotic cell counts are not affected. (C) Introduction of pif-pocket mutant of PDK1 (L155E) to L-*Pdk1*KO improved liver regeneration even in the 70% PH model. (D) Phosphorylation of p70^{S6K} (Thr389) suppressed by sodium salicylate (NaSal), an inhibitor of p70^{S6K} phosphorylation, in phorbol myristate acetate (PMA)-stimulated AML12 cells. NaSal was added to the culture media 16 hours before the experiment, and then AML12 cells were stimulated by PMA for 1 hour. Inhibition did not affect cell size, but slightly suppressed cell proliferation in phorbol myristate acetate-stimulated AML12 cells. Data are means ± SEM. Each blot is representative of at least three independent experiments.

proteins in cell proliferation and cell growth in liver regeneration have not been well delineated. In the current study, we investigated the roles of PI3-K/PDK1-mediated signaling pathways in liver regeneration after PH from the perspective of cell growth and proliferation. PI3-K signaled STAT3 for cell proliferation and PDK1/Akt for cell growth. We propose that PDK1/Akt-mediated signals (but not p70^{S6K}/S6) are essential contributors to physical and functional liver regeneration mainly by regulating cell size, but not cell proliferation.

PDK1-deficiency in liver had a critical effect on both physical and functional post-PH liver regeneration. Seventy percent PH was lethal to L-*Pdk1*KO mice; remaining liver tissue did not regenerate in either 70% or 30% PH models in L-*Pdk1*KO mice despite normal cell proliferation. The increase of cell size (cell growth) that occurred after PH in control mice was completely absent in L-*Pdk1*KO mice and in fact was even reduced (Fig. 4A). This finding may account for the impaired liver regeneration in L-*Pdk1*KO mice.

Regarding the molecules downstream of PDK1, Akt and mTOR were very weakly phosphorylated at quiescence, whereas they were markedly phosphorylated 4 hours after PH. In L-*Pdk1* KO mice, phosphorylation of these molecules was significantly reduced. In contrast, S6 (and p70^{S6K}) were phosphorylated even at quiescence in control mice, were phosphorylated 4 hours after PH, and had recovered to pre-PH levels by 72 hours post-PH. In L-*Pdk1* KO mice, phosphorylation of S6 was suppressed 4 hours after PH but recovered to pre-PH levels despite lack of recovery of liver mass. Considering these findings, it seems that PDK1 signals weakly to Akt/mTOR under

