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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · JANUARY 2003

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Protective Role of Kupffer Cells in Acetaminophen-Induced Hepatic Injury in Mice

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Received August 13, 2002

Hepatic injury induced by various toxic agents, including acetaminophen (APAP), has been attributed, in part, to the production of proinflammatory cytokines and other mediators by resident Kupffer cells within the liver. However, recent evidence from our laboratory has demonstrated that hepato-protective factors, such as interleukin (IL)-10 and cyclooxygenase-derived mediators, are also upregulated in response to hepatic damage to help protect against exacerbated injury, and Kupffer cells have been suggested to be a source of these modulatory factors. In other models, Kupffer cells also serve important regulatory functions in pathophysiological states of the liver. Therefore, we reevaluated the role of Kupffer cells in a murine model of APAP-induced liver injury using liposome-entrapped clodronate (liposome/clodronate) as an effective Kupffer cell-depleting agent. We show that in contrast to pretreatment of mice with a widely used macrophage inhibitor, gadolinium chloride, which did not deplete Kupffer cells but moderately protected against APAP-induced hepatotoxicity as reported previously, the intravenous injection of liposome/clodronate caused nearly complete elimination of Kupffer cells and significantly increased susceptibility to APAP-induced liver injury as compared with mice pretreated with empty liposomes. This increased susceptibility was apparently unrelated to the metabolism of APAP since liposome/clodronate pretreatment did not alter APAP−protein adduct levels. Instead, Kupffer cell depletion by liposome/clodronate led to significant decreases in the levels of hepatic mRNA expression of several hepato-regulatory cytokines and mediators, including IL-6, IL-10, IL-18 binding protein and complement 1q, suggesting that Kupffer cells are a significant source for production of these mediators in this model. Our findings indicate that, in addition to their protoxicant activities, Kupffer cells can also have an important protective function in the liver through the production of a variety of modulatory factors which may counteract inflammatory responses and/or stimulate liver regeneration.

Introduction

Drug-induced liver disease causes significant morbidity and mortality and is a major detriment to new drug development (*1*). Unfortunately, its study remains difficult because of the inability to reproduce toxicity from most drugs in animal models. This inability may be due to protective molecular and cellular mechanisms that normally counteract the adverse effects of drugs within the liver. Studies from our laboratory have demonstrated that hepato-regulatory factors including interleukin (IL)-10[1] and cyclooxygenase (COX)-derived prostaglandins may play critical protective roles against exacerbated toxicity caused by the model hepatotoxic drug, acetaminophen (APAP) (*2*, *3*). Kupffer cells have been suggested to be a source of these modulatory factors in various models of liver injury, including bile duct ligation (*4*), acute alcoholic liver damage (*5*), and endotoxin-induced septic shock (*6*). Furthermore, it has been shown that Kupffer cells clearly provide important regulatory functions within pathophysiological states of the liver (*7*). For instance, although Kupffer cells can act as antigen presenting cells, they are poor stimulators of allogeneic T cells (*8*) due to insufficient expression of co-stimulatory molecules and/or production of antiinflammatory and immunosuppressive cytokines and mediators such as IL-10 and prostaglandin E_2 (*9*, *10*). A number of studies have also shown that Kupffer cells play an important role in portal vein tolerance (*11*) and prolonged liver allograft survival (*12*).

However, APAP hepatotoxicity has previously been attributed in part to the activation of Kupffer cells secondary to hepatocyte damage initiated by the formation of a hepatotoxic reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (*13*). It is believed that Kupffer cell activation results in the release of a wide range of proinflammatory mediators capable of causing further

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[1] Abbreviations: IL, interleukin; COX, cyclooxygenase; APAP, acetaminophen; liposome/clodronate, liposome-entrapped clodronate; ALT, alanine transaminase; RT-PCR, reverse transcription-polymerase chain reaction; TNF, tumor necrosis factor; IL-18BP, IL-18 binding protein; C1q, complement 1q.

10.1021/tx0255976
Published on Web 11/19/2002

hepatic injury (*14*, *15*). The most convincing evidence for this role of Kupffer cells in APAP-induced liver injury is based upon findings that pretreatment of rats and mice with gadolinium chloride results in a significant decrease in APAP hepatotoxicity (*16*, *17*) despite continued controversy as to whether gadolinium chloride actually causes depletion of Kupffer cells (*17*−*19*) or has other effects such as stimulating hepatocyte proliferation and regeneration (*20*, *21*) or reducing cytochrome P450 activity (*22*, *23*).

The dichotomy of Kupffer cell activities suggests that their precise role may vary with the specific model and pathophysiological conditions. Therefore, we set out to reevaluate the role of Kupffer cells in APAP-induced acute hepatotoxicity with the use of liposome-entrapped clodronate (liposome/clodronate), which has definitively been shown to cause nearly complete depletion of Kupffer cells from the liver (*24*, *25*). We show that Kupffer cells are a predominant source of several hepato-protective cytokines and mediators, which appear to override the proinflammatory, adverse effects of Kupffer cells and can protect against APAP-induced liver injury.

