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Endogenous Interleukin-4 Regulates Glutathione Synthesis Following Acetaminophen-Induced Liver Injury in Mice

Pauline M. Ryan, Mohammed Bourdi, Midhun C. Korrapati, William R. Proctor, Ronald A. Vasquez, Steven B. Yee, Timothy D. Quinn, Mala Chakraborty, and Lance R. Pohl*

Molecular and Cellular Toxicology Section, Laboratory of Molecular Immunology, Immunology Center, National Heart, Lung and Blood Institute, National Institutes of Health, 9000 Rockville Pike, Building 10, Room 8N110, Bethesda, Maryland 20892.

Abstract

In a recent study, we reported that interleukin (IL)-4 had a protective role against acetaminophen (APAP)-induced liver injury (AILI), although the mechanism of protection was unclear. Here, we carried out more detailed investigations and have shown that one way IL-4 may control the severity of AILI is by regulating glutathione (GSH) synthesis. In the present studies, the protective role of IL-4 in AILI was established definitively by showing that C57BL/6J mice made deficient in IL-4 genetically ($IL-4^{-/-}$) or by depletion with an antibody, were more susceptible to AILI than mice not depleted of IL-4. The increased susceptibility of $IL-4^{-/-}$ mice was not due to elevated levels of hepatic APAP-protein adducts, but was associated with a prolonged reduction in hepatic GSH that was attributed to decreased gene expression of γ -glutamylcysteine ligase (γ -GCL). Moreover, administration of recombinant IL-4 to $IL-4^{-/-}$ mice post-acetaminophen treatment diminished the severity of liver injury and increased γ -GCL and GSH levels. We also report that the prolonged reduction of GSH in APAP-treated $IL-4^{-/-}$ mice appeared to contribute towards increased liver injury by causing a sustained activation of c-Jun-N-terminal kinase (JNK), since levels of phosphorylated JNK remained significantly higher in the $IL-4^{-/-}$ mice up to 24 hours after APAP treatment.

Conclusion—Overall these results show for the first time that IL-4 has a role in regulating the synthesis of GSH in the liver under conditions of cellular stress. This mechanism appears to be responsible at least in part for the protective role of IL-4 against AILI in mice and may have a similar role not only in AILI in humans, but also in pathologies of the liver caused by other drugs and etiologies.

Keywords

Interleukin-4; GSH; AP-1; Nrf-2; drug-induced liver injury; risk factors

Introduction

Drug-induced liver injury (DILI) is a serious and often life-threatening adverse effect of drug-treatment that presents a major hindrance to new drug development (1). Efforts to identify hepatotoxic drugs early in development and patients who are susceptible to DILI have been impeded in a large part by the lack of appropriate animal models and insufficient understanding of the mechanisms and nature of risk factors contributing to DILI (2). Nevertheless, fundamental mechanisms and potential susceptibility factors that may

*To whom correspondence should be addressed: Lance R. Pohl, Pharm. D., Ph.D., Molecular and Cellular Toxicology Section, Laboratory of Molecular Immunology, Immunology Center, National Heart Lung and Blood Institute, National Institutes of Health, 10 Center Drive, Bldg 10, Room 8N110, Bethesda, MD 20892, USA. Telephone: 301-451-1097, Fax: 301-480-4852, pohl@nih.gov.

contribute to the idiosyncratic nature of liver injury caused by most drugs have been largely uncovered in murine studies of acetaminophen-induced liver injury (AILI), the leading cause of acute-liver failure in the United States (3). For example, it is known that AILI is initiated by cytochrome (CYP) P450-mediated bioactivation of acetaminophen (APAP) to form the reactive and toxic metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) (4). This metabolite depletes hepatic glutathione (GSH) and covalently modifies liver proteins and their activities (5) causing oxidative stress, mitochondrial dysfunction and ultimately, centrilobular necrosis (6). These findings have led other investigators to consider the possibility that underlying genetic and acquired mitochondrial abnormalities may be important susceptibility factors contributing to the idiosyncratic nature of DILI in many cases (7).

Other studies of AILI in mice suggest that additional susceptibility factors may have a role in promoting the incidence and severity of DILI. These include, but are not limited to pro-inflammatory cytokines and chemokines (8), cells of the innate immune system (9, 10) and other prototoxicant effectors (11, 12). In contrast, other factors reduce susceptibility to AILI, including glycoproteins (13), antioxidants (14, 15) and hepatoprotective cytokines and chemokines (8). It appears that the balance between the activities of prototoxicant and protective factors, which may be determined genetically and environmentally, play a role in determining the severity of AILI in mice and possibly of DILI in humans (7).

In a related matter, preliminary findings within our laboratory have suggested that interleukin (IL)-4 also has a protective role against AILI (16). We reported at a single time point that mice genetically deficient in IL-4 ($IL-4^{-/-}$) were more susceptible than wild type (WT) mice to AILI. Moreover, mice genetically deficient in IL-10 and IL-4 ($IL-10/4^{-/-}$) were highly susceptible to AILI even at low doses of APAP and had a prolonged depletion of hepatic GSH, although the mechanism of IL-4 protection was unclear (16). In the present study, we show for the first time that IL-4 has a role in regulating the hepatic synthesis of GSH under conditions of cellular stress, and provide evidence for this pathway having a protective role against AILI in mice.

Experimental Procedures

Materials

All reagents were of analytical or molecular grade and were supplied by Sigma-Aldrich, St Louis, MO, unless otherwise indicated.

Animals and Treatment

Eight to nine week old male (20–25g) WT and $IL-4^{-/-}$ mice on a C57BL/6J background from Jackson Laboratories (stock# 000664 and 002253, respectively, Bar Harbour, ME) were used in these studies. Animals were acclimatized for 1 week at the National Institutes of Health (NIH) animal facilities. Experiments were conducted with the approval of the National Heart Lung and Blood Institute Animal Use and Care Committee, and all animals received humane care according to the criteria outlined in the National Academy of Sciences 'Guide for the Care and Use of Laboratory Animals' and published by the NIH (NIH publication 86-23 revised 1985). Prior to each study, animals were fasted overnight (16 h) to deplete hepatic GSH levels (17). The following morning, food was restored after a single intraperitoneal injection of warm saline vehicle or APAP at 200 or 300 mg/kg prepared in warm saline. In some studies, WT mice were injected intraperitoneally with 5 mg of anti-IL-4 neutralizing monoclonal antibody (11B11, National Cancer Institute, Frederick, MD) or normal rat immunoglobulin G (IgG) 24 h prior to APAP treatment. In other studies, 100 pg of recombinant mouse IL-4 (rIL-4) (BD Biosciences, San Jose, CA) was administered

intravenously through the lateral tail vein of IL-4^{-/-} mice 3 h following APAP treatment. Recombinant IL-4 was dissolved in sterile PBS, pH 7.4, containing 1% bovine serum albumin Cohn Fraction V for protein stabilization.

Sera and Tissue Collection

Blood samples were obtained by retro-orbital sinus bleed or from the inferior vena cava followed by sera collection using microcontainer serum separator tubes (Becton Dickinson and Co., Franklin Lakes, NJ). Sera was flash frozen and stored at -80 °C until further analysis. Sections of the left and right lateral liver lobes were fixed in 10% buffered formalin (Fischer Scientific, Fair Lawn, NJ) and remaining tissue was flash frozen in liquid nitrogen and stored at -80 °C for further study. Fixed tissue was embedded in paraffin, mounted onto glass slides and stained with hematoxylin and eosin (H&E) (American Histolabs, Gaithersburg, MD).

Assessment of Hepatotoxicity

Liver injury was evaluated by measuring serum alanine aminotransferase (ALT) activity using a microtiter plate adaptation of a commercially available kit (Teco Diagnostics, Anaheim, CA) and by histopathological examination of H&E stained liver sections and scoring of lesion size as described (9).

Cytokine Serum Levels

IL-4 serum levels were measured using an enzyme-linked immunoabsorbent assay (BD OptEIA™, BD Biosciences, San Jose, CA), while IL-13 serum levels were measured commercially using SearchLight Proteome Arrays (Pierce Biotechnology, Woburn, MA).

Isolation of Liver Homogenates and Nuclear Protein Extraction

Liver homogenates were prepared as described previously (9) except that the homogenization buffer included 1 mM sodium orthovanadate (Calbiochem, San Diego, CA) to prevent dephosphorylation of liver proteins (18). Nuclear extracts were isolated from whole liver using a nuclear extraction kit (Panomics, Fremont, CA) with minor protocol modifications. Briefly, 0.5 g of tissue per mouse was homogenized in 1.5 ml of ice-cold hypotonic buffer with 15–20 strokes in a dounce homogenizer on ice. Disrupted cells were allowed to swell on ice for 15 minutes and then centrifuged at 850 × g for 10 minutes at 4°C. The supernatant was discarded and 1.5 ml of ice-cold hypotonic buffer was added to the pellet and homogenized for 15–20 strokes in a dounce homogenizer on ice. Following 15 minutes incubation on ice, the homogenate was centrifuged at 14,000 × g for 3 minutes at 4 °C and the supernatant discarded. To isolate the nuclear protein extract, 400 µl of ice-cold high-salt buffer was added to each pellet and vortexed vigorously for 10 seconds to detach the pellet from the wall of the microcentrifuge tube. The pellet was then incubated on ice on a rocking platform at 150 rpm for 2 h. Following centrifugation at 14,000 × g for 5 minutes at 4 °C, the supernatant containing the nuclear protein extract was concentrated using a speed vacuum concentrator (Savant, Instruments Inc., Farmingdale, NY), flash frozen in liquid nitrogen, and stored at -80 °C until further analysis.