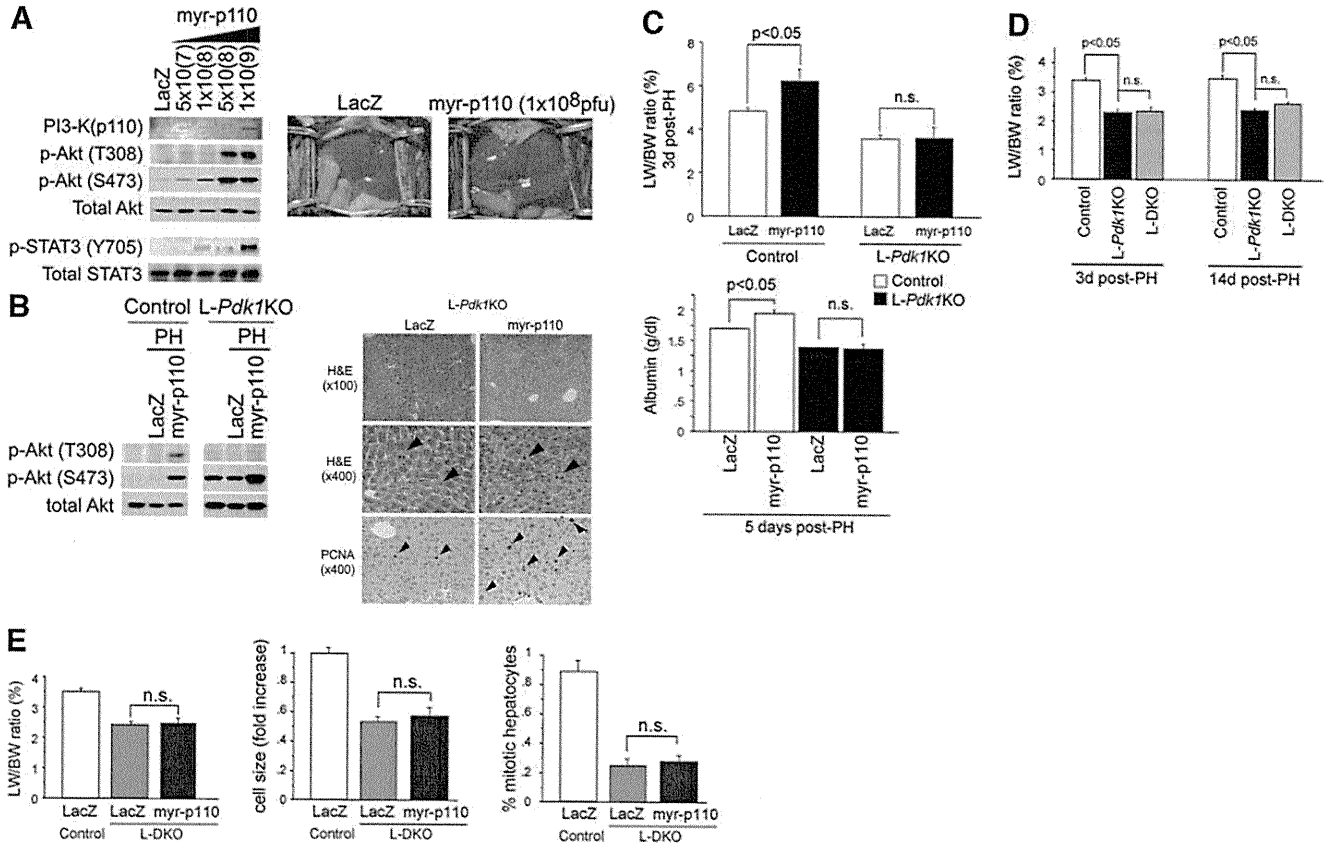

Fig. 6. Effects of PI3-K on liver regeneration in L-*Pdk1*KO mice. (A) Expression of constitutively active PI3-K (myr-p110) induced phosphorylation of Akt (Thr308 and Ser473) and STAT3 (Tyr705) in the liver and enlarged the liver mass. The amount of adenovirus was adjusted to 1×10^9 plaque-forming units in total by adding LacZ in each mouse. (B) Expression of constitutively active PI3-K (myr-p110) did not phosphorylate Akt at Thr308 and more strongly at Ser473 72 hours post-PH in L-Pdk1KO liver, and induced more hepatocyte proliferation after PH in L-*Pdk1*KO mice (Hematoxylin-eosin staining and immunostaining for PCNA. Arrowheads indicate mitotic hepatocytes. Original magnification ×200). (C) Activation of PI3-K failed to regenerate L-*Pdk1*KO liver after PH, despite efficacy in control liver. It also increased serum albumin levels in control mice, but not in L-*Pdk1*KO mice. Adenovirus vector encoding myr-p110 was intravenously injected at the time of surgery in this experiment (1×10^8 plaque-forming units/body). (D) Additional deletion of STAT3 in PDK1-deficient liver (L-DKO mice) did not further exacerbate liver regeneration in L-*Pdk1*KO mice. (E) Rescue experiment using myr-p110 showed that activation of PI3-K did not affect post-PH cell size, mitosis, or liver regeneration in L-DKO mice.

quiescent conditions but signals strongly immediately after PH in a transient manner for the liver regeneration response. In contrast, S6 (and $p70^{S6K}$) may be phosphorylated even when PDK1/$p70^{S6K}$ signals are not fully activated. S6 was phosphorylated after PH but returned to pre-PH levels regardless of liver mass recovery. These dynamics of protein phosphorylation may indicate that PDK1/Akt is more responsive than $p70^{S6K}$ to PH and plays more roles in liver regeneration. Canceling PDK1-mediated signals other than Akt in L-*Pdk1*KO liver led to full recovery of liver mass and responsive cell growth after PH. These findings suggest that PDK1/$p70^{S6K}$ signals may not be directly involved in cell growth–associated liver regeneration.

Stimulation of PI3-K induced activation of STAT3 and Akt, which enlarged liver and increased levels of serum albumin. This, however, failed to regenerate post-PH liver physically and functionally in L-*Pdk1*KO mice, despite marked proliferation of hepatocytes (Fig. 6B, C). Furthermore, liver regeneration of *Pdk1*KO and DKO mice was equally suppressed after PH (Fig. 6D). These findings clearly indicate the potential importance of PDK1 and PDK1-mediated cell growth in liver regeneration but suggest that STAT3-mediated cell proliferation is not involved. It was reported previously that Akt and associated signals are strongly activated immediately after PH in L-*Stat3*KO mice, in which the post-PH mitotic response is greatly suppressed,[6] and it was suggested that activation of these signals may be responsible for compensation of the impaired liver regeneration. Recent evidence indicates an essential role of certain molecules in determining cell size in genetically manipulated animals. Akt, mTOR, $p70^{S6K}$, and glycogen synthase kinase-3β are all good candidates for determining the original (inborn) cell size in various cells or organs.[19-21,23] This is consistent with our proposal that PDK1/Akt and associ-

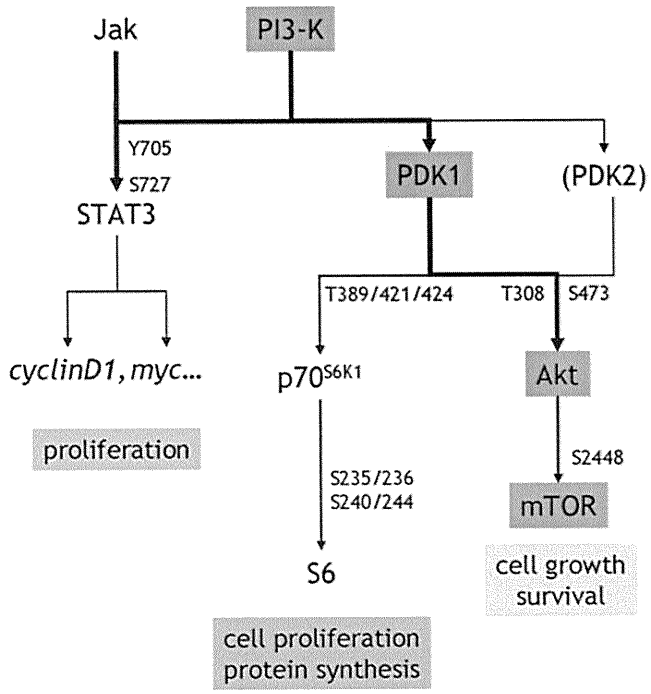

Fig. 7. Schematic illustration of the pivotal signaling pathways in liver regeneration. Janus kinase and PI3-K signaling to STAT3 for hepatocyte proliferation. PDK1/Akt plays a central role in cell growth-mediated liver regeneration and anti-apoptosis. $p70^{S6K}$ mediates protein synthesis and cell proliferation but may not be essential for liver regeneration.

ated signals contribute to liver regeneration mainly by inducing cell growth (cell size), but not by inducing cell proliferation.

