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*J Immunol* 2014; 193:2512-2518; Prepublished online 25 July 2014;
doi: 10.4049/jimmunol.1400588
http://www.jimmunol.org/content/193/5/2512

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/07/25/jimmunol.1400588.DCSupplemental

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Acute and Chronic Effects of IL-22 on Acetaminophen-Induced Liver Injury

Dechun Feng,* Yan Wang,* Hua Wang,* Honglei Weng,† Xiaoni Kong,* Brittany V. Martin-Murphy,‡ Yongmei Li,* Ogyi Park,* Steven Dooley,† Cynthia Ju,‡ and Bin Gao*

Acetaminophen (APAP)-induced liver injury (AILI) accounts for half of the acute liver failure cases in the United States. A better understanding of the underlying mechanisms of AILI is necessary for the development of novel antidotes. We found that pretreatment with IL-22 protected mice from APAP-mediated hepatotoxicity. The protection was dependent on STAT3, as IL-22 failed to reduce APAP hepatotoxicity in liver-specific STAT3 knockout mice. In contrast to the acute exposure to IL-22, the endogenous chronic overexpression of IL-22 in IL-22 transgenic (TG) mice or IL-22 adenovirus treatment for 6 wk resulted in a markedly increased susceptibility to AILI. Furthermore, the hepatic expression levels of cytochrome 2E1 (Cyp2E1) and Cyp1A2 were much higher in IL-22TG mice. Ablation of Cyp2E1 but not hepatic STAT3 abolished AILI and protein-adduct formation in IL-22TG mice. Finally, hepatic expression of HNF-1α, a transcriptional factor that is known to control Cyp2E1 expression, was elevated in IL-22TG mice compared with wild-type mice. Upregulation of hepatic Cyp2E1 was only observed in mice with constitutive overexpression of IL-22 but not with short-term treatment with one dose of IL-22 or multiple doses of IL-22 for 2 wk. In conclusion, short-term acute IL-22 exposure protects mice against AILI through STAT3 activation; however, chronic constitutive overexpression of IL-22 exacerbates AILI by increasing Cyp2E1 and toxic reactive APAP metabolite production. These findings may not only enhance our understanding of the effects of chronic inflammation on AILI in patients with liver disease, but are also helpful to identify novel therapeutic targets for the treatment of AILI.

Received for publication March 7, 2014. Accepted for publication June 27, 2014.

Acetaminophen (APAP) is one of the most widely used drugs to relieve mild to moderate pain and reduce fever (1). APAP is generally safe at therapeutic doses; however, an overdose of APAP may lead to severe liver damage (2, 3). In the past four decades, the mechanism of APAP hepatotoxicity has been extensively studied in a murine model. APAP is bioactivated to a toxic reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), by cytochrome 2E1 (Cyp2E1) and, to a much lesser extent, Cyp1A2 in the liver. NAPQI depletes glutathione (reduced glutathione [GSH]) and subsequently binds to liver proteins, leading to oxidative stress, mitochondrial dysfunction, and necrotic cell death (3–7).

Cyp2E1 is critically involved in the bioactivation of APAP to form NAPQI and, thus, APAP hepatotoxicity (8). The expression and activity of Cyp2E1 are regulated by many factors. For example, alcohol and isoniazid can upregulate Cyp2E1 and enhance APAP-induced liver injury (AILI) (9–12). In contrast, Cyp2E1 expression and APAP hepatotoxicity are usually reduced by the inflammatory mediators induced by LPS (13–16) or polysinosinic-polycytidylic acid (17–19). For example, IL-6, TNF-α, and IFNs have been shown to suppress Cyp2E1 (13, 20–22). These studies clearly demonstrate an interaction between immune cell functions and drug metabolism. However, the majority of the studies have focused on the short-term or acute effects of inflammatory cytokines on Cyp2E1 expression. It is not known how the long-term elevation of cytokines in chronic inflammatory conditions, which occurs in chronic liver disease, influences Cyp2E1 expression and Cyp2E1-mediated drug metabolism.