Determination of Protein Concentrations and Immunoblotting

Protein concentrations were determined by the Bradford method using a Coomassie Plus Protein Assay Reagent kit (Pierce, Rockford, IL). Immunoblotting was performed as described previously (9). APAP-protein adducts were detected by probing membranes with rabbit anti-APAP antibody, which was prepared as described previously (19), anti-β-actin (Millipore Corporation, Temecula, CA), anti-phosphorylated-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology, Danvers, MA) or anti-γ-GCL (detects the catalytic subunit of γ-

GCL, Thermo Scientific, Charlottesville, VA), followed by incubation with the appropriate peroxidase-labeled secondary antibody. Bound antibody was detected by chemiluminescence (Millipore Corporation, Billerica, MA). Images were captured using a Kodak image station 2000RT imager and software (Eastman Kodak, Rochester, NY). Image intensities were normalized to those of β -actin to give a value that was multiplied by a factor of 10 for plotting purposes.

CYP2E1 Activity in Liver Microsomes

The activity of CYP2E1 in liver microsomes from fasted untreated WT and IL4^{-/-} mice was assessed by the conversion of the prototypical CYP2E1 substrate, 4-nitrophenol, to 4-nitrocatechol. Briefly, microsomes were diluted in 50 mM Tris-Cl (pH 7.4) to a final reaction concentration of 1mg/ml and pre-incubated with 4-nitrophenol (0.1 mM or 1.0 mM) for 5 min at 37°C. The metabolic reactions (0.5 mL total volume) were initiated by addition of nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system (BD Gentest, Sparks, MD, USA). Reactions were stopped at 10min (within the linear range of conversion with respect to time) with 100 μ L of 20% w/v trichloroacetic acid, vortexed, and centrifuged at 1,000 g for 5min. A portion of the supernatant (500 μ L) was treated with 600 μ L of 2N NaOH and 4-nitrocatechol was detected by UV absorption ($\lambda=520\text{nm}$) on a 96-well plate reader (Molecular Devices, Sunnyvale, CA).

Determination of Reduced and Oxidized GSH

Reduced GSH was measured in liver homogenates from individual mice using a DTNB assay based on Tietze's method (20) described previously (12). A commercial assay kit (Cayman Chemicals, Ann Arbor, MI) was used to measure oxidized GSH (GSSG) in liver homogenates prepared from individual mice.

γ -GCL and CYP2E1 Messenger RNA Expression

RNA isolation and complementary DNA synthesis for each mouse was performed as previously described (10). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using commercially available probes and primers for γ -GCL, and β -actin and a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). The levels of gene expression were calculated relative to the housekeeping gene, β -actin.

Transcription Factor Nuclear DNA Binding Activity

Nuclear factor erythroid-2 related factor-2 (Nrf-2) and activator protein-1 (AP-1) nuclear DNA binding activities were determined in liver nuclear extracts using the TransAM™ Nrf-2 or TransAM™ AP-1/ phospho-Jun enzyme-linked immunosorbent assay kits, respectively (Active Motif, Carlsbad, CA), according to the manufacturer's instructions. The detection antibody provided in this assay is anti-phospho-c-Jun which detects c-Jun in the AP-1 complex when bound to its nuclear DNA binding site (21).

Statistical Analysis

Data for single comparisons were analyzed by Student's *t*-test. Multiple comparisons of homogenous data were analyzed by one-way or two-way analysis of variance (ANOVA) as appropriate and group means were compared using the Student-Newmann-Keuls post-hoc test. Data expressed as percentages were subject to arcsine square root transformations prior to analyses by ANOVA. Results are expressed as mean \pm SEM and were considered significant at $p<0.05$.

Results

Deletion of IL-4 Increased Susceptibility of Mice to AILI

In a preliminary study, IL-4^{-/-} mice were found to be more susceptible than WT mice to AILI 8 h after APAP treatment at 300 mg/kg. When IL-4^{-/-} and WT mice were administered APAP at 300 mg/kg in the present study, 62% of the IL-4^{-/-} mice died, while only 17% of WT mice died within 24 h of drug treatment (Figure 1A). Although no animals died in either group at the lower dose of 200 mg/kg of APAP (Figure 1A), IL-4^{-/-} mice were more susceptible than WT mice to AILI, as determined by increased serum ALT activity (Figure 1B) and by the extent of hepatic coagulative necrosis (Figure 2 and Table 1). To further demonstrate the hepatoprotective role of endogenous IL-4 in AILI, WT mice were pretreated with an IL-4 neutralizing antibody or isotype control antibody prior to APAP (300 mg/kg) treatment. Pretreated WT mice exhibited lower IL-4 serum levels (Figure 1C) and were significantly more susceptible to AILI as assessed biochemically (Figure 1D) and histopathologically (data not shown) relative to WT mice pretreated with the isotype control antibody 24 h post-APAP treatment. Together, these findings firmly establish a protective role for IL-4 against AILI in mice.

IL-4^{-/-} Mice Had Reduced Hepatic APAP-Protein Adducts, GSH and γ -GCL Protein Following APAP Treatment

Since 62% of the IL-4^{-/-} mice succumbed to massive liver injury and death with APAP administered at 300 mg/kg, subsequent mechanistic studies were conducted using APAP at the lower toxic dose of 200 mg/kg. To determine if increased susceptibility of the IL-4^{-/-} mice to AILI was due to enhanced metabolic activation of APAP to its toxic metabolite, NAPQI, hepatic levels of APAP-protein adducts and GSH were compared between APAP-treated WT and IL-4^{-/-} mice. Considerably lower levels of APAP-protein adducts were found in liver homogenates of IL-4^{-/-} mice relative to WT mice 2 h following APAP treatment (Figure 3A). Similar differences were seen at 1 h post-APAP (Figure 3A), when AILI would be minimal (Figure 1B), suggesting that reduced levels of adducts in livers from APAP treated-IL-4^{-/-} mice were not due to their enhanced release during hepatic parenchymal cell injury.

As found in other studies of AILI in mice (22), hepatic GSH was depleted in WT mice at 2 h, but returned to normal levels by 8 h post-APAP treatment (Figure 3B). In contrast, GSH in livers from APAP-treated IL-4^{-/-} mice was depleted lower than WT mice at 2 h, partially recovered at 8 h, but remained lower than WT mice at 24 h (Figure 3B). This prolonged depletion of GSH in the APAP-treated IL-4^{-/-} mice did not appear to result from enhanced formation of oxidized GSH, as hepatic GSSG was not significantly different between APAP-treated WT and IL-4^{-/-} mice at 8 h after drug administration (data not shown). Moreover, there was no increase in GSSG levels throughout the 24 hour time period in either the IL-4^{-/-} or WT mice. This finding is in contrast to other studies of AILI, which have reported increased GSSG levels in WT mice after APAP-treatment. This discrepancy to earlier published reports may be explained by a variety of factors including, the relatively low dose of APAP we used (200 mg/kg) compared to 300 mg/kg and higher doses employed by other investigators (23, 24), the strain of mice used (23, 24), and the length of time starving the mice (24). On the other hand, the increases in total GSSG observed by other researchers were relatively small and the only major relative increase has been observed in the mitochondrial fraction of liver cells not in the whole cell (25).

We next determined whether depressed levels of hepatic GSH and enhanced susceptibility to AILI in the IL-4^{-/-} mice is a result of a deficiency in γ -GCL, which is the rate limiting enzyme in GSH synthesis (26). Immunoblotting of liver homogenates from WT mice

showed a time-dependent increase in expression of γ -GCL relative to untreated mice at 2, 8 and 24 h following APAP treatment (Figure 4A and B). APAP treatment also increased levels of γ -GCL protein in the IL-4^{-/-} mice at 2 h, similar to that found in WT mice, which then significantly dropped relative to both untreated IL-4^{-/-} mice and APAP-treated WT mice at 8 h and remained reduced up to 24 h (Figure 4A and B).

To exclude the possibility that depressed levels of hepatic γ -GCL found in the IL-4^{-/-} mice were due to enhanced loss of this enzyme from more injured hepatocytes, WT and IL-4^{-/-} mice were administered APAP at equally hepatotoxic doses of 300 mg/kg to WT mice and 200 mg/kg to IL-4^{-/-} mice (Figure 5A). Immunoblotting of liver homogenates showed that IL-4^{-/-} mice still had considerably lower γ -GCL levels compared to those detected in WT mice at 8 h following APAP treatment (Figure 5B). Moreover, prolonged depletion of GSH in the APAP-treated IL-4^{-/-} mice did not appear to be simply due to these mice having significantly more liver damage than WT mice, since we reported earlier that hepatic levels of GSH in both IL-13^{-/-} mice and WT mice were identical following APAP treatment albeit the IL-13^{-/-} mice were considerably more susceptible to AILI (9). These findings, however, did not eliminate the possibility that the relatively low protein levels of γ -GCL found in livers of APAP-treated IL-4^{-/-} mice were due in part to enhanced protein degradation.