Inhibition of $p70^{S6K}$ by salicylic acid mildly suppressed hepatocyte proliferation, although it was not seen to be involved in cell growth *in vitro* (Fig. 5C). It has been reported that $p70^{S6K}$/S6 deletion suppresses cell proliferation in different types of cells,[40-42] consistent with our results *in vitro*. However, hepatic suppression of $p70^{S6K}$ by deletion of PDK1 did not suppress hepatocyte proliferation after PH. Because PI3-K/STAT3 mitotic signals possibly function well enough to initiate/maintain cell proliferation in the post-PH liver, inhibition of $p70^{S6K}$ may not affect post-PH hepatocyte proliferation. Interestingly, mitotic responses and STAT3 activation after PH occurred to the same degree in L-*Pdk1*KO and control mice (Fig. 3A-D), despite the marked difference in liver regeneration (Fig. 2A, B). This indicates that, at least in the impaired liver regeneration induced by deficient PDK1, the interleukin-6/STAT3 pathway is activated but not sufficient to maintain normal liver regeneration.

Seventy percent PH was lethal in L-*Pdk1*KO mice (Fig. 2A) but not in L-*Stat3*KO mice.[6] Most L-*Pdk1*KO mice died within 12 hours of PH with no signs of liver regeneration. Serum markers of liver damage (glutamic oxaloacetic transaminase/glutamate pyruvate transaminase/lactate dehydrogenase and bilirubin) were moderately elevated 72 hours after PH even in 30% PH L-*Pdk1*KO mice (Fig. 2D), suggesting the possibility of post-PH extended liver failure in 70% PH L-*Pdk1*KO mice. The exact cause of death is not yet clear; however, remaining liver function certainly seems responsible for any survival. Liver function assessed by serum albumin level showed persistent reduction 14 days after PH. Reduced liver function of L-*Pdk1*KO mice may therefore be one of the major causes of death.

In conclusion, we have demonstrated a pivotal role for PDK1/Akt-mediated acute responsive cell growth in liver regeneration, and for PI3-K/STAT3-mediated mitotic pathway in a rodent PH model (Fig. 7). PDK1/Akt signals are especially crucial for avoiding post-PH liver failure as well as for maintaining normal liver regeneration capacity. Although further studies are necessary, the current report has begun to elucidate the molecular mechanisms underlying liver regeneration.

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Low prevalence of juvenile-onset Behçet's disease with uveitis in East/South Asian people

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ABSTRACT

Aim: There is little information on the demographic and clinical characteristics of Behçet's disease in children in different parts of the world. We sought to provide this information through a questionnaire survey of specialist eye centres.

Methods: Descriptive questionnaires were collected from 25 eye centres in 14 countries. The questionnaire surveyed details of juvenile-onset Behçet's disease with uveitis. Ethnic groups, clinical features, treatments and prognosis of paediatric-age Behçet's disease were examined on a worldwide scale.

Results: The clinical data of 135 juvenile-onset and 1227 adult-onset patients with uveitis were collected. The average age of disease diagnosis in the children was 11.7 years old. Of the ethnic groups identified 54% were from Middle East, 43% from Europe, but only 2% from East/South Asian countries. By contrast, 19.2% of adult patients were from East or South Asia. The frequency of genital ulcers in juvenile patients was 38.7%, which was significantly lower than in adult cases (53.5%; $p<0.01$).

Conclusions: Behçet's disease with uveitis was less common in children than in adults in East/South Asia. Although the clinical features of the systemic disease were similar in children and adults, there was a lower frequency of genital ulceration in children.

Behçet's disease is a chronic multisystem disorder characterised by oral aphthous ulcers, genital ulcers, skin lesions, ocular lesions, gastrointestinal involvement, vascular lesions and neurological manifestations. The mean age of disease onset is 25–30 years old,[1] and initial presentation in children is uncommon. Details of the presentation and clinical characteristics of juvenile-onset Behçet's disease have been limited to case reports or small case series.[2–8]

One of the manifestations of Behçet's disease is uveitis. In general, uveitis in childhood is uncommon with an incidence of 4–7/100 000 children per year.[9–11] Unlike adults, juvenile idiopathic arthritis (JIA) is the most common identified cause of uveitis in children.[12–14] Even in Turkey, one of the countries of the highest prevalence of Behçet's disease, JIA is the most common cause of uveitis in children, followed by idiopathic uveitis and pars planitis.[15] Behçet's disease is one of the three most frequent diagnoses in patients with uveitis in Japan.[16–18] Although the prevalence of Behçet's disease in the Japanese population overall has been estimated to be 10–15/100 000,[19] juvenile-onset Behçet's disease is uncommon.

Accordingly, there is little information on the demography and clinical characteristics of Behçet's disease with ocular lesions in children. Against this background, we administered a descriptive questionnaire survey in a large-scale international collaborative study and compared the epidemiology, clinical phenotype and visual outcome in children and adults. A summary of the clinical features of the whole cohort has been recently reported.[1]

METHODS

Descriptive questionnaires were sent to 132 ophthalmology centres by email and airmail in 2005–2006. We encouraged the centres to provide clinical data on as many patients as possible. We gave no details of juvenile-onset Behçet's disease in the survey to avoid bias in the recruitment of patients in their institutions. Responses were collected from 25 eye centres in 14 countries; Australia, Germany, Greece, India, Iran, Italy, Japan, Jordan, Morocco, Portugal, Turkey, Saudi Arabia, Tunisia and the UK. The race of each patient was also ascertained. The inclusion criterion was development of the disease at less than 16 years of age. The age of disease onset was recognised as the timing of meeting the classification criteria based on the estimation of the responding doctors. Statistical analysis was performed using the χ^2 test or F test. Values of $p<0.01$ were considered statistically significant.