Materials and Methods

Chemicals and Reagents. The following chemicals and reagents were purchased commercially: APAP, dichloromethylene diphosphonate (clodronate), gadolinium chloride, and alanine transaminase (ALT) kits (Sigma, St. Louis, MO); xylene and 10% neutral buffered formalin (Fisher, Fairlawn, NJ); rat immunoglobulin G2b anti-mouse F4/80 antibody (Serotec, Raleiph, NC); peroxidase-conjugated anti-rabbit immunoglobulin G (Roche, Indianapolis, IN); enhanced chemiluminescence reagents, Ready-To-Go reverse transcription-polymerase chain reaction (RT-PCR) beads and Vistra Green nucleic acid dye (Amersham Pharmacia Biotech, Piscataway, NJ); RNeasy kits (Qiagen, Valencia, CA).

Animal Treatment. Female 6−8 weeks old C57BL/6J and IL-10$^{-/-}$mice (on C57BL/6J background) were purchased from Jackson Laboratory (Bar Harbor, ME). Upon arrival, the animals were acclimated for approximately 1 week to a 12 h light-dark cycle in a humidity- and temperature-controlled specific-pathogen-free environment in autoclaved, microisolator cages in accordance with the National Institutes of Health standards and the *Guide for the Care and Use of Laboratory Animals*. Animals were fasted overnight (16−18 h) before treatment with APAP (200 or 300 mg/kg in saline) or saline by intraperitoneal injection. At various times thereafter, blood was collected by retro-orbital puncture and the livers were removed. Blood samples were allowed to clot overnight at 4 °C. Sera were separated for measurement of ALT activity. Upon removal, a portion of each excised liver was fixed in buffered formalin and the remainder was snap-frozen and stored at −80 °C for subsequent RNA isolation and immunoblot analyses. Tissue sections were embedded in paraffin and were subsequently either mounted onto glass slides and stained with hematoxylin/eosin, or mounted onto poly (L-lysine)-treated glass slides for use in immunohistochemical analyses (Histoserv Inc., Gaithersburg, MD).

Assessment of Hepatotoxicity. Liver injury was determined by measuring serum levels of ALT using a diagnostic kit and by histological examination of hematoxylin and eosin-stained tissue sections using light microscopy. The degree of centrilobular necrosis following treatment with APAP was graded from ± to +++ according to a previously described grading scheme (*26*).

Effect of Gadolinium Chloride Pretreatment on APAP−Induced Hepatotoxicity. Mice were dosed intravenously with gadolinium chloride (7 mg/kg in saline), the livers were removed 24 h later and poly (L-lysine)-treated slides were prepared (Histoserv Inc., Gaithersburg, MD). The effect of gadolinium chloride treatment on the number of Kupffer cells in the liver was determined immunohistochemically with the use of a murine macrophage marker, F4/80, following an established procedure (*24*). The phagocytotic activity in the liver was determined by intravenously administering India ink (100 μL) to mice (*27*). After 20 min, the livers were removed and hematoxylin/eosin slides were prepared (Histoserv Inc., Gaithersburg, MD). To determine the effect of gadolinium chloride on APAP toxicity, mice were dosed intravenously with gadolinium chloride (7 mg/kg in saline) or saline 24 h before the treatment with APAP (*17*).

Effect of Kupffer Cell Depletion by Liposome/clodronate on APAP−Induced Hepatotoxicity. Liposome/clodronate were prepared as previously described (*24*). To determine the effect of Kupffer cell depletion on APAP toxicity, mice were injected intravenously with 0.1 mL of liposome/clodronate or with empty liposomes 48 h prior to treatment with APAP.

RNA Isolation and RT-PCR Analysis. Total RNA was isolated from approximately 80 mg of frozen liver tissue using RNeasy kits as described by the manufacturer (Qiagen). The concentration and purity of isolated RNAs were determined by measuring the UV absorbance at 260 nm (A_{260}) and the A_{260}/A_{280} ratio, respectively. RNA integrity was confirmed by electrophoretic separation in 1.25% agarose gels and visualization with Vistra Green nucleic acid dye. RNA (2 μg) was reverse transcribed at 42 °C for 30 min using Ready-To-Go RT-PCR beads (Amersham). After enzyme inactivation (95 °C, 5 min), cDNA fragments were amplified for 22−36 cycles using gene-specific primers for IL-10 (sense 5′-ATGCAGGACTTTAAGGGTTACTTGGGTT-3′; antisense 5′-ATTTCGGAGAGAGGTACAAACGAGGTTT-3′), COX-2 (sense 5′-ACTCACTCAGTTTGTTGAGTCATTC-3′; antisense 5′-TTTGATTAGTACTGTAGGGTTAATG-3′), IL-6 (sense 5′-ATGAGGTTCCTCTCTGCAAGAGACT-3′; antisense 5′-CACTAGGTTTGCCGAGTAGATCTC-3′), IL-18 binding protein (IL-18BP, sense 5′-CACCTCAGACAACTGCCACT-3′; antisense 5′-GCCACTTGTCCAGGATCCAC-3′), complement 1q (C1q, sense 5′-ACAGTGGCTGAAGATGTCTG-3′; antisense 5′-CTGGTCCCTGATATTGCCTG-3′), and TNF-α (sense 5′-AGCCCACGTCGTAGCAAACCACCAA-3′; antisense 5′-ACACCCATTCCCTTCACAGAGCAAT-3′). Amplification of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (sense 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′; antisense 5′-CATGTAGGCCATGAGGTCCACCAC-3′), served as a control for the normalization of results. All PCR products were resolved in 2% agarose gels in Tris-borate buffer and visualized using Vistra Green nucleic acid dye. RNA expression was determined by measuring band intensities with the use of a fluoroimager, and normalized relative to the glyceraldehyde-3-phosphate dehydrogenase expression levels for each sample.