IL-4^{-/-} Mice Had Reduced Hepatic γ -GCL mRNA Expression and Reduced Nrf-2 and AP-1 Nuclear DNA Binding Activities Following APAP Treatment

To examine if a deficiency in IL-4 lead to a reduced rate in the protein synthesis of γ -GCL, mRNA levels of γ -GCL were compared between WT and IL-4^{-/-} mice prior to and at various times after APAP treatment. Although hepatic mRNA levels of γ -GCL in both WT and IL-4^{-/-} mice increased time-dependently at 2, 4 and 8 h after APAP treatment, expression in IL-4^{-/-} mice was significantly lower at each time point, before returning to near control levels after 24 h (Figure 6A). This result suggested that the prolonged depletion of hepatic GSH in IL-4^{-/-} mice following APAP treatment (Figure 3B) might be due to inhibition of γ -GCL transcription. This hypothesis was supported by determining that the DNA binding activities of transcription factors, Nrf-2 and AP-1/phospho-c-Jun, established activators of hepatic γ -GCL transcription (27–29), were significantly lower in hepatic nuclear extracts of IL-4^{-/-} mice compared to WT mice as early as 1 h after APAP treatment (Figure 6B and C). However, at 2 h, Nrf-2 DNA binding did not differ between WT and IL-4^{-/-} mice (Figure 6B), while DNA binding activity for AP-1/phospho-c-Jun remained significantly depressed at this time point in the IL4^{-/-} mice (Figure 6C).

rIL-4 Reduced Susceptibility of IL-4^{-/-} Mice to AILI and Restored Hepatic Levels of GSH, γ -GCL mRNA and γ -GCL Protein

IL-4^{-/-} mice were treated intravenously with rIL-4 or vehicle 3 h after APAP treatment to provide further confirmation for the protective role of IL-4 in acetaminophen-induced liver injury. Indeed, at 8 h after APAP treatment, rIL-4 reduced the extent of AILI (Figure 7), while also enhancing GSH (Figure 8A) and γ -GCL mRNA and protein to levels that were even higher than those of control mice (Figure 8B and C), as did rIL-4 when it was administered alone to naïve IL-4^{-/-} mice (Figure 8B and C).

IL-4^{-/-} Mice Had Higher Levels of Prolonged JNK Activation Following APAP Treatment

As it is known that prolonged JNK activation is mediated by oxidative stress and plays a pathogenic role in AILI (30, 31), we determined whether the prolonged depleted levels of GSH seen in APAP-treated IL-4^{-/-} mice correlated with higher levels of phosphorylated JNK, the activated form of JNK, compared to WT mice following a hepatotoxic dose of APAP. We investigated this effect by examining the time course of phosphorylated-JNK1 and -JNK2 levels between WT and IL-4^{-/-} mice following APAP treatment. Relative to

their respective untreated mice, protein levels of phosphorylated-JNK2 and -JNK1 were significantly increased in both WT and IL-4^{-/-} mice at 2, 8 and 24 h after treatment with APAP (Figure 9A, B and C). Although maximum levels of JNK phosphorylation were reached early at 2 h in the WT mice and plateaued, levels of phosphorylated-JNK2 and JNK1 in the IL-4^{-/-} mice continued to rise and reached maximum levels at 8 h and remained at these high levels up to 24 h after APAP treatment. Moreover, activation of JNks in the IL-4^{-/-} mice was significantly higher than in WT mice at 8 and 24 h following treatment with APAP (Figure 9A, B and C), indicating that the prolonged and severely depleted levels of GSH in the APAP treated IL-4^{-/-} mice likely lead to increased JNK activation and increased liver injury (Figure 1B and Figure 2).

Discussion

Several studies indicate IL-4 can have a pathological or a protective role in a variety of liver diseases. IL-4 acts as a major proinflammatory cytokine in concanavalin A-induced hepatitis (18), causes lethal hepatitis by a mechanism involving apoptosis of hepatocytes (32), contributes to hapten-induced hepatitis (33) and is associated with increased susceptibility of patients to diclofenac-induced liver injury (34). In contrast, IL-4 is protective in liver injury caused by Schistosomiasis through regulating formation of highly reactive oxygen and nitrogen intermediates (35) and in hepatitis induced by ischemia reperfusion injury as a result of suppressing inflammation caused by tumour necrosis factor (TNF)- α secretion and neutrophil accumulation (36). Previous studies of AILI in mice lacking both IL-4 and IL-10, suggest IL-4 may also protect mice from inflammation associated with AILI (16). In the current study, we have made the unique discovery indicating IL-4 can additionally protect mice from AILI by preventing prolonged loss of hepatic GSH.

Several criteria were used to establish that IL-4 protected mice from AILI. First, IL-4 neutralizing antibody (Figure 1C) exacerbated AILI in WT mice (Figure 1D). We believe the diminished AILI at 24 hours, but not at 8 hours following APAP treatment at 300 mg/kg, is due to the neutralizing antibodies having to compete with the binding of IL-4 to its receptors. As IL-4 is highly potent due to its high affinity receptors (37), it is possible that IL-4 is tightly bound to its cell surface receptors at 8 hours and earlier. As the majority of IL-4 receptors become occupied and internalized (38), anti-IL-4 can then neutralize increasing levels of serum IL-4 and increase susceptibility of WT mice to AILI. The neutralization study was not done at 200 mg/kg of APAP because at this dose, serum IL-4 levels were below the detection limits of our ELISA, making it impossible to confirm that the neutralizing antibody depleted serum IL-4 levels. Second, IL-4^{-/-} mice were more susceptible than WT mice to AILI (Figures 1B and 2 and Table 1). Third, the high susceptibility of IL-4 mice to AILI was significantly reduced when mice were injected with rIL-4 at 3 hours after APAP treatment (Figure 7). This dose of rIL-4 approximated the serum concentrations of IL-4 found in WT mice treated with APAP (Figure 1C) and those found in a previous study of AILI (39), based upon 20 g and 25 g mice having average total blood volumes of 1.6 ml and 2.0 ml, respectively (The Jackson Laboratory, <http://jaxmice.jax.org/faq/withdrawingbloodamounts.html>).

From a mechanistic perspective, increased susceptibility of the IL-4^{-/-} mice to AILI was not due to enhanced bioactivation of APAP to its reactive NAPQI metabolite, as total APAP-protein adducts, a measure of NAPQI levels (19) were significantly lower in livers from APAP-treated IL-4^{-/-} mice compared to those detected in APAP-treated WT mice (Figure 3A). This was not unexpected given that IL-4 is known to induce mRNA and protein levels of CYP2E1, a major CYP isoform responsible for the bioactivation of APAP to NAPQI (4), in human primary hepatocytes (40) and liver cell lines (41). However, CYP2E1 activity in liver microsomes of WT and IL-4^{-/-} mice did not differ, nor were mRNA levels of CYP2E1

lower in IL-4^{-/-} compared to WT mice (data not shown), suggesting that factors other than CYP2E1 accounted for the relatively low levels of APAP-protein adducts found in livers of IL-4^{-/-} mice.

An unexpected finding of our study was the extensive and prolonged depletion of hepatic GSH in IL-4^{-/-} mice relative to WT mice following a hepatotoxic dose of APAP (Figure 3B), despite APAP-protein adduct formation being significantly lower in the IL-4^{-/-} mice (Figure 3A). The basis for this discrepancy and kinetics of GSH recovery in livers of IL-4^{-/-} mice is yet unclear, but is likely due to many factors, which in some cases, may be regulated by IL-4. We speculate that glutathione transferase (GST)-*pi* in livers of IL-4^{-/-} mice may catalyze the conjugation of GSH with NAPQI more rapidly than in livers of WT mice (42). GSH may also form more conjugates with other electrophiles in livers of IL-4^{-/-} mice including those with 4-hydroxy-2-nonenal (43), 15-deoxy-^{12,14}-PGJ2 (44), and perhaps oxidized proteins forming S-glutathionylated protein adducts (45).

Our findings indicate that the prolonged depletion and slow recovery of hepatic GSH in IL-4^{-/-} mice after APAP treatment is also due at least in part to a dysfunction in γ -GCL and GSH homeostasis in the liver as a result of an IL-4 deficiency. This is illustrated by the relatively low levels of γ -GCL protein (Figure 4) and GSH (Figure 3B) in livers of APAP-treated IL-4^{-/-} mice as compared to WT mice and the findings that administration of rIL-4 to IL-4^{-/-} mice reversed these trends at 8 hours after APAP treatment (Figure 8A and C). Although rIL-4 dosing of both APAP-treated and naïve IL-4^{-/-} mice increased the levels of hepatic γ -GCL protein above those of control IL-4^{-/-} mice (Figure 8C), similar elevations in GSH were not reached (Figure 8A). One possible explanation for these findings is that the catalytic activity of γ -GCL was slowed down as a result of non-allosteric feedback inhibition by rising levels of GSH (46).