RESULTS

Clinical data on 1465 Behçet's disease patients were successfully collected. From these, the age of disease onset was reported for 1362 patients: 1227 patients were considered adult-onset and 135 were juvenile-onset. Boys accounted for 65.2% of the cohort of juvenile-onset patients (table 1). HLA-B51 was detected in 68.6% of children and 61.6% of adult patients. The mean age of disease diagnosis in children was 11.7 years old, and the mean follow-up period was 13.5 years (table 1). We were able to identify the ethnic groups of 127 children: 53.5% (68 cases) were from Middle Eastern countries, 43.3% (55 cases) from Europe, and only 2.4% (three cases) from East/South Asia (fig. 1). One case was reported as Cuban of mixed race. Among adults, 19.2% of patients were from East/South Asia (fig. 1). This difference in ethnicity was significantly different between adult-onset and juvenile-onset ($p<0.004$, F test).

Among children, there were recurrent oral aphthous ulcers in 94.8%, skin lesions in 67.4% and genital ulcers in 38.5% (fig. 2). In adults, recurrent oral aphthous ulcers in 94.5%, skin lesions in 70.0% and genital ulcers in 53.5% (fig. 2). The frequency of genital ulcers in juvenile patients was significantly lower than that in adult

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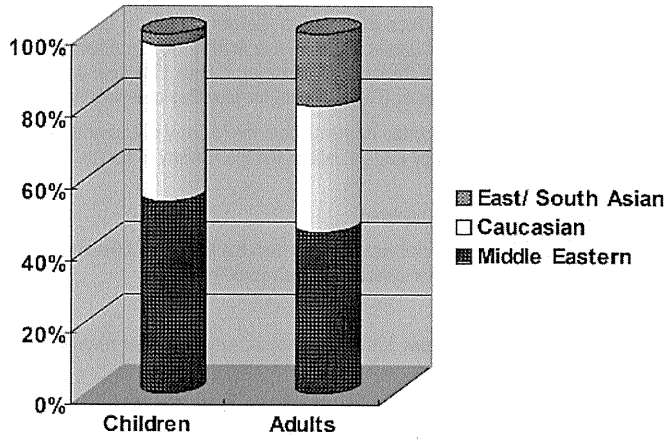

Figure 1 Ethnic groups of patients with Behçet's disease. Middle Eastern, Caucasian and East/South Asian people were the three major ethnic groups of Behçet's disease among adults. However, only a few East/South Asian patients suffered from the disease in childhood ($p<0.01$).

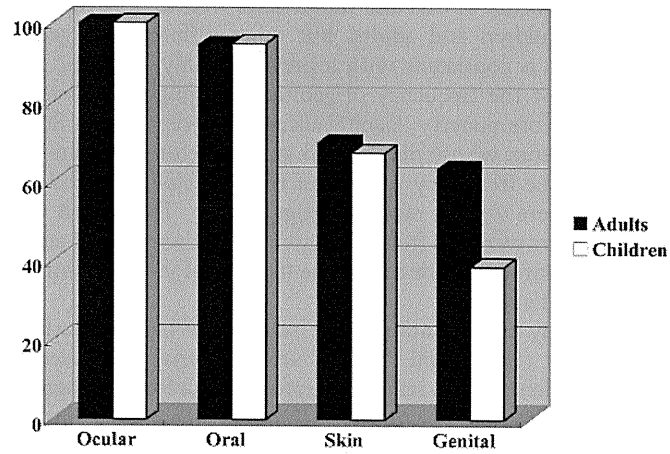

Figure 2 Frequency (%) of the major symptoms of Behçet's disease. The frequency of genital ulcers was significantly lower in children than in adults ($p<0.01$).

cases ($p<1\times10^{-8}$, χ^2 test). More than 90% (93.9%) of juvenile patients developed combined anterior and posterior segment intraocular inflammation (CAPSII)/panuveitis as adults. Most of the children suffered from bilateral recurrent CAPSII/panuveitis (table 1). The percentage of eyes achieving a final visual acuity of 0.1 (20/200) or better in the better eye was 92.6% of children (table 1) and 86.3% of adults. Thus, 7.4% of children but 13.7% of adult patients were legally blind. A good visual prognosis (≥20/200) was more frequent in children than in adult cases ($p<0.033$, χ^2 test).

The most frequently prescribed systemic therapy was corticosteroids (57 cases, 42.2%), followed by cyclophosphamide (27 cases, 20.0%), methotrexate (25 cases, 18.5%), colchicine (18 cases, 13.3%), azathioprine (12 cases, 8.9%), ciclosporin (11 cases, 8.1%) and interferon-α (two cases, 1.5%) (table 2). Three-quarters (73.6%) of patients received more than one drug.

DISCUSSION

In the present study, we successfully performed an international collaborative survey of the phenotypes of children with Behçet's disease and uveitis. Although several diagnostic criteria are used to make the diagnosis of Behçet's disease in adults,[20 21] there are no diagnostic criteria in children.[22] This means that the epidemiology of paediatric Behçet's disease is difficult to evaluate because there is no general agreement about either the age of onset or the age of full diagnosis. Previous studies have shown that the proportion of patients in whom the onset of symptoms occurred under the age of 16 years varies.[3 23 24] A study of juvenile-onset Behçet's disease from France reported that the mean age of disease onset was 7.5 years old, but the mean age at which patients met the criteria for Behçet's disease was 11.6 years old.[4] Since the mean age of disease diagnosis in our study was 11.7 years old (table 1), our present results from an international study confirm these previous findings.