Microarray Analysis. Global gene expression within the liver was evaluated with high-density oligonucleotide microarrays (Murine U74 arrays) as detailed by the manufacturer (Affymetrix, Santa Clara, CA). RNA samples from four to six APAP-treated and two saline-treated mice were pooled to obtain a representative gene expression profile for each treatment condition. RNA pooling for microarray analyses has previously been shown to be a viable experimental approach based upon the reproducibility of microarray results by RT-PCR in individual mice (*28*) and the high correlation between microarray results from individual and pooled RNA samples (*29*). Data analysis was performed using Gene Chip Analysis Suite (version 4.0, Affymetrix), and scaled to allow direct comparisons among arrays. Comparisons of gene expression were performed using one selected array as a baseline measurement for another's analysis and data filtered using the exclusion criteria outlined previously (*28*).

Other Methods. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis and immunoblotting of whole liver homogenates were performed as previously described (*30*). Rabbit anti-serum used for the detection of APAP−protein adducts was generously

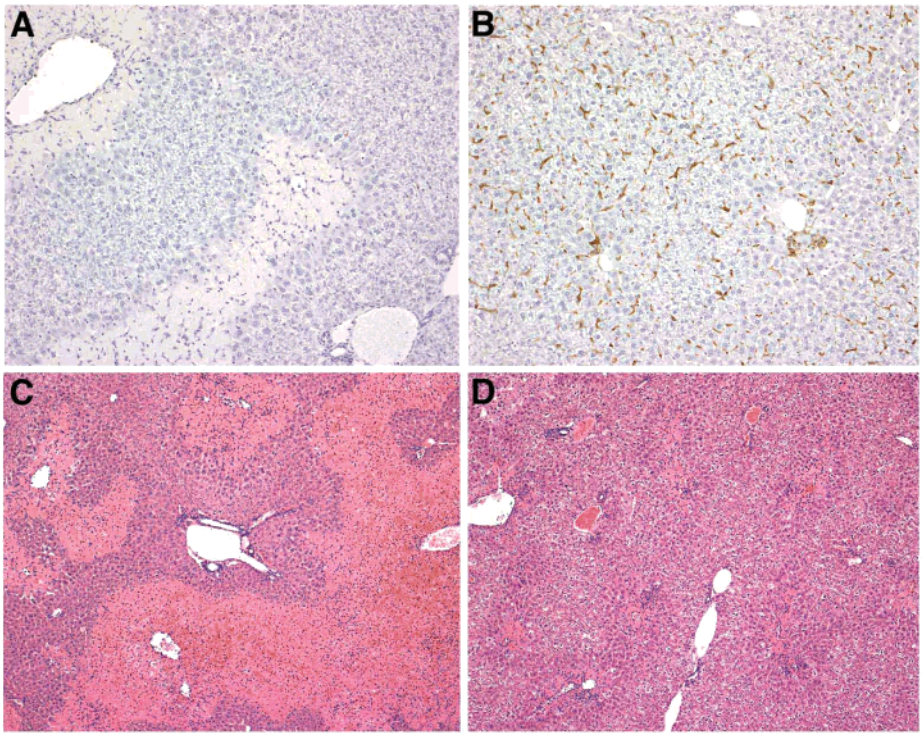

Figure 1. Depletion of Kupffer cells by liposome/clodronate pretreatment and potentiation to APAP-induced histopathology. C57BL/6J female mice were injected intravenously with either liposome/clodronate (panels A and C) or empty liposomes (panels B and D) 2 days prior to APAP treatment (200 mg/kg). After an additional 24h (post-APAP), animals were killed and liver sections were stained for Kupffer cells with F4/80 antibody (diluted 1 to 50, panels A and B, original magnification 100×), or with hematoxylin and eosin (panels C and D, original magnification 50×).