Other studies we carried out indicated that IL-4 regulated γ -GCL protein at the transcriptional level. For example, hepatic mRNA levels of γ -GCL were appreciably lower in IL-4^{-/-} mice compared to WT mice after APAP treatment (Figure 6A), while rIL-4 treatment reversed this trend (Figure 8B). Furthermore, the transcription factors Nrf-2 and AP-1, shown by other researchers to up-regulate the promoter activity of the γ -GCL gene (27–29) and increase GSH synthesis (29), had lower nuclear DNA binding activities in IL-4^{-/-} mice compared to WT mice as early as 1 hour following APAP administration (Figure 6). Although the mechanisms responsible for the reduced DNA binding activities of these transcription factors in the IL-4^{-/-} mice remain to be determined, they may be related to defects in their nuclear translocation or retention (47). Additionally, these effects may be related to a deficiency of phospho-c-Jun in the AP-1 complex potentially caused by low levels of the kinase that phosphorylates c-Jun in the nucleus and activates AP-1. JNK-c-Jun activation is commonly known to be involved in AP-1 mediated gene regulation (48). However, the finding of increased and sustained JNK activation in our studies indicates upstream activation of c-Jun by a protein kinase other than JNKs. Currently, several kinases have been found that can phosphorylate c-Jun, including protein kinase C (PKC), ERKs, C-terminal Src kinase, CKII, DNA-PK, p34cdc2 and cAb1 (49, 50). Among these kinases, PKC would be a potential candidate of interest to investigate since phosphorylation by PKC is required for IL-4 signaling in other cell systems such as monocytes (51).

While we did not observe increased GSSG levels in the IL-4^{-/-} mice following APAP treatment, this did not necessarily indicate the absence of an oxidative stress response occurring in the livers of these mice (52), as the prolonged depletion of hepatic GSH in the APAP-treated IL-4^{-/-} mice coincided with considerably higher levels of JNK activation (Figure 9) that were sustained for much longer time periods than those reported in earlier studies (1–6 h) of AILI (30, 31). Upstream events such as increased production of reactive

oxygen species (ROS) generated by GSH-depleted mitochondria (52), consequently altering the redox status within cells (52), possibly contributed to enhanced JNK activation during AILI (30, 31) in the IL-4^{-/-} mice (Figure 9) by targeting upstream kinases, such as ASK1 (53) and possibly GSK-3 β (54). These events in turn allow JNK translocation to the mitochondria to promote the mitochondrial permeability transition pore opening with collapse of the membrane potential and hepatocyte death (7). Hence, perhaps the protective role of IL-4 in regulating GSH synthesis during AILI may also involve suppressing JNK signaling, since inhibition of JNK has been shown to protect in one other hepatotoxicity model, ischemia reperfusion injury (55) a model in which IL-4 also plays a protective role (33).

Although we have shown that IL-4 can regulate GSH synthesis during AILI, the signaling pathways involved in this process remain to be determined. Two types of heterodimeric IL-4 receptors have been found on the surface of cells. The type I receptor consists of an IL-4R α and a common γ -chain and is expressed primarily on hematopoietic cells (37), while the type II receptor exists as a IL-4R α and IL-13R α 1 dimer and is found on non-hematopoietic cells (56). IL-4 activates both type I and type II receptors, while IL-13 activates the type II receptor, and as a consequence, IL-4 and IL-13 can have overlapping activities. Since it is has been reported that the type II receptor can be expressed on the surface of hepatocytes (56), it is possible that this receptor mediates IL-4 induction of γ -GCL protein synthesis in hepatocytes. If this were the case, then IL-13 might also be expected to modulate GSH homeostasis in hepatocytes. However, this is unlikely in the case of AILI, because although IL-13 serum levels in the IL-4^{-/-} mice were significantly higher compared to WT mice at 8 hours after APAP treatment (578 ± 116 pg/ml versus 66 ± 140 pg/ml, corresponding to mean \pm SEM of 5 mice per group, $P < 0.05$), IL-13 still could not compensate for the absence of IL-4 in these animals. Nevertheless, it is possible that much higher serum levels of IL-13 are required before it can activate the type II IL-4 receptors on hepatocytes, as it has been reported that IL-4 can have a much higher affinity for this receptor than IL-13 (37). Conversely, the non-redundant nature of the activity of IL-4 in GSH synthesis may be due to it signaling through type I IL-4 receptors expressed on the surface of hepatocytes. Although this needs to be determined, expressions of both type-I and -II IL-4 receptors have been detected on the surface of non-hematopoietic cells (56).

In conclusion, we have shown that IL-4 has a previously unrecognized role in regulating the synthesis of GSH under conditions of cellular stress and as such, may have a non-redundant role in lowering susceptibility of mice to AILI by maintaining antioxidant defenses within the liver. These findings may have important implications in human medicine. Polymorphisms in the promoter region of the IL-4 gene that are associated with low IL-4 expression (57), might predispose a subpopulation of patients to AILI or to liver disease caused by other drugs. A similar effect may be achieved by polymorphisms in genes encoding the IL-4 receptors (57) and by pharmacological inhibition of IL-4 due to anti-IL-4 immunotherapy in asthma (58) and other diseases (59) as well as by drug treatments (60). On a broader scope, a deficiency in IL-4 signaling might also be relevant to the pathology of other diseases of the liver (61) and other organ systems (61) involving cellular stress and disruptions in GSH homeostasis, including those involving cells of the immune system that require GSH for normal functioning (62).

Abbreviations

IL	interleukin
APAP	acetaminophen

AILI	acetaminophen-induced liver injury
GSH	glutathione
IL-4^{-/-}	mice genetically deficient in IL-4
γ-GCL	γ-glutamylcysteine ligase
DILI	drug-induced liver injury
CYP	cytochrome P-450
NAPQI	<i>N</i> -acetyl- <i>p</i> -benzoquinone imine
WT	wild type
IgG	immunoglobulin G
rIL-4	recombinant IL-4
IL-10/4^{-/-}	mice genetically deficient in IL-10 and IL-4
H&E	hematoxylin and eosin
ALT	alanine aminotransferase
JNK	c-Jun <i>N</i> -terminal kinase
GSSG	oxidized GSH
qRT-PCR	quantitative real-time polymerase chain reaction
Nrf-2	nuclear factor erythroid 2-related factor
AP-1	activator protein-1
CV	central vein
P-p-46-JNK1	phosphorylated JNK1
P-p-54-JNK2	phosphorylated JNK2
TNF-α	tumour necrosis factor