We showed that the visual prognosis was better in juvenile-onset patients than in adult-onset subjects. More than 20% of eyes of Behçet's patients of all ages become legally blind as reported recently.[1 25] In the present study, 93% of the juvenile patients had final visual acuity of 0.1 (20/200) or better in their better eyes (table 1), and visual prognosis was significantly better among children than adults. However, this statistical result may not always mean that the ocular involvement in children is milder than adults. The visual prognosis of patients from East/South Asia was poor compared with other countries in all age groups, as we reported.[1] In addition, the prevalence in East/South Asia was significantly lower than that in the Middle East and European countries (fig 1). Thus, only a few East/South Asian children, a group with a poor visual prognosis, were included in the population of the present study. Furthermore, we did not have information on ocular co-morbidity (eg cataract, glaucoma and/or macular degeneration) as a confounding factor, which might be expected to be more common in adults. It should be noted that cyclophosphamide was one of the most commonly used immunosuppressive agents in this study. Although it is true that this old drug is cheap and familiar in many countries, it is not recommended for children because of its toxicity.

Table 1 Summary of juvenile-onset Behçet's disease in eye centres (n = 135)

Variable	Incidence (%)
Boys	65.5
HLA-B51	68.6
Age of disease onset	11.7*
Mean follow-up period	13.5*
CAPSII/panuveitis	93.9
Bilaterality	83.6
Recurrence of ocular inflammation	96.3
Poor visual prognosis (<20/200, better eyes)	7.4

CAPSII, combined anterior and posterior segment intraocular inflammation.
*Years.

Table 2 Initial systemic therapies for children

Treatment	%
Corticosteroids	42.2
Cyclophosphamide	20.0
Methotrexate	18.5
Colchicine	13.3
Azathioprine	8.9
Ciclosporin	8.1
Interferon-α	1.5

Three-quarters of patients received two or more drugs as their initial therapies.

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The prevalence of oral and skin lesions were almost the same between children and adults, but quite different for genital ulcers. This is consistent with a previous study in Israel.[6] It is possible that the frequency of genital ulcer might be lower in patients before puberty. Genital ulcers were reported in 30.9% of the patients whose disease had started before 10 years old. Although the frequency was lower than that of older children (42.7%), there was no statistical significance. The reason why genital ulcers were less frequently seen in children than in adults is still unclear. It may be one of the features of younger patients with Behçet's disease.

Our study may contain some sources of bias as outlined previously.[1] (1) We did not ask what criteria were used to include patients in the study. Accepted criteria for the diagnosis of Behçet's disease include those of the Japanese Committee,[21] the International Study Group[20] and O'Duffy.[26] The use of different standardised criteria may lead to misclassification when comparing the frequencies of systemic features. However, since most of the colleagues were members of International Committee of Behçet's Disease as well as uveitis specialists, the false-positive rate of diagnosis should be quite low. In addition, each had used the same criteria for inclusion of both juvenile and adult patients. (2) We also had only a limited response rate to the questionnaire from 25/132 eye centres. Therefore, the response may not have been representative of all countries and ethnic groups. (3) There may have been reporting bias as the population was taken from tertiary referral centres and the cases may have been more severe. (4) Access to uveitis clinic may be another source of bias. Healthcare systems are different in each country. There may be some regional problems relevant to the paediatric age group. However, in Japan, children can seek immediate medical attention, as can adults. This may partially explain the low prevalence of juvenile-onset Behçet's disease in East Asia. Japanese paediatricians previously monitoring Behçet's disease among children were able to collect the medical data of only 31 cases from 1290 hospitals throughout the country.[27] It may that the prevalence of the patients without ocular symptoms is also low in Japan.

Although there were some sources of bias that may distort the results, the authors consider that data from a sufficient number of patients with a relatively uncommon type of disease were analysed. The present results provide an indication that the clinical features of juvenile-onset Behçet's disease with ocular lesions do differ from adult-onset disease in some respects.

In conclusion, there were racial differences in the frequency of Behçet's disease in children. Only a few children suffered from Behçet's disease in contrast to the high prevalence in adults in East and South Asian. The clinical features of Behçet's disease with uveitis were different in adult-onset and juvenile-onset patients: the prevalence of genital ulcers was less in children than in adults.

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Association of *TLR4* polymorphisms with Behçet's disease in a Korean population

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Objectives. HLA-B51 is strongly associated with Behçet's disease (BD) in any ethnic background. We recently reported that another gene, Toll-like receptor-4 (*TLR4*) is also implicated in BD in a Japanese population. To confirm these results, we investigated polymorphisms in the *TLR4* gene in Korean patients with BD.

Methods. In this study, 119 patients with BD and 141 healthy controls were enrolled; every participant was a Korean. Nine single nucleotide polymorphisms previously detected in *TLR4* by direct sequencing were analysed for an association with BD.

Results. The most frequent haplotype, TAGCGGTAA, was significantly increased in *HLA-B*51*-positive BD patients (49.5%), compared with healthy control participants [32.3%; $P = 0.029$; odds ratio (OR) = 2.01; 95% CI 1.25–3.23]. This haplotype was also significantly increased in BD patients with arthritis (48.2%; $P = 0.003$; OR = 1.96; 95% CI 1.26–3.26). There were no significant differences in the allele and genotype frequencies of patients and controls for each single nucleotide polymorphism.