Table 1. Gene Expression in the Livers of Kupffer Cell-Depleted versus Nondepleted Mice by Microarray Analysis[a]

gene description	GenBank or SwissProt ID	fold change[b]		
		saline	8 h APAP	24 h APAP
Mus bacteria binding macrophage receptor MARCO	u18424	−6.8	−16.7	−21.8
Kupffer cell receptor	d88577	−24.1	−30.4	−15.3
major histocompatibility complex II, alpha	x52643	−6.2	−7.5	NC[c]
IL-18BP	ab019505	−4.6	−5.6	−7.2
C1q, c polypeptide	x66295	−5.5	−7.1	−3.3
C1q, B chain	m22531	−12.4	−20.1	−3.8
C1q, alpha polypeptide	x58861	−14.2	−7.4	−3.8

[a] Microarray studies were performed for discovery purposes using pooled samples as outlined in the methods. All results are complementary to other presented results, are consistent across time and drug treatment, and/or were confirmed via independent experiments (RT-PCR). [b] Fold changes were taken directly from microarray analysis (Affymetrix) using preset exclusion criterion (*28*) that compared Kupffer cell-depleted mice against nondepleted mice following saline treatment, or 8 and 24 h after APAP treatment. [c] NC, no change based on the preset exclusion criterion.

provided by Drs. Neil R. Pumford and Jack A. Hinson (University of Arkansas, Little Rock, AR).

Statistical Analysis. Data are presented as mean ± SEM and analyzed by using ANOVA and Student's *t*-test. Differences were considered significant when $p < 0.05$.

Results

Differential Effects of Gadolinium Chloride and Liposome/Clodronate Pretreatments on APAP–Induced Hepatotoxicity. We previously reported (*24*) that Kupffer cells were virtually eliminated from the liver 24 h after intravenous injection of liposome/clodronate and did not begin to reappear until the third day after the treatment as reported by other investigators (*31*, *32*). Hence, this reagent was used to investigate the role of Kupffer cells in APAP-induced hepatotoxicity. Female C57BL/6J mice were pretreated with liposome/clodronate and, then after 2 days, were treated with 200 mg/kg APAP. Twenty-four hours later, immunohistochemical staining for F4/80, a murine macrophage marker, showed that Kupffer cells remained depleted from the liver (Figure 1A). In contrast, F4/80 stained numerous macrophages in the livers of mice pretreated with empty liposomes (Figure 1B). The depletion of Kupffer cells by liposome/clodronate was further supported by microarray results revealing decreased mRNA levels of macrophage and Kupffer cell receptors and Kupffer cell-related molecules, such as the major histocompatibility complex class II, in the livers of mice pretreated with liposome/clodronate both before as well as 8 and 24 h after APAP treatment (Table 1). Liver sections from mice depleted of Kupffer cells with liposome/clodronate showed massive, bridging, perivenous hepatocyte necrosis following 200 mg/kg APAP in contrast to the much milder damage seen in the livers of empty liposome-pretreated nondepleted mice (Figure 1, panels C and D, respectively, and Table 2). In line with this observation, biochemical analysis of serum ALT activity showed that APAP caused signifi-

Table 2. Histopathological Grading of Liver Injury in Kupffer Cell-Depleted and Nondepleted Mice at 24 h Following APAP Treatment

grade[a]	Kupffer cell-depleted animals*	Kupffer cell-nondepleted animals
±	0/6[b]	1/6
+	1/6	2/6
++	0/6	3/6
+++	5/6	0/6

[a] Upon the basis of a previously described histopathological grading scheme of liver damage (*26*): (±) degeneration of perivenous hepatocytes; (+) slight perivenous hepatocyte necrosis (<3-cell layers); (++) moderate perivenous hepatocyte necrosis (in more than 3-cell layers from the central vein but limited to less than half of the liver section); (+++) serious perivenous hepatocyte necrosis (necrotic areas occupying more than half of the liver section). [b] Results shown represent the number of mouse livers classified into a given grade of damage out of the total number of mouse livers inspected for a particular treatment group. (*) $P < 0.05$ compared with Kupffer cell-nondepleted mice using Mann−Whitney nonparametric analysis.

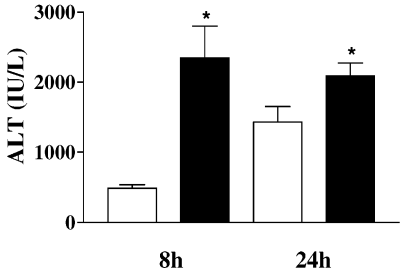

Figure 2. Increased ALT activities in sera of APAP-treated mice depleted of Kupffer cells with liposome/clodronate. Serum ALT activities in mice pretreated with empty liposomes (clear bars) or liposome/clodronate (black bars) were determined at 8 and 24 h after APAP (200 mg/kg) treatment. Results shown represent mean ± SEM of 10 mice per group and individual samples were assayed in triplicate. (*) $P < 0.05$ compared with empty liposome-pretreated, Kupffer cell-nondepleted mice. Serum ALT values for saline-treated mice were typically in the 5−10 IU/L range, regardless of pretreatment.