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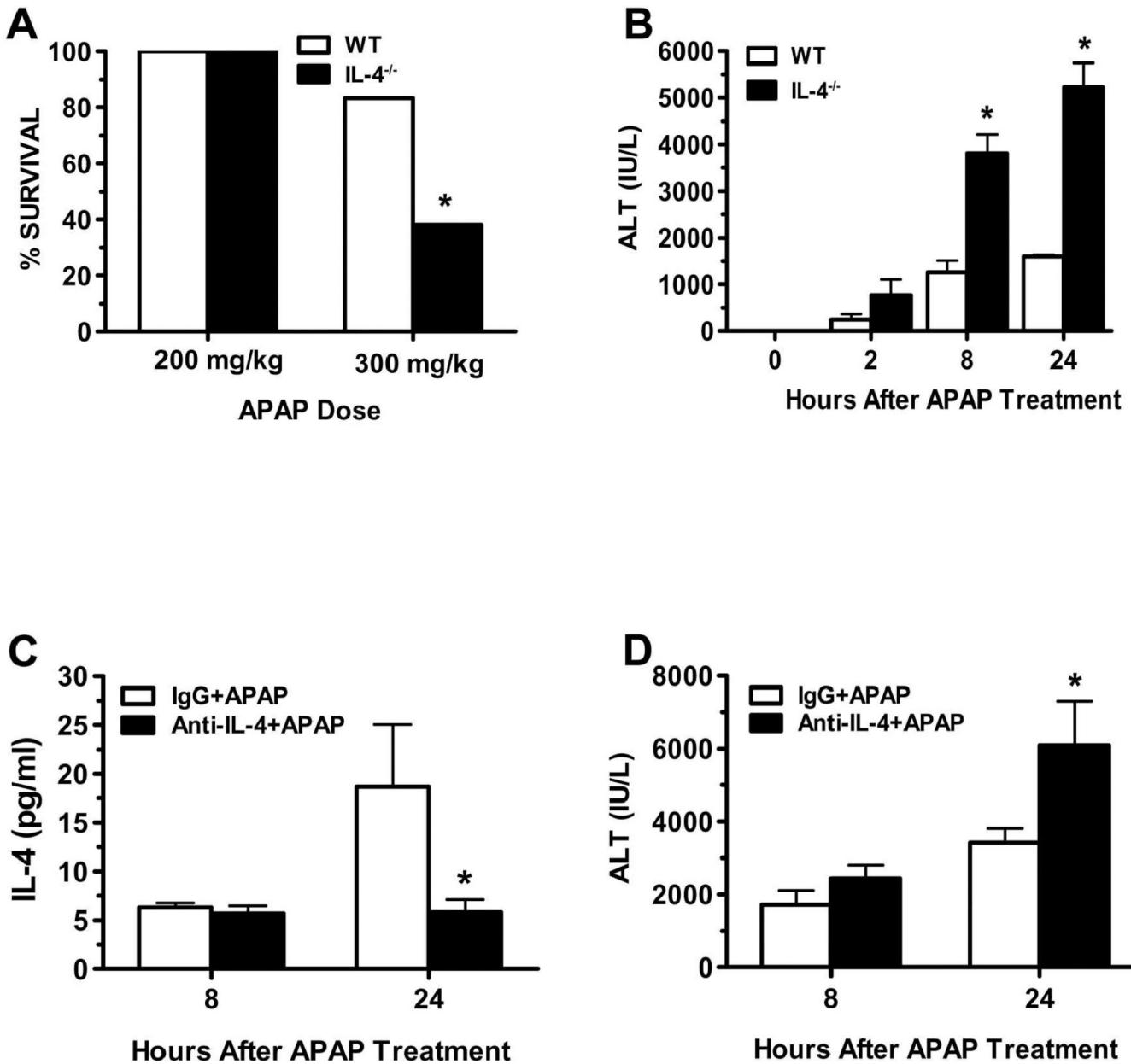
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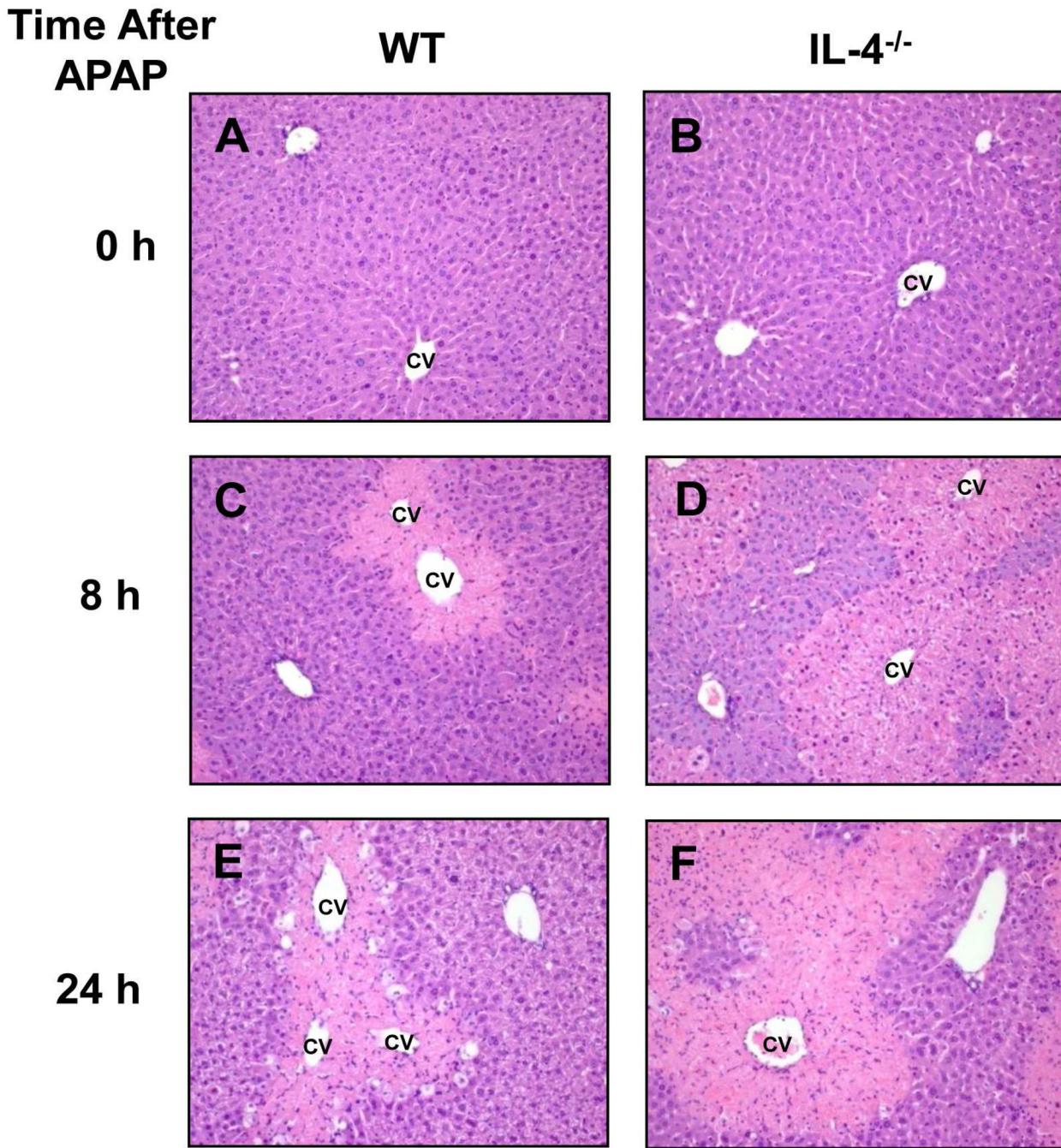
**Figure 1.**

Interleukin-4 deficient mice were more susceptible than wild type mice to acetaminophen-induced death and liver injury. (A) Percent survival rates for WT and IL-4^{-/-} mice 24 h after APAP (200 mg/kg or 300 mg/kg) treatment. Data represent cumulative percent deaths following APAP treatment from two independent experiments (10 to 12 mice per group).

*P<0.05 versus APAP-treated WT mice at the same dose. (B) Serum ALT activities in WT and IL-4^{-/-} mice before and after APAP (200 mg/kg) treatment. Results represent the mean ± SEM of 10 to 12 mice per group from two combined studies. *P<0.05 versus APAP-

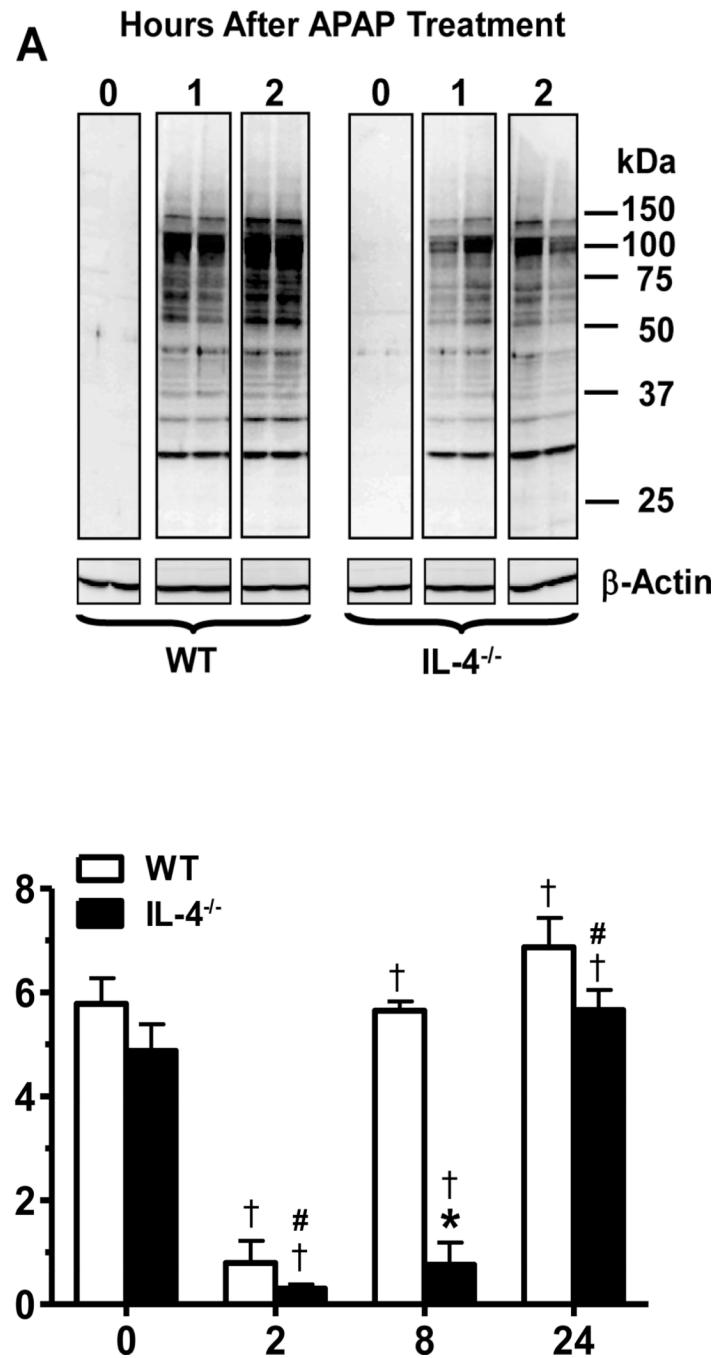
treated WT mice at the same time point. (C and D) WT mice were pre-treated with anti-IL-4 neutralizing antibody (anti-IL-4) or control antibody (IgG) 24 h prior to APAP (300 mg/kg) treatment. (C) serum IL-4 levels and (D) serum ALT activities were measured in WT mice treated with anti-IL-4+APAP or IgG+APAP. Results represent the mean ± SEM of 10 to 12

mice per group from two combined studies. * $P<0.05$ versus IgG+APAP-treated WT mice at the same time point.