Conclusions. The haplotype of *TLR4* may increase the risk for developing BD and the complication of arthritis in the Korean population.

Key words: Behçet's disease, Toll-like receptor 4, Korea, Polymorphism.

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Introduction

Behçet's disease (BD) is a refractory, multisystemic inflammatory disorder characterized by oral aphthous ulcers, ocular lesions, skin lesions and genital ulcers [1]. This disease is occasionally associated with inflammation throughout the body including the vascular system and joints [2]. In patients with BD, serum TNF and IFN-γ are significantly elevated [3, 4]. The strong association of HLA-B51 with BD was first described in 1973 and has been confirmed in patients from many ethnic groups [5, 6]. Epidemiologically, BD is scattered throughout the world but a higher prevalence has been found among Asian populations along the Silk Route, stretching into the countries of the Mediterranean region [6]. BD appears to be influenced by many susceptible genes, including endogenous and exogenous elements, because most HLA-B51-positive individuals do not suffer from BD throughout their lives, and ~50% of the BD patients are negative for HLA-B51 [6, 7]. These observations suggest that there are other susceptible genes in addition to those that have been reported, which include *TNF-α*, *IFN-γ*, *IL-1α*, *-1β*, *-2*, *-8*, *-10* and *-12* and *CD28* [5, 8, 9].

The Toll-like receptor (*TLR*)4 gene on chromosome 9q32–33 spans ~13 kb and contains three exons encoding a protein consisting of 222-amino acid protein. The TLR proteins are a family of phylogenetically conserved receptors that recognize both self and non-self molecular patterns and play an important role in both the innate and adaptive immune systems [10–12]. Among TLR family members, TLR4 has been the most exhaustively investigated; it has been shown to be a principal receptor for lipopolysaccharide (LPS)-recognition. Single nucleotide polymorphisms (SNPs) in *TLR4* have been reported to be associated with endotoxin hyporesponsiveness and Gram-negative infections, and they affect the risk for various inflammatory diseases, such as atherosclerosis, Crohn's disease, ulcerative colitis, RA and prostate cancer [13–20].

Recently, we reported that *TLR4* is significantly associated with BD in Japanese patients [21]. To confirm the reproducibility of this result, and to extend the investigation into the Korean population, we explored the association of *TLR4* with BD in Korean patients using the same nine SNPs examined in the Japanese study. The information on linkage disequilibrium (LD) and haplotype structure defined by SNPs in a population is essential for the design of disease association studies. Genetic structure constructed by LD patterns and haplotype structure may be different among populations. Although differences in genetic structure between Korean and Japanese populations remain unclear, recent reports have presented strong genetic affinities [22, 23]. In the context of genetic similarity between Koreans and Japanese, we attempted to analyse disease susceptibility of the *TLR4* gene for BD in Korean patients and differences in the haplotype structure in those SNPs examined.

Materials and methods

Participants

We recruited 119 Korean patients with BD from Seoul National University Hospital, Seoul, Korea. All the patients fulfilled the diagnostic criteria of the International Study Group for BD (ISGBD) [24], and the definition of the clinical manifestations followed the criteria by ISGBD [24]. Arthritis was defined as any episode of joint pain not related to direct trauma that was confirmed by a physician as the presence of either swelling or tenderness or pain on motion of the involved joints. The healthy blood donors were randomly recruited as healthy controls. Informed consents were obtained from all of the individuals. This study was approved by the ethical committee of Seoul

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University Hospital. The procedures used conformed to the tenets of the Declaration of Helskinki.

Genotyping

DNA was prepared from peripheral blood specimens using the QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan). Nine previously described SNPs (rs10759930, rs1927914, rs1927911, rs12377632, rs2149356, rs11536889, rs1554973, rs7037117 and rs7045953) from the *TLR4* region were examined [21]. Each PCR product for the nine SNPs was amplified by standard PCR reactions (Table 1).

After purification using ExoSAP-IT (USB Corporation, Cleveland, OH, USA), the PCR products were sequenced with Big Dye terminator v3.1 (Applied Biosystems, Foster City, CA, USA) using either sense or anti-sense primers (Table 1). The BigDye XTerminator Purification Kit was used to purify the DNA from the sequencing reactions. The sequencing reactions were analysed using an ABI3130 sequencer (Applied Biosystems).

Statistical analysis

Differences in genotype frequency differences between case and control genotypes were assessed by using the χ^2-test and Fisher's exact test. The strength of LD between SNPs was quantified by the standardized disequilibrium (D′) [25]. The maximum likelihood estimates of haplotype frequencies were estimated by pairs of unphased genotypes using the expectation–maximization (EM) algorithms and *P*-values were corrected by using the 1000 permutations test in Haploview software [26]. All *P*-values were derived from a two-sided test, and those that were <0.05 were considered to be statistically significant.

TABLE 1. PCR primers, product sizes and sequence primers for *TLR4* polymorphisms

SNP	ID	Forward primer Reverse primer	Product size (bp)	Sequence primer
rs10759930	1	ATGGCACTATGTAGGCTGAT ACCCTCTTTTATCCTTGGAC	295	Forward
rs1927914	2	GCTTTTAGGACAGTGTCTGG CTTTGACATGGAGGTTGTTT	251	Reverse
rs1927911	3	GATGACACACAAATCCATGA GGGAAAGTAGGCTAGAAAGC	400	Forward
rs12377632	4	AATCGATACCATCACTCAGG GCCCTAATTCAGAATCTCCT	473	Forward
rs2149356	5	AAGCATGTAATAGCCTTGGA TGTTCACTTCACCTCCTTTT	419	Forward
rs11536889	6	CTCACTGCCAGGAGAACTAC GAAGAAGGGTTCCAATTTCT	341	Reverse
rs1554973	7	GGAGCTTCAAACAAAGGATA ACTCACCCATAAGGACAGTG	330	Reverse
rs7037117	8	TGCAAATGCAGTTTTGTTAC ATTCTTAAGCCAAGCATTTG	470	Forward
rs7045953	9	CCCCATTGAGTTATAGGTGA TATTGGAGGCTCTGTCTCAT	196	Forward

Results

The patient group included 61 males (51.3%) and 58 females (48.7%). Oral aphthous ulcers were observed in all patients. Genital ulcers, skin lesions, ocular lesions, deep vein thrombosis and arthritis were observed in 77.3, 80.7, 37.8, 16.8 and 47.9% of the patients, respectively. We found 39.5% of the patients to be *HLA-B*51* positive (Table 2).