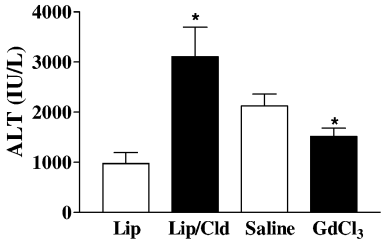

Figure 3. Comparison of the effects of pretreatment with liposome/clodronate or gadolinium chloride on APAP-induced liver damage. Serum ALT activities in mice pretreated with empty liposome (Lip), liposome/clodronate (Lip/Cld), saline, or gadolinium chloride ($GdCl_3$) were determined at 8h after APAP (300 mg/kg) treatment. Results shown represent mean ± SEM of 10 mice/group and individual samples were assayed in triplicate. (*) $P < 0.05$ compared with mice pretreated with the respective vehicles, empty liposomes or saline. Serum ALT values for saline-treated mice were typically in the 5−10 IU/L range, regardless of pretreatment.

cantly greater increases of ALT levels in the sera of Kupffer cell-depleted mice at 8 and 24 h compared with the nondepleted mice (Figure 2). Similar results were observed at 8 h after 300 mg/kg of APAP (Figure 3). In contrast, however, gadolinium chloride (7 mg/kg) treatment did not cause a significant decrease in the number of F4/80 positive cells in the liver compared with that in saline-treated control mice (Figure 4, panels A and B, respectively). Moreover, phagocytotic activity in liver determined by the uptake of carbon particles was not inhibited by the treatment of mice with gadolinium chloride (Figure 4, panels C and D). Despite this lack of Kupffer cell depletion in contrast to the nearly complete elimination by liposome/clodronate treatment, pretreatment of mice with gadolinium chloride protected against

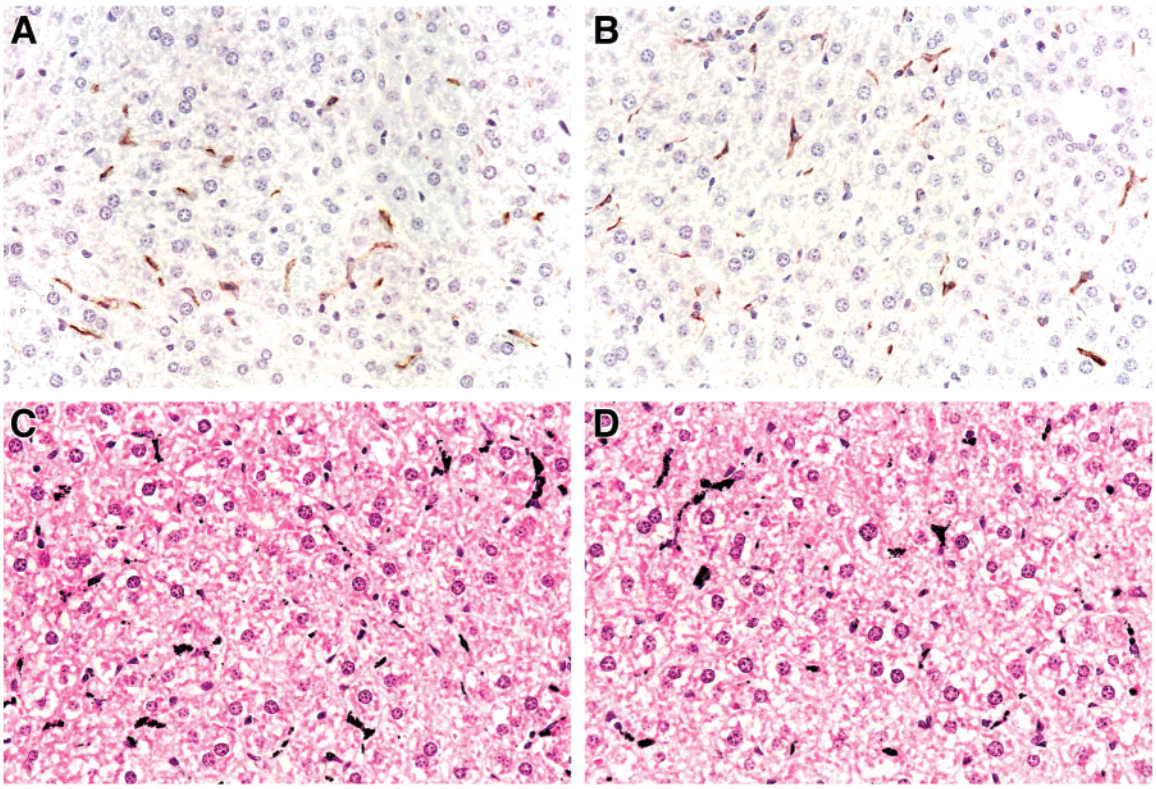

Figure 4. Lack of Kupffer cell depletion by gadolinium chloride treatment. Animals were injected intravenously with either saline (panels A and C, 400×) or gadolinium (7 mg/kg, panels B and D, 400×). After 24 h, some animals were killed and the liver sections were stained with F4/80 antibody (diluted 1 to 100) for Kupffer cells (panels A and B), while other mice were killed 20 min after injection of India ink for the determination of carbon particle uptake by Kupffer cells in hematoxylin/eosin stained sections (panels C and D).