**Figure 2.**

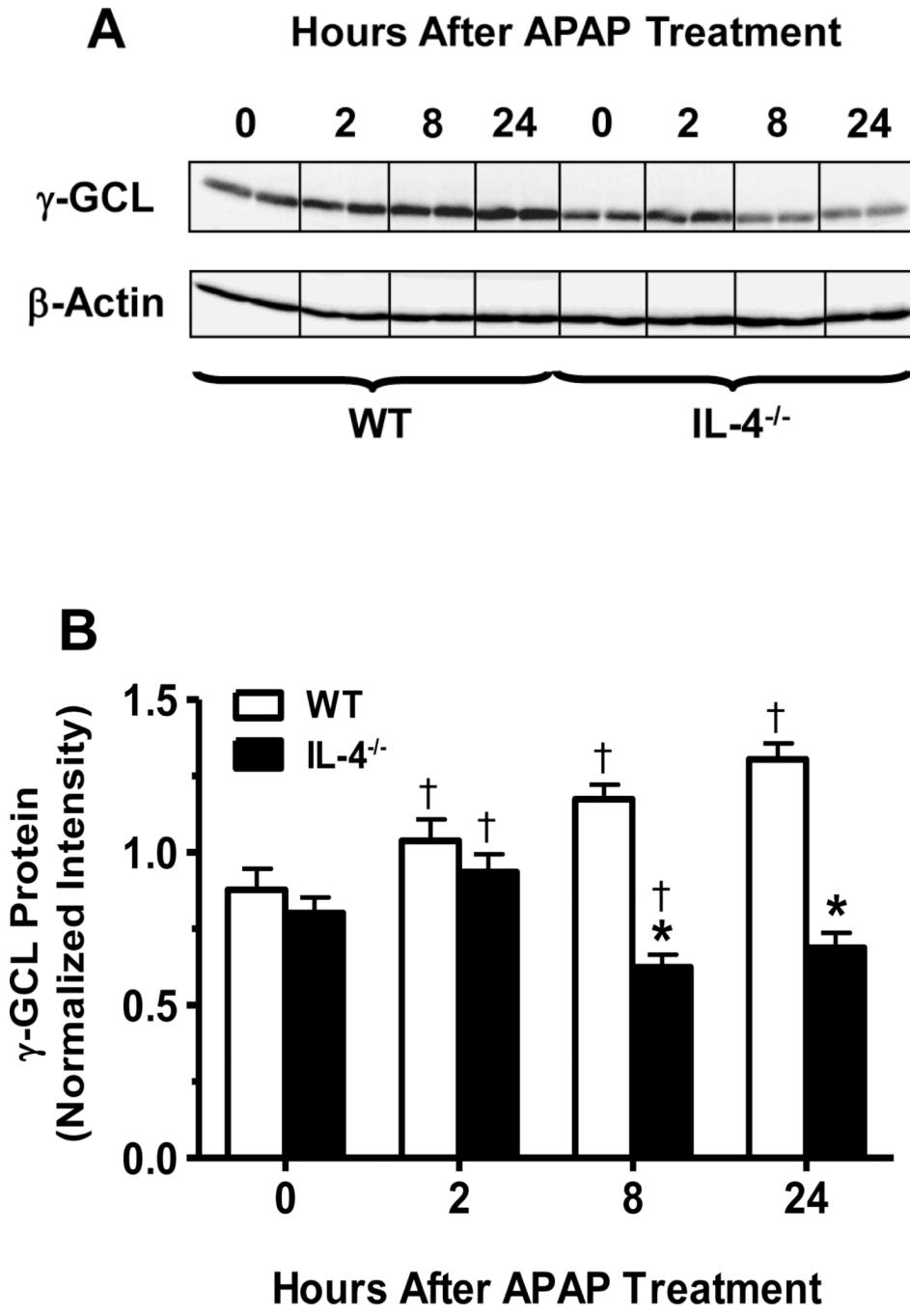
Livers of interleukin-4 deficient mice showed more histopathological injury than wild type mice treated with acetaminophen. Representative photomicrographs of H&E stained liver sections from WT and IL-4^{-/-} mice before and after treatment with APAP (200 mg/kg). Liver sections of WT (A) and (B) IL-4^{-/-} mice before APAP treatment both exhibited normal liver architecture with no histopathological changes. (C) Liver section from a WT mouse at 8 h after APAP treatment showed moderate centrilobular coagulative necrosis with hemorrhaging at the lesion periphery. (D) Liver section from an IL-4^{-/-} mouse at 8 h after APAP treatment demonstrated marked to severe centrilobular coagulative necrosis with lesions expanding into the midzonal area, causing bridging between intact central veins

along with moderate hemorrhaging in the lesion periphery, minimal ballooning hydropathy and moderate sinusoidal congestion. (E) Liver section from a WT mouse at 24 h after APAP treatment showed moderate to marked centrilobular coagulative necrosis with definite ballooning hydropathy at the lesion periphery and minimal sinusoidal congestion. (F) Liver section from an IL-4^{-/-} mouse at 24 h after APAP treatment was histopathologically similar to that of an 8 h APAP-treated IL-4^{-/-} mouse (D). CV = central vein. Original magnification, 40×.

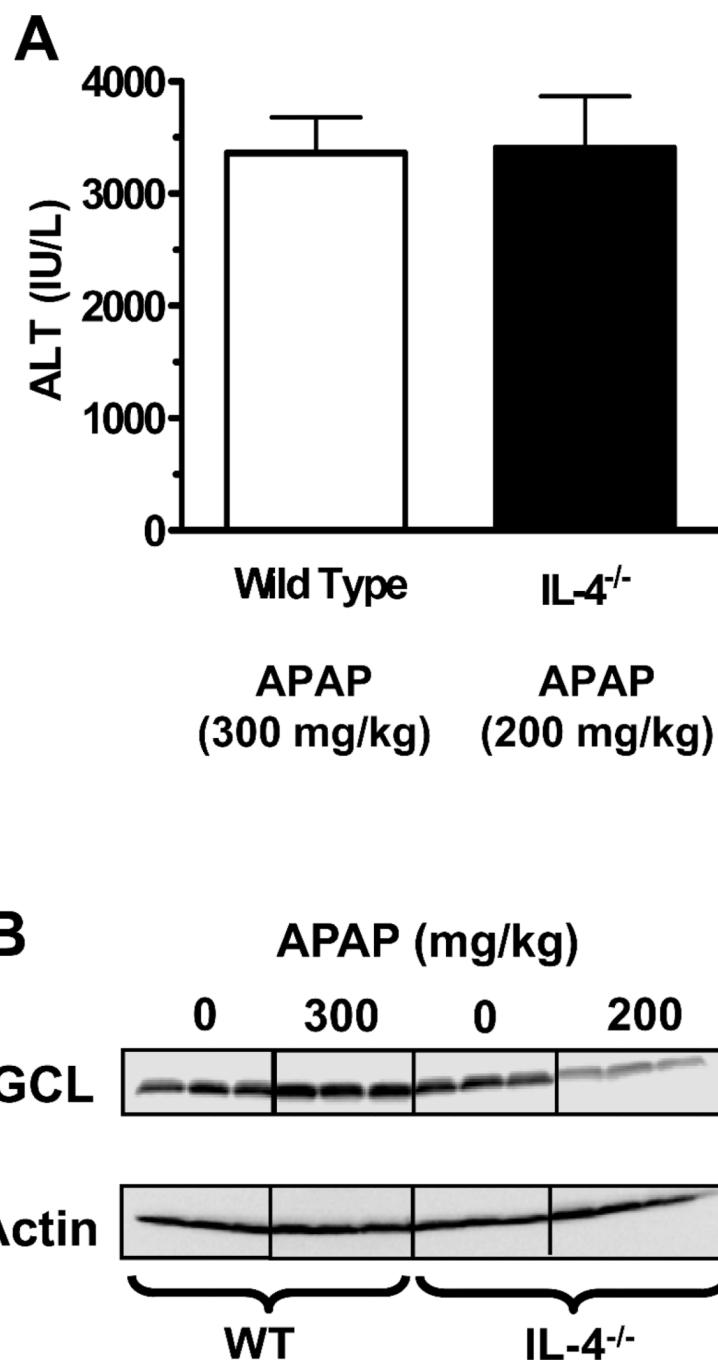
**Figure 3.**

Hepatic levels of protein adducts and glutathione were lower in interleukin-4 deficient mice than wild type mice after acetaminophen treatment. (A) Representative immunoblots showing APAP-protein adducts in livers (70 µg homogenate/lane) from WT and IL-4^{-/-} mice at 1 and 2 h after APAP (200 mg/kg) treatment. Immunoblots shows 2 mice per group, which are representative of a total of 5 mice used per group in this experiment. β-actin was used as a protein loading control. (B) Reduced GSH levels in APAP-treated WT and IL-4^{-/-} mice. Results for GSH analysis represent the mean ± SEM of 10 to 12 mice per group from two combined studies. † $P < 0.05$ versus the earlier time point within the same treatment