The magnitude of LD between any two of the nine SNPs showed an extremely high value among all SNPs, with pair-wise LD valued at D′ > 0.68 in controls and > 0.74 in cases (Fig. 1). There was no significant difference between the allele or genotype frequencies of the cases and controls. In our clinically stratified analysis, we investigated the presence of some of the clinical features, such as oral aphthous ulcers, genital ulcers, skin lesions, ocular lesions, deep vein thrombosis and arthritis. None of these clinical findings was significantly associated with the nine SNPs (data not shown).

Table 3 shows haplotype frequencies of the patients with BD and healthy controls. Only possible haplotypes were estimated to have a frequency > 0.05 in both case and control groups using an EM algorithm. The patients with BD were divided into two groups, those with or those without *HLA-B*51* and arthritis. The healthy controls were also divided into with or without *HLA-B*51*.

Table 4 summarizes the analysis of the haplotypes, comparing BD patients with controls. The most frequent haplotype, TAGCGGTAA, was significantly increased in *HLA-B*51*-positive patients compared with healthy controls [odds ratio (OR) = 2.01; 95% CI 1.25, 3.23; $P = 0.029$]. This haplotype was also increased in patients with BD complicated by arthritis when compared with healthy controls (OR = 1.96; 95% CI 1.26, 3.05; $P = 0.0030$) or BD patients without arthritis (OR = 2.01; 95% CI 1.19, 3.40; $P = 0.037$) (Table 4).

Discussion

The TLR4 protein is expressed in a wide variety of human cells, and acts as a receptor in the activation of the innate/adaptive immune system in response to both endogenous and exogenous ligands. This transmembrane signalling receptor is the primary

TABLE 2. Clinical features of the Korean patients with BD

	Cases, *n* (%)
Sex	
Male	61
Female	58
Oral aphthous ulcers	119 (100)
Genital ulcers	92 (77.3)
Skin lesions	96 (80.7)
Ocular lesions	45 (37.8)
Deep vein thrombosis	20 (16.8)
Arthritis	57 (47.9)
*HLA-B*51*	47 (39.5)

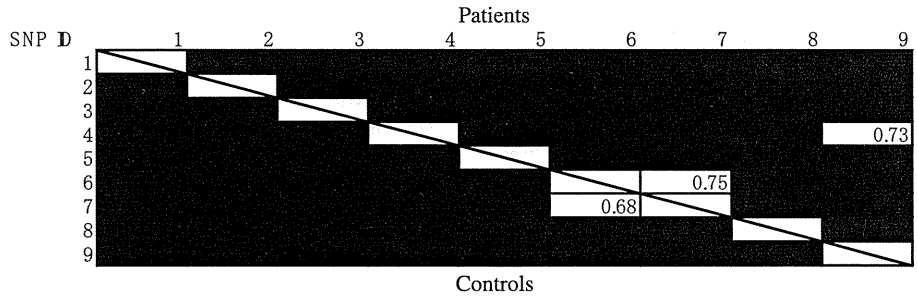

FIG. 1. D'score for the nine SNPs studied across the *TLR4* haplotype. Upper triangle, patient population; lower triangle, control population. A black cell means D′ > 0.8.

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TABLE 3. Haplotype frequencies for TLR4 in BD patients and healthy controls

Haplotype		BD (%; $n=119$)	BD *B*51(+)* (%; $n=47$)	BD *B*51(−)* (%; $n=72$)	BD Arthritis(+) (%; $n=57$)	BD Arthritis(−) (%; $n=62$)	Controls (%; $n=141$)	Controls *B*51(+)* (%; $n=28$)	Controls *B*51(−)* (%; $n=113$)
Hap 1	TAGCGGTAA	39.7	49.5	35.4	48.2	32.2	32.3	30.4	33.5
Hap 2	TAGCGCTAA	22.4	13.3	26.4	20.2	24.1	26.1	23.1	25.0
Hap 3	CGATTGTAA	11.7	5.8	13.2	9.6	13.7	13.8	12.5	15.0
Hap 4	CGATTGTGA	8.1	9.6	7.6	7.0	8.9	11.4	16.1	11.2
Hap 5	CGATTGCGG	8.0	9.5	6.9	7.9	8.0	7.8	8.9	7.2