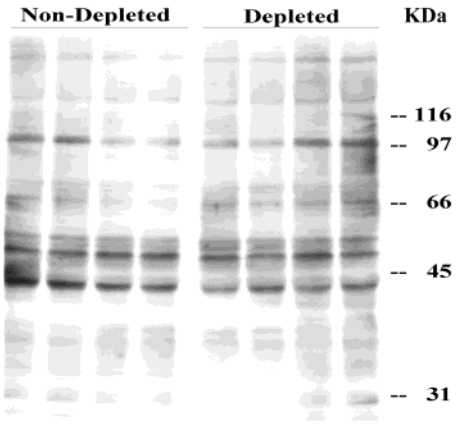

Figure 5. Lack of effect of Kupffer cell depletion by liposome/clodronate on APAP−protein adducts in mouse liver homogenates. At 4 h after APAP (200 mg/kg) treatment, liver homogenates from 4 mice pretreated with either empty liposomes (Nondepleted) or liposome/clodronate (Depleted) were prepared and used for immunoblot detection of APAP−protein adducts (75 μg/lane, from individual mice) with anti-APAP sera. Molecular mass marker (kDa) migrations are shown on the right.

APAP-induced liver injury at a dose of 300 mg/kg (Figure 3), as reported by other investigators (*17*).

Analysis of APAP−Protein Adducts. Immunoblotting was performed to determine whether the susceptibility to APAP hepatotoxicity in Kupffer cell-depleted mice might be due to elevated levels of APAP−protein binding secondary to the administration of liposome/clodronate. This did not appear to be the case because the patterns (Figure 5) and the levels of APAP-protein adducts quantified by densitometry of the molecular mass regions between 40 and 90 kDa (data not shown) revealed no significant difference at 4 h after APAP treatment in the liver homogenates of mice pretreated with empty liposomes or liposome/clodronate. Thus, the increased APAP hepatotoxicity caused by Kupffer cell depletion was not likely due to an increased metabolic activation of APAP or decreased detoxification of its reactive metabolite. Moreover, similar levels of APAP−protein adducts were observed in the livers of mice pretreated with gadolinium chloride or saline (data not shown) as reported earlier (*17*), indicating that, in this model, hepatoprotection was not due to inhibition of cytochrome P450-mediated bioactivation of APAP.

Effect of Kupffer Cell Depletion on APAP−Induced IL-10 mRNA Expression in the Liver. APAP treatment has been shown to cause increased levels of IL-10 mRNA expression in the livers of mice, a response which appears to play a central role in protecting against APAP-induced hepatic damage (*2*). To determine whether Kupffer cells are a major hepato-cellular source of this protective cytokine in this system, the levels of IL-10 mRNA expression were compared between Kupffer cell-depleted and -nondepleted mice following APAP treatment (200 mg/kg). Baseline levels of hepatic IL-10 expression following saline treatment were significantly decreased in mice depleted of Kupffer cells with liposome/clodronate as compared to nondepleted mice (Figure 6B). In line with a previous report (*2*), a trend toward increased levels of IL-10 expression following APAP treatment was also observed in Kupffer cell-nondepleted mice (Figure 6B). In contrast, the APAP-induced increase of IL-10 expression within the liver was significantly inhibited in Kupffer cell-depleted mice for at least 24 h