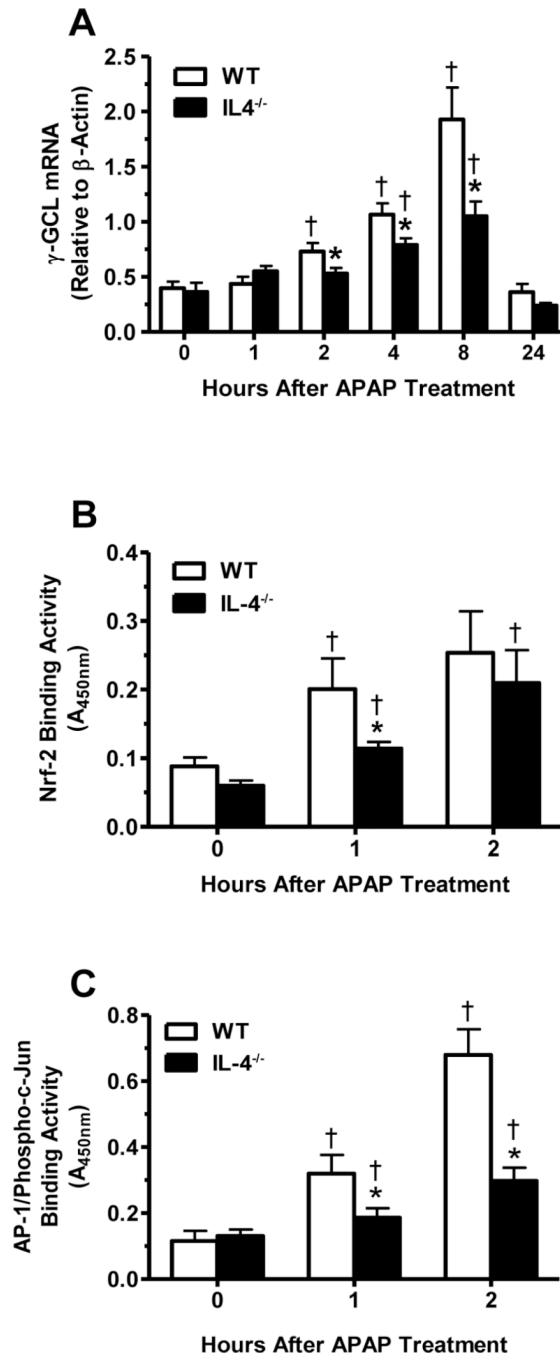
group. * $P<0.05$ versus APAP-treated WT mice at the same time point analyzed by ANOVA, while # $P<0.05$ versus WT mice at the same time point analyzed by Student's *t*-test.

**Figure 4.**

Hepatic levels of γ -glutamylcysteine ligase protein were lower in interleukin-4 deficient mice than wild type mice after acetaminophen treatment. (A) Representative immunoblot showing γ -GCL levels in the liver (100 μ g homogenate/lane) of 2 mice per time point before and after APAP (200 mg/kg) treatment of WT and $IL-4^{-/-}$ mice. β -actin was used as a protein loading control. (B) Densitometric analysis of γ -GCL protein levels from (A) normalized to β -actin levels. Results represent the mean \pm SEM of 4 mice per group from two independent experiments. $\dagger P < 0.05$ versus the earlier time point within the same group. $*P < 0.05$ versus WT mice at the same time point.

**Figure 5.**

Hepatic levels of γ -glutamylcysteine ligase were lower in interleukin-4 deficient mice than wild type mice at 8 h after treatment with equivalent hepatotoxic doses of acetaminophen. APAP was administered to WT or IL-4^{-/-} mice at doses of 300 or 200 mg/kg, respectively. (A) ALT activities; results are representative of two independent experiments and represent the mean \pm SEM of 5 mice per group. (B) Representative immunoblot showing hepatic levels (100 μ g of homogenate/lane) of γ -GCL. Equal protein loading was confirmed by blotting for β -Actin.

**Figure 6.**

Hepatic levels of γ -glutamylcysteine ligase mRNA and nuclear DNA binding activity of transcription factors were lower in livers of interleukin-4 deficient mice than wild type mice after acetaminophen treatment. WT and IL-4^{-/-} mice were treated with APAP (200 mg/kg). (A) qRT-PCR determination of γ -GCL mRNA levels. Enzyme-linked immunosorbant assay determinations of nuclear DNA binding activities of (B) Nrf-2 and (C) AP-1. Results represent the mean \pm SEM of 7 to 9 mice per group. † $P < 0.05$ versus the earlier time point within the same group. * $P < 0.05$ versus APAP-treated WT mice at the same time point. Results represent the mean \pm SEM of 6 mice per group. † $P < 0.05$ versus the earlier time point within the same group. * $P < 0.05$ versus APAP-treated WT mice at the same time point.

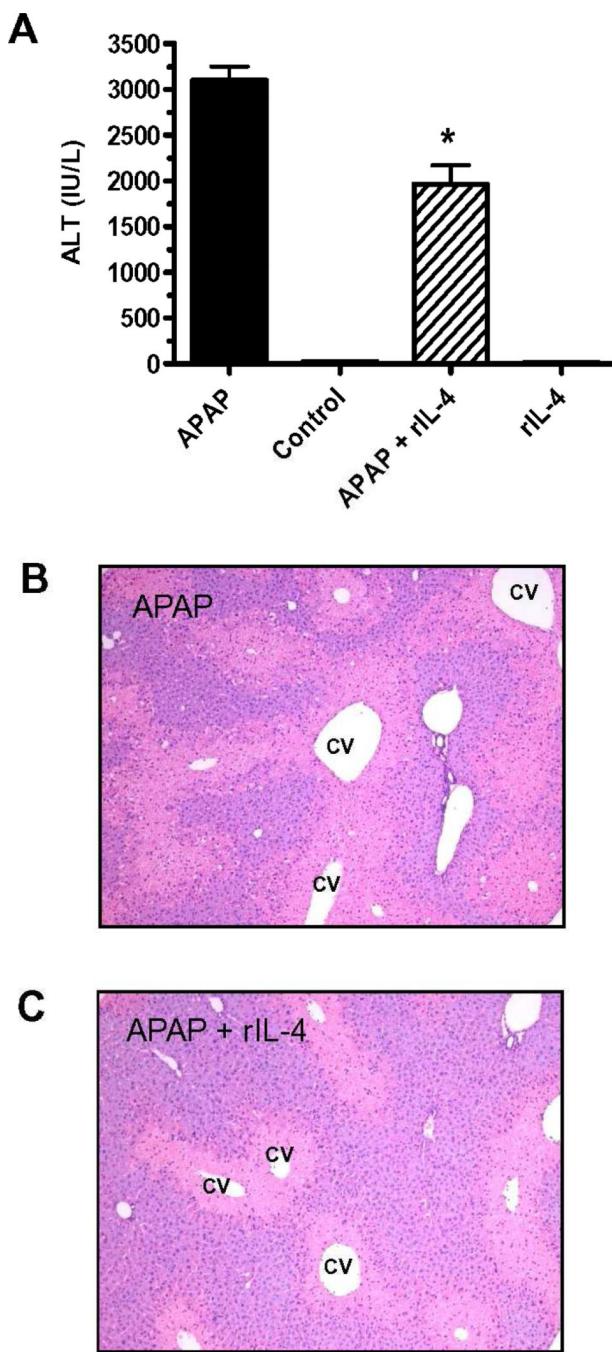
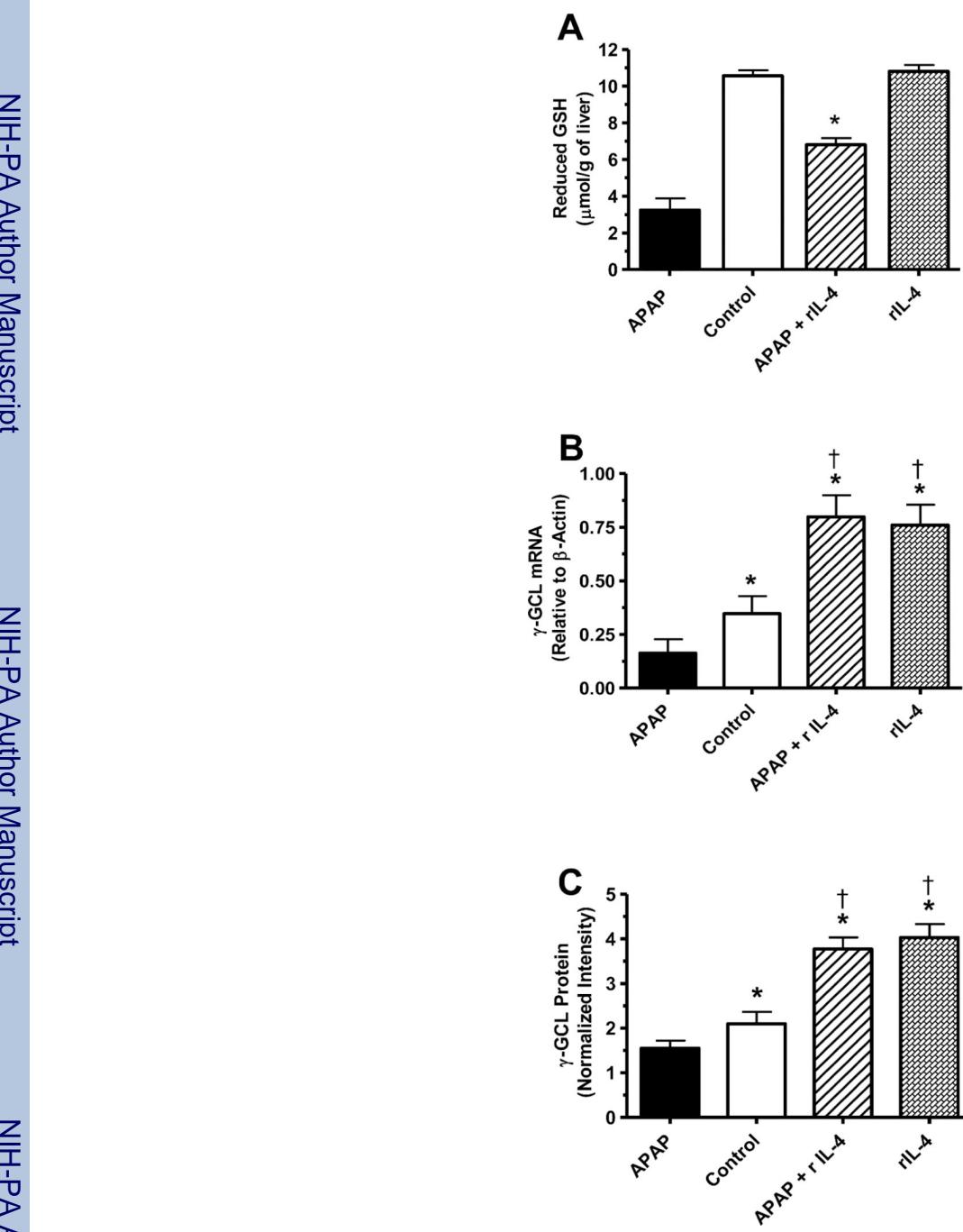