IL-22 is a member of the IL-10 family of cytokines. IL-22 is mainly produced by Th17 cells, NK, and NKT cells and plays critical roles in controlling bacterial infection and tissue repair (23–25). The hepatoprotective effects of IL-22 have been well documented in various mouse models (26–31). Clinical trials evaluating the therapeutic potential of rIL-22 on alcoholic hepatitis and acute liver failure are under consideration. Our previous studies revealed that the hepatic expression of IL-22 is elevated in patients with a chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection (32). To better understand the effects of chronic IL-22 elevation in the pathogenesis of liver diseases, we have generated liver-specific IL-22–transgenic (IL-22TG) mice, in which both hepatic and serum IL-22 levels are elevated (33). This model of IL-22TG mice serves as an excellent tool to further elucidate the impact of the chronic elevation of IL-22 on liver diseases as well as the liver’s response to drug therapies.

In the current study, we evaluated: 1) whether a single dose of IL-22 exerts a hepatoprotective function in AILI; and 2) how the long-term elevation of IL-22 affects AILI. Our data demonstrate that the injection of a single dose of IL-22 has a prophylactic protective effect against AILI, which is in agreement with a previous report.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Ad-GFP, GFP adenovirus; Ad–IL-22, IL-22 adenovirus; AILI, acetaminophen-induced liver injury; ALT, alanine aminotransferase; APAP, acetaminophen; Cyp2E1, cytochrome 2E1; GSH, reduced glutathione; HBV, hepatitis B virus; HCV, hepatitis C virus; IL-22TG, IL-22–transgenic; KO, knockout; NAPQI, N-acetyl-p-benzoquinone imine; NIH, National Institutes of Health; STAT3KO, liver-specific STAT3 knockout; WT, wild-type.
The protective effect of acute IL-22 treatment on AILI is mediated through STAT3 activation because the disruption of hepatic STAT3 abolished the protective function of acute IL-22 treatment. To our surprise, the chronic elevation of IL-22 markedly exacerbates AILI. Further investigation suggested that the detrimental effect of chronic IL-22 exposure on AILI is mediated by upregulating the hepatic expression of Cyp2E1 and to a lesser extent Cyp1A2. Upregulation of hepatic Cyp2E1 was not observed in mice with short-term treatment with one dose of IL-22 or multiple doses of IL-22 for 2 wk.

Materials and Methods

Materials

Recombinant mouse IL-22 protein was provided by Dr. Xiaqiang Yan (Generon Corporation).

Animal experiments

Male C57BL/6N mice were purchased from the National Cancer Institute (Frederick, MD). The Cyp2E1 knockout (KO) mice were provided by Dr. Frank Gonzalez (National Cancer Institute, National Institutes of Health [NIH]) and backcrossed to a C57BL6/N background for at least eight generations in our facility. Two strains of IL-22 TG mice (designated as IL-22TG8 and IL-22TG6) were generated as previously described (32). The IL-22TG8 mice with Cyp2E1 KO double-mutant mice were generated through several steps of crossing the IL-22TG8 hepat-specific STAT3 KO (STAT3flox/flox) mice with Alb CreSTAT3flox/flox mice. The liver-specific STAT3 KO mice (AbCreSTAT3flox/flox) mice were described previously (35). IL-22TG8 mice with Cyp2E1 KO double-mutant mice were generated by dissolving the compound in warmed PBS. All of the mice were housed in a pathogen-free environment. The IL-22 adenovirus (Ad–IL-22) and a GFP adenovirus (Ad-GFP) were kindly provided by Drs. M. Zhang and J. Kolls (Louisiana State University, Piscataway, NJ) or enhanced fluorescence and analyzed with the Typhoon analyzer (GE Healthcare). The Abs against JNK, p-JNK, Bcl-2, and Bcl-XL were purchased from Cell Signaling Technology. The Abs against Cyp2E1 and Cyp1A2 were purchased from Millipore and Sigma-Aldrich, respectively. The Ab against HNF-1α was purchased from BD Biosciences. The Ab for the APAP adducts was kindly provided by Dr. Lance R. Pohl from the National Heart, Lung, and Blood Institute, NIH.

Statistical analysis

The data are expressed as the means ± SD. To compare values obtained from three or more groups, a one-factor ANOVA was used, followed by Tukey post hoc test. To compare values obtained from two groups, the Student t test was performed. Statistical significance was set at the p < 0.05 level.