*B*51: HLA-B51.*

TABLE 4. Analysis of the haplotypes in *TLR4*

	Hap 1 OR (95% CI); *P*-value	Hap 2 OR (95% CI); *P*-value	Hap 3 OR (95% CI); *P*-value	Hap 4 OR (95% CI); *P*-value	Hap 5 OR (95% CI); *P*-value
BD *vs* Control	1.35 (0.95, 1.94); 0.173	0.84 (0.56, 1.26); 1	0.75 (0.45, 1.25); 1	0.63 (0.35, 1.15); 0.763	1.13 (0.59, 2.18); 1
*B*51(+)* BD *vs* Control	**2.01 (1.25, 3.23); 0.029**	0.46 (0.24, 0.88); 0.17	0.60 (0.28, 1.29); 0.744	0.77 (0.36, 1.68); 0.845	0.81 (0.31, 2.16); 1
*B*51(−)* BD *vs* Control	1.09 (0.71, 1.67); 1	1.12 (0.71, 1.76); 1	0.86 (0.48, 1.55); 1	0.61 (0.30, 1.23); 0.817	0.98 (0.45, 2.16); 1
*B*51(+)* BD *vs B*51(−)* BD	1.84 (1.08, 3.13); 0.228	0.41 (0.20, 0.83); 0.202	0.70 (0.30, 1.61); 1	1.28 (0.51, 3.21); 0.76	0.83 (0.28, 2.43); 1
*B*51(+)* Control *vs B*51(−)* Control	0.87 (0.46, 1.64); 1	0.89 (0.45, 1.77); 1	1.04 (0.47, 2.30); 1	1.16 (0.47, 2.84); 0.76	1.39 (0.48, 4.01); 1
*B*51(+)* BD *vs B*51(+)* Control	2.25 (1.12, 4.52); 0.071	0.51 (0.21, 1.20); 0.887	0.55 (0.21, 1.49); 0.945	0.74 (0.26, 2.11); 0.869	0.63 (0.18, 2.22); 1
*B*51(−)* BD *vs B*51(−)* Control	1.06 (0.68, 1.65); 1	1.09 (0.68, 1.75); 1	0.82 (0.45, 1.50); 1	0.67 (0.32, 1.41); 1	1.06 (0.46, 2.43); 1
Arthritis(+) BD *vs* Control	**1.96 (1.26, 3.05); 0.003**	0.73 (0.43, 1.25); 0.843	0.61 (0.30, 1.23); 0.764	0.55 (0.25, 1.23); 0.787	1.13 (0.50, 2.57); 1
Arthritis(+) BD *vs* Arthritis(−) BD	**2.01 (1.19, 3.40); 0.037**	0.77 (0.41, 1.42); 1	0.67 (0.30, 1.51); 1	0.77 (0.30, 1.99); 1	0.98 (0.38, 2.52); 1

P-values were corrected by 1000 permutation test. Significant *P*-values are in bold.

recognition molecule for LPS and HSP, both of which are reported to trigger BD. HSPs, widely distributed in nature, are highly immunogenic molecules. Human HSPs are expressed on cell membranes in response to physiological stress and microbial challenge. Recent reports have indicated that HSPs of microorganisms were frequently found in the oral flora and serum of BD patients and they were considered to be implicated in the development of BD through cross-reactive immunopathological responses [27–30]. Notably, the expression of HSPs is up-regulated at lesions of BD and the serum level of HSPs is significantly higher in BD patients than in controls [31–33]. Furthermore, HSPs stimulated $\gamma\delta$T-cell responses from BD patients but not in controls *in vitro* [31, 34]. TLR4 reportedly recognizes and interacts with HSP and LPS, regarded as antigens in BD. Recent studies have reported *TLR4* polymorphisms and the risk of various diseases including inflammatory diseases [16, 18, 35]. It has been reported that two non-synonymous *TLR4* SNP sites, Asp299Gly (rs4986790) and Thr399Ile (rs4986791), are associated with an elevation of serum cytokines in the Caucasian population [18]. However, no other studies have detected these non-synonymous mutations in Asian populations, including Koreans [36]. Thus, we investigated nine intronic SNPs, the same SNPs described in our earlier work focused on a Japanese population [21].

In the present study, we examined *TLR4* because it was implicated in that previous study as a candidate gene in Japanese patients with BD [21]. It is widely accepted that *HLA-B*51* affects the development of BD, and the frequency of *HLA-B*51* is much higher in male patients, patients with ocular lesions and complete-type BD patients [6]. The association of the nine SNPs with BD could not be explained by any single SNP, but the data in this study show that the frequency of the TAGCGGTAA haplotype is significantly increased in *HLA-B*51*-positive BD. *TLR4* is located on the long arm of chromosome 9, whereas *HLA* is on the short arm of chromosome 6; thus, to elucidate the genetic influence of *HLA-B*51* on *TLR4*, we divided *HLA-B*51*-positive participants from controls. Among healthy controls, there were no significant differences between healthy participants with and without *HLA-B*51*. In addition, there were no significant differences between *HLA-B*51*-positive patients and controls. These results suggest that the most frequently occurring haplotype, TAGCGGTAA, may be influenced by *HLA-B*51* and dependently associated with *HLA-B*51-positive* BD patients.

The *TLR4* gene has been well investigated in the context of RA [18, 35, 37], and arthritis classified as one of the minor symptoms of BD. In fact, 40% of the BD patients have the complication of arthritis, and it is the most common minor symptom [38]. In the present study, we successfully examined 57 BD patients (47.9%) with arthritis. When we examined the haplotype frequencies for BD patients with and without arthritis, the most frequently occurring haplotype, TAGCGGTAA, which is associated with a higher risk of BD, was significantly increased in patients with arthritis. Although the pathogenesis of BD and RA is still unknown, the arthritis in RA is chronic inflammation accompanied by constitutional symptoms such as fever, fatigue, malaise, myalgia, decreased appetite and weight loss. In most cases, RA is a chronic progressive disease that can cause extensive joint damage and deformity [39]. On the other hand, the arthritis in BD is usually self-limiting and non-erosive [40]. Nevertheless, these two diseases may share some genetic or immunological mechanisms.

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