Figure 7.

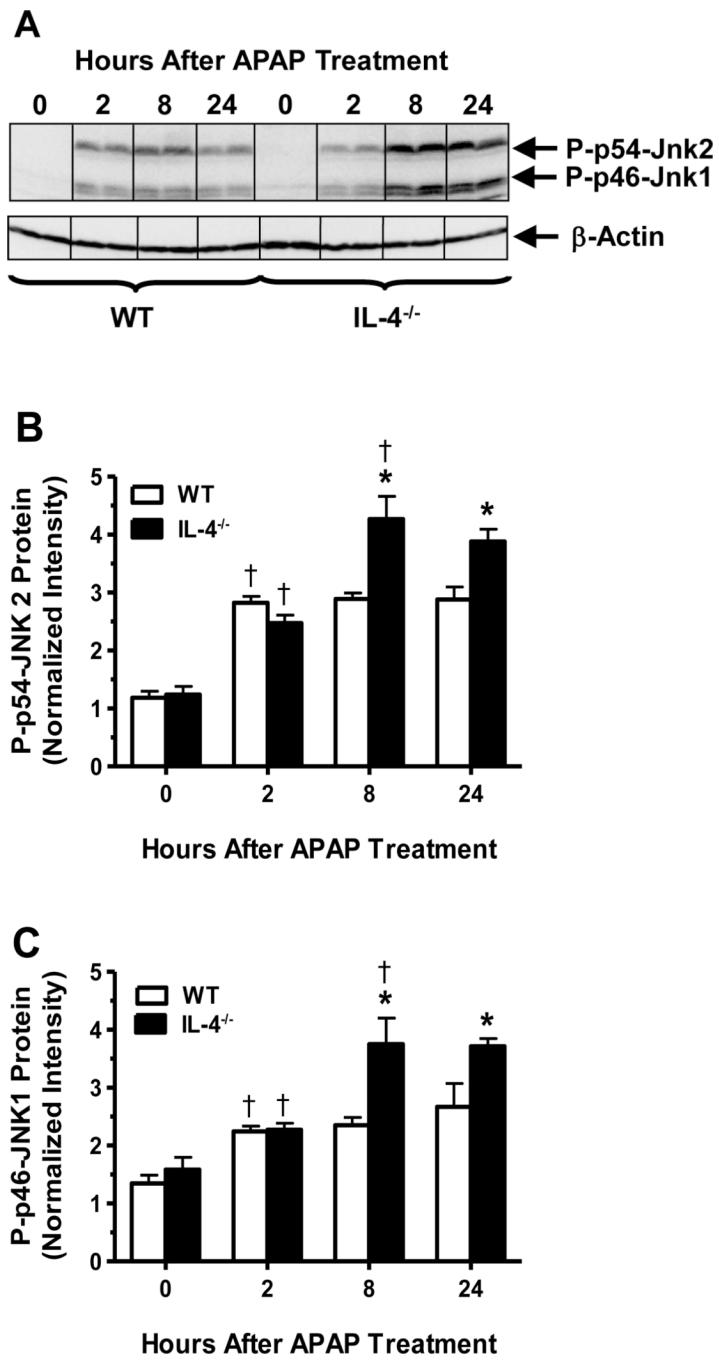
Recombinant IL-4 reduced susceptibility of interleukin-4 deficient mice to liver injury at 8 hours after acetaminophen treatment. IL-4^{-/-} mice were administered APAP (200 mg/kg) or saline vehicle, 3 h later mice were administered 100 pg of rIL-4 or vehicle (PBS, pH 7.4 containing 1% bovine serum albumin (PBS+BSA). (A) ALT activity; results represent the mean ± SEM of 6 mice per group. *P<0.05 versus APAP-treated mice. The APAP group was treated with APAP and PBS+BSA vehicle; the control group was treated with saline and PBS+BSA vehicles; the APAP+ rIL-4 group was treated with APAP and rIL-4; the rIL-4 group was treated with saline and rIL-4. Representative photomicrographs of H&E stained liver sections from mice treated with (B) APAP or (C) APAP + rIL-4 where n=6 for

each group. The APAP + rIL-4 treatment group showed less centrilobular coagulative necrosis than the APAP treatment group. CV= central vein. Original magnification, 20 \times .

**Figure 8.**

Recombinant IL-4 increased γ -glutamylcysteine ligase and GSH synthesis in interleukin-4 genetically deficient mice. $\text{IL-4}^{-/-}$ mice were administered APAP (200 mg/kg) or saline vehicle, 3 h later, mice were administered 100 pg of rIL-4 or vehicle, PBS, pH 7.4 containing 1% bovine serum albumin (PBS+BSA). Eight hours after APAP treatment, hepatic (A) GSH levels and (B) γ -GCL mRNA levels were determined as was (C) densitometric analysis of γ -GCL protein levels analyzed by immunoblotting and normalized to β -actin levels. Results represent the mean \pm SEM of 6 mice per group. * $P < 0.05$ versus APAP-treated mice. The APAP group was treated with APAP and PBS+BSA vehicle; the

control group was treated with saline and PBS+BSA vehicles; the APAP+ rIL-4 group was treated with APAP and rIL-4; the rIL-4 group was treated with saline and rIL-4.

**Figure 9.**

Enhanced hepatic levels of phosphorylated-JNK2/1 in livers from IL-4^{-/-} mice relative to levels seen in WT mice after APAP-treatment. Liver homogenates were isolated from WT and IL-4^{-/-} mice before and after APAP treatment (200 mg/kg) and analyzed (100 µg/lane) by immunoblotting. Representative immunoblots of phosphorylated (P)-p54 JNK2 and P-p46 JNK1 protein levels. (A) P-p54 JNK2 and P-p46 JNK1 protein levels in livers from WT and IL-4^{-/-} mice before and after APAP. β-actin was used as a protein loading control. Scanned intensity for (B) P-p54 JNK2 and (C) P-p46 JNK1 normalized to β-actin levels. Results are presented as the mean ± SEM of 6 mice per group. Results represent the mean ±

SEM of 6 mice per group. † $P<0.05$ versus the earlier time point within the same group.
* $P<0.05$ versus APAP-treated WT mice at the same time point.

Table 1Morphometric Analysis of Liver Lesions from Acetaminophen-Treated WT and IL-4^{-/-} Mice

<i>Percent Lesion Area (Time After Treatment)</i>			
Treatment	2 hours	8 hours	24 hours
WT APAP	11.0 ± 1.7	20.8 ± 6.1	32.4 ± 3.9
IL-4 ^{-/-} APAP	16.5 ± 3.7	57.3 ± 6.1*	53.5 ± 3.4*

Acetaminophen (APAP) was administered to WT and IL-4^{-/-} mice at a dose of 200 mg/kg. Livers were excised from mice after APAP treatment, fixed in formalin and processed for light microscopy. Morphometric analysis was carried out as described in the Experimental Procedures. Results represent the mean ± SEM of 5 to 7 animals per group.

* $P<0.05$ versus APAP-treated WT mice.