Results

Short-term treatment with a single dose of IL-22 protects mice from AILI

The protective role of IL-22 in various models of acute liver injury including AILI has been well documented (26–31, 34); in this study, we also confirmed that treatment with a single dose of IL-22 prevents AILI. As shown in Fig. 1A, IL-22 pretreatment significantly blocked the elevation of alanine aminotransferase (ALT) induced by the injection of APAP. Similar to the ALT results, H&E staining of the liver and TUNEL staining revealed a significant improvement regarding the development of necrotic areas in the liver tissue (Fig. 1B, 1C). However, treatment of the mice with IL-22 1.5 h post–APAP injection failed to alleviate the liver damage (data not shown). To test whether the protective effect of IL-22 on AILI was due to the alteration of APAP metabolism, we analyzed APAP adducts in the liver by using Western blotting. As illustrated in Fig. 1D, treatment with a single dose of IL-22 did not affect APAP metabolism in the liver.

It has been reported that IL-22 rapidly activates STAT3 in the liver, thereby protecting against Con A–induced liver injury (27). To investigate the involvement of STAT3 in the IL-22–induced protection against AILI, we measured pSTAT3 levels in the liver.
between APAP-treated STAT3\textsuperscript{LKO} and WT mice. The injection of a single dose of IL-22 prevented the APAP-induced elevation of serum ALT, liver necrosis, and apoptosis in WT mice but failed to attenuate AILI in the STAT3\textsuperscript{LKO} mice. These data suggest that the protective effect of IL-22 is mediated through the activation of STAT3.

**Chronic overexpression of IL-22 exacerbates AILI**

To further study the impact of IL-22 on AILI, we generated IL-22 TG mice to mimic the long-term high-level IL-22 expression that is found in chronic viral hepatitis patients (32). Two different TG strains, IL-22\textsuperscript{TG6} (serum IL-22 levels reach \(\sim 600\) pg/ml) and IL-22\textsuperscript{TG8} (serum IL-22 levels reach \(\sim 6000\) pg/ml), with different serum levels of IL-22 were used. To our surprise, when mice were treated with 300 mg/kg of APAP, all of the IL-22\textsuperscript{TG8} mice (n = 6) died within 12 h, whereas all of the WT (n = 6) and IL-22\textsuperscript{TG6} (n = 5) mice survived until 24 h after the APAP injection. When the dose of APAP was reduced to 160 mg/kg, the WT mice developed only mild liver injury; however, the IL-22\textsuperscript{TG6} and IL-22\textsuperscript{TG8} mice exhibited much more severe tissue damage, as evidenced by a marked elevation of ALT levels and increased cell death (Fig. 3A–C). Notably, the extent of the AILI correlated with the level of IL-22 in the serum because the liver injury was much more severe in the IL-22\textsuperscript{TG8} mice than in the IL-22\textsuperscript{TG6} mice. JNK activation is reported to play a crucial role in the hepatotoxicity induced by APAP-associated oxidative stress (38, 39). In agreement with the elevation of ALT activity, hepatic JNK phosphorylation was strongly induced in the IL-22\textsuperscript{TG6} and IL-22\textsuperscript{TG8} mice, but not in WT mice after administration of a low dose of APAP (Fig. 3D).

In addition to using IL-22 TG mice, we achieved long-term high levels of IL-22 expression by treating WT mice with Ad–IL-22 once every 2 wk for a total of 6 wk. Control mice were treated with Ad-GFP. Two weeks after the last adenovirus injection, all of the mice were fasted overnight and treated with 160 mg/kg APAP. Similar to the IL-22 TG mice, the Ad–IL-22–treated mice also developed much more severe liver damage compared with the Ad-GFP–treated control mice (Fig. 3E, 3F). Taken together, these data suggest that, in contrast to the protective effect of a single dose of IL-22, chronically high levels of IL-22 actually exacerbate AILI.
Effects of chronic overexpression of IL-22 on APAP bioactivation and protein adduct formation

Because the degree of AILI is closely associated with the amount of NAPQI, we compared the levels of NAPQI–protein adducts in WT and IL-22TG mice. The data reveal a marked increase of protein adduct formation in both the IL-22TG6 and the IL-22TG8 strains of mice (Fig. 4A). The data suggest that the increased susceptibility to AILI in the IL-22TG mice is due to a greater amount of NAPQI generation. The major pathway for NAPQI detoxification is conjugation with GSH. Therefore, we measured liver GSH levels in the WT and IL-22TG mice before and after APAP treatment (160 mg/kg). Prior to the APAP treatment, the GSH levels were similar between the WT and IL-22TG mice. After the APAP challenge, the WT mice exhibited a faster recovery of GSH levels than the IL-22TG mice (Fig. 4B), reflecting a smaller burden of toxic NAPQI in the WT mice than in the IL-22TG mice.