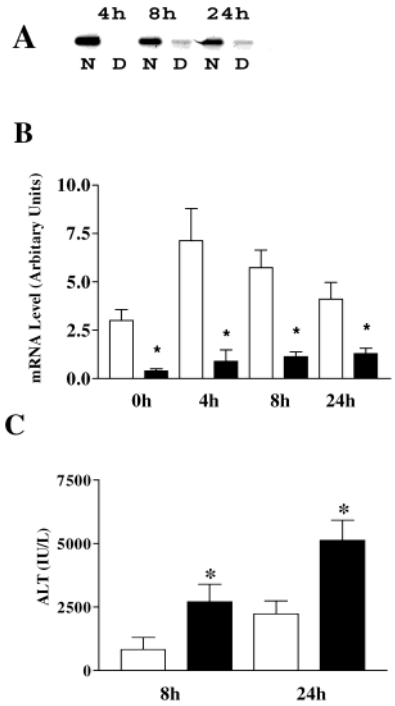

Figure 6. Effect of Kupffer cell depletion by liposome/clodronate on the level of IL-10 mRNA expression and on susceptibility of IL-10$^{-/-}$mice to APAP-induced liver injury. Mice were pretreated with empty liposomes or liposome/clodronate prior to the treatment with APAP (200 mg/kg). After various times, IL-10 message levels or serum ALT activities were determined. Animals pretreated with empty liposomes are designated by N or clear bars while those pretreated with liposome/clodronate are represented by D or black bars. (A) IL-10 RT-PCR results from representative wild-type mice. (B) Relative mRNA expression levels of IL-10, where results represent mean ± SEM of groups of 5, 6, 5, and 9 wild-type mice, respectively, at times following treatment with saline (0 h) or APAP. (C) Serum ALT activities of IL-10$^{-/-}$ mice with or without Kupffer cell depletion, where results represent mean ± SEM of 10 mice per group and individual samples were assayed in triplicate. (*) $P < 0.05$ compared with Kupffer cell-nondepleted IL-10$^{-/-}$ mice at each time point.

(Figure 6, panels A and B). To determine whether the increase of APAP hepatotoxicity observed in Kupffer cell-depleted mice might be due exclusively to the inhibition of APAP-induced elevations in IL-10 production, we further investigated the effect of Kupffer cell depletion on APAP hepatotoxicity in IL-10$^{-/-}$ mice. Similar to wild-type mice, the severity of APAP hepatotoxicity was greater in IL-10$^{-/-}$ mice depleted of Kupffer cells with liposome/clodronate compared with nondepleted mice (Figure 6C).

Effect of Kupffer Cell Depletion on mRNA Expression Levels of Other Potential Hepato-Protective Mediators in the Liver. In addition to IL-10, APAP has also been shown to cause elevated mRNA expression levels of other potential protective cytokines and mediators including COX-2 (*3*) and IL-6 (*2*). To determine whether Kupffer cells play an important role in the production of these factors, the levels of their hepatic mRNA expression in response to APAP treatment were compared between Kupffer cell-depleted and -nondepleted mice (Figure 7). Depletion of Kupffer cells with liposome/clodronate caused significant decreases of IL-6 expression levels following saline treatment and at 8 h following APAP treatment. In addition, a trend toward decreased levels of IL-6 expression was clearly evident

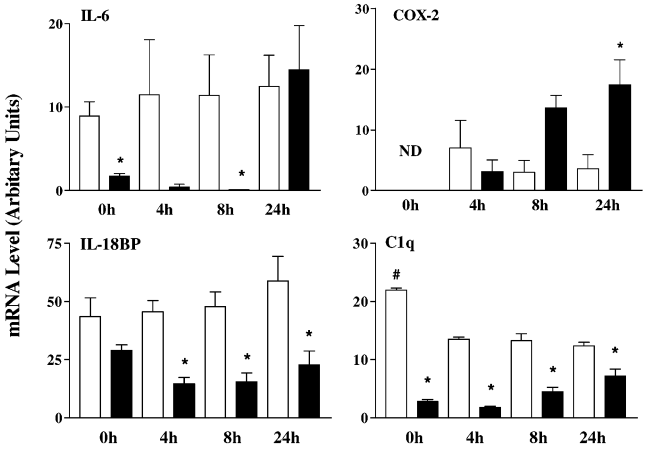

Figure 7. Effect of Kupffer cell depletion by liposome/clodronate on hepatic mRNA expression of other protential hepatoprotective factors following APAP treatment. Hepatic mRNA expression of IL-6, COX-2, IL-18BP, and C1q in Kupffer cell-depleted (black bars) and nondepleted mice (clear bars) were determined by RT-PCR over a 24 h time period following APAP treatment (200 mg/kg). Results shown represent mean ± SEM of groups of 5, 6, 5, and 9 mice, respectively, at times following treatment with saline (0 h) or APAP. (*) $P < 0.05$ compared with Kupffer cell-nondepleted mice. (#) $P < 0.05$ compared with APAP-treated Kupffer cell-nondepleted mice at all time points.

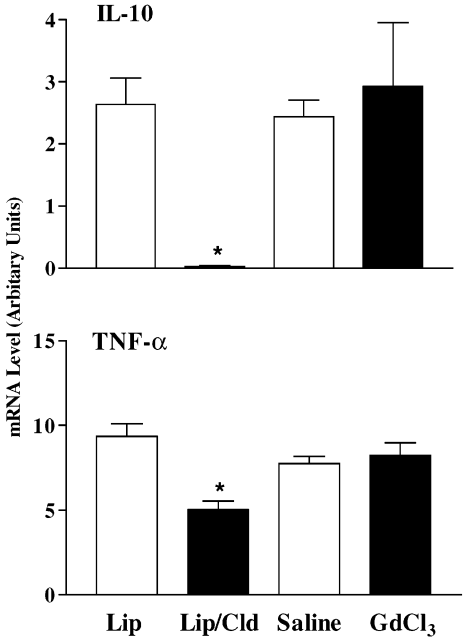

Figure 8. Comparison of the effects of pretreatment with liposome/clodronate or gadolinium chloride on APAP-induced cytokine expression levels in the liver. Mice were pretreated with empty liposomes (Lip), liposome/clodronate (Lip/Cld), saline, or gadolinium chloride ($GdCl_3$). Hepatic mRNA levels of IL-10 and TNF-α were determined by RT-PCR 8 h following APAP (300 mg/kg) treatment. Results shown represent mean ± SEM of 10 mice/group. (*) $P < 0.05$ compared with mice pretreated with empty liposomes.