The data indicate that the increased NAPQI generation in the IL-22 TG mice was not due to impaired detoxification in these mice. Hence, we measured the hepatic expression levels of Cyp2E1 and Cyp1A2, two major enzymes that mediate the bioactivation of APAP to NAPQI. As illustrated in Fig. 5A and 5B, the levels of Cyp2E1 and Cyp1A2 mRNA and protein were significantly higher in the IL-22TG8 and IL-22TG mice compared with those in the WT mice. Immunohistochemical staining further confirmed that the areas of hepatic Cyp2E1 and Cyp1A2 protein staining were larger in the IL-22TG6 and IL-22TG8 mice than in the WT mice (Fig. 5C, 5D).

We previously demonstrated that serum IL-22 levels in 2-wk-old mice were ~30% of the peak level observed in mice at ≥4 wk of age (32). As illustrated in Supplemental Fig. 1, the hepatic expression of the Cyp2E1 and Cyp1A2 proteins was comparable in 2-wk-old WT and IL-22TG mice. These data suggest that the upregulation of hepatic Cyp2E1 and Cyp1A2 by IL-22 requires long-term exposure (a 2-wk exposure is inadequate).

The deletion of Cyp2E1 abrogates the increased susceptibility to APAP hepatotoxicity in IL-22 TG mice

To further confirm that the upregulation of Cyp2E1 by the chronic expression of IL-22 causes increased AILI in the IL-22TG mice, we generated IL-22TG Cyp2E1KO double mutant mice via several steps of crossing the IL-22TG mice with Cyp2E1KO mice. Compared with the IL-22TG mice, the double-mutant mice had similar levels of IL-22 in the serum, but the AILI was almost completely abolished (Fig. 6A–D). Moreover, similar to the Cyp2E1KO mice, no APAP adducts were detected in the double mutant mice (Fig. 6E). Of note, although Cyp1A2 is not deleted in the IL-22TG Cyp2E1KO mice, an APAP dose of 160 mg/kg did not cause liver injury. This result suggests that Cyp1A2 plays a minor role in relatively low-dose AILI, which is consistent with a previous report (40).

HNF-1α, but not STAT3, is involved in the upregulation of Cyp2E1 by the chronic overexpression of IL-22

Because STAT3 is the main downstream signaling molecule for IL-22, we first examined whether STAT3 mediates the IL-22–induced upregulation of Cyp2E1 expression in the liver. We generated IL-22TGSTAT3LKO double-mutant mice (IL-22TG AlbCreSTAT3flox/flox) via several steps of crossing IL-22TG STAT3flox/flox mice with AlbCre STAT3flox/flox mice. The level of Cyp2E1 expression and the susceptibility to AILI were compared between the IL-22TG and IL-22TG STAT3LKO mice. The data revealed similar levels of hepatic Cyp2E1 expression (Fig. 7A) and serum ALT activity (Fig. 7B), as well as similar changes in liver histopathology (Fig. 7C) and TUNEL staining (Fig. 7D) between the two strains of mice. Necrotic areas in the liver (as observed after APAP administration) are usually susceptible to nonspecific staining. To rule out this possibility, we performed negative control staining (without terminal transferase) for liver slides with necrotic areas. As illustrated in Fig. 7E, no nonspecific staining was observed in negative control staining slides, which indicated the TUNEL staining was specific. These findings indicate that the effect of IL-22 on Cyp2E1 upregulation is independent of STAT3.
Several transcription factors, such as STAT6, NF-κB, and HNF-1α, have been reported to upregulate Cyp2E1 expression (41–44). We did not observe any differences in the hepatic protein levels of p-STAT6 or NF-κB p65 between WT mice and the IL-22 TG6 or IL-22 TG8 mice (Fig. 8A). In contrast, the expression of the well-documented liver-specific transcription factor HNF-1α was markedly increased in the livers of the IL-22 TG6 and IL-22 TG8 mice compared with the WT mice (Fig. 8A). Moreover, we tested the DNA binding activity of HNF-1α to the Cyp2E1 promoter via a chromatin immunoprecipitation assay. Consistent with the Western blot results, the binding of HNF-1α to the Cyp2E1 promoter was significantly increased in the IL-22 TG6 and IL-22 TG8 mice in a dose-dependent manner (binding was higher in the IL-22 TG8 than in the IL-22 TG6 mice) (Fig. 8B). These results suggest that HNF-1α may be a downstream factor that mediates the regulation of Cyp2E1 by IL-22. We then investigated whether a single dose of IL-22 also regulates HNF-1α expression. Liver protein was extracted from mice that received IL-22 for 24 and 48 h or PBS as a control. No differences were found in HNF-1α levels among these groups. Moreover, Cyp2E1 levels remained unchanged (Supplemental Fig. 2). In addition, the hepatic expression of the HNF-1α protein was comparable in 2-wk-old WT and IL-22 TG mice (supporting Fig. 1) and was not altered after injection of one dose of IL-22 or 2-wk injection of multiple doses of IL-22 (supporting Fig. 2 and supporting Fig. 3). Collectively, these findings indicate that the upregulation of HNF-1α by IL-22 requires long-term exposure.

Discussion
In the current study, we found that pretreatment with a single dose of IL-22, through the activation of STAT3, protected against AILI. However, chronic constitutive exposure to IL-22, through the upregulation of Cyp2E1, exacerbated AILI.

The hepatoprotective effects of STAT3 activation have been well documented (27, 45–48). STAT3 activation in the liver has also been observed after APAP challenge (49, 50), and STAT3 is believed to stimulate hepatocyte compensatory proliferation, thereby protecting against APAP hepatotoxicity (49). In addition to

**FIGURE 6.** Increased Cyp2E1 contributes to the increased susceptibility of IL-22 TG mice to AILI. (A) Serum levels of IL-22 were measured in the IL-22 TG mice and the IL-22 TG Cyp2E1 KO double-mutant mice. (B–E) Overnight-fasted WT, IL-22 TG, IL-22 TG Cyp2E1 KO, and Cyp2E1 KO mice were injected with 160 mg/kg APAP. Sera and livers were obtained 6 and 24 h post–APAP injection. (B) Serum ALT levels were measured. (C) H&E staining (original magnification ×40). (D) TUNEL staining (original magnification ×100). (E) Western blot analyses of APAP adducts in the liver protein 6 h post–APAP injection. ***p < 0.001.

**FIGURE 7.** The increased expression of Cyp2E1 is not dependent on STAT3 activation. (A) Immunohistochemical analysis of hepatic Cyp2E1 protein from overnight-fasted IL-22 TG and IL-22 TG STAT3 KO mice (original magnification ×40). (B–D) Overnight-fasted IL-22 TG, STAT3 KO and IL-22 TG STAT3 KO mice received a 160-mg/kg APAP injection. Serum ALT levels were measured 6 h post–APAP injection (B). Representative H&E (original magnification ×40) (C) and TUNEL staining (original magnification ×100) (D) are shown. Negative control staining for TUNEL assay is shown in (E) (original magnification ×100). NS, p > 0.05.
STAT3, several other signaling pathways, which are known to regulate cell survival, are also activated by IL-22. These include STAT1, STAT5, ERK1/2, and AKT (51). During the preparation of our manuscript, Scheiermann et al. (34) reported that the pretreatment of mice with IL-22 prevented AILI; however, the underlying mechanisms were not elucidated. In the current study, we demonstrated that the protective effect of IL-22 is abrogated in liver-specific STAT3 KO mice. This suggests that STAT3, not other signaling pathways, play an important role in mediating the hepatoprotection by IL-22 (Figs. 1, 2). Notably, the treatment of mice with IL-22 1.5 h post–APAP injection had no protective effect against AILI (data not shown). Our previous study revealed that an IL-22 injection rapidly induced STAT3 activation in vivo with a peak time 1 h after IL-22 administration (27). Nevertheless, the induction of STAT3 downstream survival genes, such as Bcl2 and Bcl-xL, is likely delayed. In contrast, the onset of AILI in mice is very rapid because massive APAP adducts form as early as 2 h after APAP injection (52). This may explain why IL-22 pretreatment is protective, whereas treatment at 1.5 h post–APAP challenge fails to protect against AILI.

In contrast to the hepatoprotective effect of a single dose of IL-22, the chronic expression of high levels of IL-22, which is observed in viral hepatitis patients, significantly exacerbates AILI in mice with IL-22 (Fig. 8). This suggests that STAT3, not other signaling pathways, play an important role in mediating the hepatotoxicity induced by IL-22. In the current study, we used two lines of IL-22TG mice, including IL-22TG mice with 6000 ng/ml and IL-22TG mice with 600 ng/ml. Both lines of TG mice had increased sensitivity to APAP-induced liver injury. The ~600 ng/ml serum IL-22 levels in IL-22TG mice were similar to those from HBV patients as reported by Zhang et al. (57). Therefore, we believe that the data from IL-22TG TG6 mice are clinically relevant. It will be interesting to investigate whether hepatic expression of Cyp2E1 in HCV patients is elevated and whether such elevation correlates with IL-22 levels and the increased susceptibility of these patients to hepatotoxicity induced by APAP overdose. However, the situation in patients may be more complex because both viral factors and immune responses may regulate the expression of CYPs in HCV patients. In contrast to IL-22, many cytokines involved in liver inflammation have been shown to be negative regulators of Cyp2E1 expression (13, 20–22). Thus, hepatic CYP levels in HCV patients are likely upregulated by IL-22 but downregulated by many other proinflammatory cytokines. Individual CYP levels are a result of the net effect of the two opposing factors and may vary significantly. When these patients are treated with drugs that are metabolized by CYPs, variations in pharmacodynamics and pharmacokinetics as well as drug toxicity will likely occur.

Because of the beneficial effects of IL-22 on liver damage, rIL-22 is currently being developed to treat inflammatory liver disease and promote liver regeneration after injury. Although our current study revealed that chronic overexpression of IL-22 via transgenic expression or injection of Ad–IL-22 upregulates Cyp2E1 and Cyp1A2 expression levels, short-term treatment with one dose of IL-22 or multiple doses of IL-22 for 2 wk did not alter Cyp2E1 (supporting Fig. 2 and supporting Fig. 3). Therefore, it is plausible to speculate that treatment of patients with multiple doses of IL-22 unlikely affects hepatic Cyp2E1 expression.

Acknowledgments
We thank Drs. M. Zhang and J. Kolls (Louisiana State University) for providing the Ad-GFP and Ad–IL-22, and Dr. Lance R. Pohl (National Heart, Lung, and Blood Institute, NIH) for providing the APAP adduct Ab.

Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Fig. S1**

**Fig. S1: Comparable levels of hepatic cyp2E1 and cyp1A2 expression in young WT and IL-22 TG mice.** Livers were obtained from 4-week old overnight-fasted WT, and IL-22TG^8^ mice. (A) Immunohistochemical analysis of liver Cyp2E1 and Cyp1A2. (B) Western blot analysis of hepatic Cyp2E1, Cyp1A2, and HNF1α proteins.
Fig. S2: A single dose IL-22 treatment does not alter cyp2E1 expression. WT mice received a single dose of IL-22 (1mg/kg). Livers were obtained 24 or 48 hours after IL-22 treatment. (A) Western blot analysis of hepatic Cyp2E1 and HNF1α proteins. (B) Immunohistochemical analysis of liver Cyp2E1.
Fig S3: Administration of IL-22 with multiple doses of IL-22 for two weeks does not alter cyp2E1 expression. WT mice were received multiple dose of IL-22 (1mg/kg, twice a week) for 2 two weeks. Livers were obtained 24 hours after last IL-22 treatment. (A) Western blot analysis of hepatic Cyp2E1 and HNF1α proteins. (B) Immunohistochemical analysis of liver Cyp2E1.