at 4 h following APAP treatment in Kupffer cell-depleted mice as compared to nondepleted mice. Interestingly,Kupffer cell depletion led to significantly increased levels of COX-2 mRNA expression at 24 h following APAP treatment. The baseline level of COX-2 expression was too low to be detected.

Furthermore, microarray experiments performed for discovery purposes indicated that IL-18BP and C1q were two noteworthy factors whose message levels were significantly decreased in the livers of Kupffer cell-depleted mice compared with that of nondepleted mice (Table 1). These alterations were observed following saline and at 8 and 24 h after APAP treatment and were confirmed by RT-PCR analysis of individual mice (Figure 7).

Differential Effects of Gadolinium Chloride and Liposome/Clodronate Pretreatments on Levels of Hepatic Cytokine Expression. To further evaluate the disparate results following liposome/clodronate versus gadolinium chloride pretreatment, their effects on hepatic cytokine production were compared. At 8 h after a dose of 300 mg/kg of APAP, significant decreases in the levels of hepatic IL-10 and TNF-α expression were observed in mice depleted of Kupffer cells with liposome/clodronate compared with empty liposome-treated control mice (Figure 8). In contrast, no significant changes in the expression levels of these cytokines were observed in gadolinium chloride-pretreated mice, as compared to saline-pretreated controls (Figure 8).

Discussion

Kupffer cells, the largest population of resident macrophages in the body, not only serve as a vanguard in host defense by phagocytosing and eliminating pathogens and particulate antigens entering the liver with portal-venous blood, but also provide important regulatory functions within the liver (*7*). Moreover, it has been shown that, in the presence of IL-4 and/or glucocorticoid, macrophages can adopt an alternative activation phenotype, distinct from that induced by interferon-γ, and play an important role in downmodulating inflammation and immunity (*33*). Despite this evidence, Kupffer cells have been linked primarily to a pathological role in liver injury induced by hepatotoxins including CCl_4 (*34*), ethanol (*35*), and APAP (*14*, *16*, *17*). Using a murine model of APAP-induced acute liver injury, results from the present study indicate that Kupffer cells may also have hepato-protective functions in drug-induced liver toxicity.

The argument that Kupffer cells have a role in the mechanism of APAP hepatotoxicity was supported by findings that rats and mice were partially protected against APAP hepatotoxicity by pretreatment with gadolinium chloride (*16*, *17*). We also found that APAP-induced hepatoxicity in mice was moderately attenuated by gadolinium chloride pretreatment (Figure 3). However, it is controversial whether gadolinium chloride is effective at actually depleting Kupffer cells (*17*−*19*, *36*−*38*). It appears that rat Kupffer cells are more responsive to gadolinium chloride treatment than mouse Kupffer cells, however, even in rats it has been reported that gadolinium chloride causes a switch in Kupffer cell phenotype or has other effects (*18*, *38*). In this regard, we showed that 7 mg/kg of gadolinium chloride did not effectively deplete Kupffer cells in mice, nor did it impair phagocytosis of carbon particles in the liver, a measure of macrophage activity (Figure 4). These results suggest that pretreatment with gadolinium chloride may protect the liver from APAP hepatotoxicity by pathways independent of that involving Kupffer cell depletion. Gadolinium chloride has been shown to cause a reduction of CYP450 activity, particularly that of the 2e1 isoform in the rat (*22*, *23*). However, consistent with observations from a previous study (*17*), we found similar levels and patterns of APAP−protein adduct formation by immunoblot analysis in the livers from gadolinium chloride-

versus saline-pretreated mice (data not shown), comparable to the lack of effect of liposome/clodronate treatment on APAP−protein adduct formation in the liver (Figure 5). In other models of liver injury, it has been suggested that gadolinium chloride causes decreased IL-10 mRNA expression in rat livers (*39*) and stimulates liver regeneration by increasing TNF-α production (*20*, *21*). However, we showed that gadolinium chloride did not significantly alter levels of cytokine expression in the liver, including IL-10 and TNF-α, following APAP treatment (Figure 8). Another recent study found that, while 10 mg/kg gadolinium chloride did deplete Kupffer cells from mice, it did not protect against cadmium-induced liver damage as was observed at higher doses of gadolinium chloride (*40*). While it is clear that gadolinium chloride protects against hepatotoxicity caused by APAP and other agents, the mechanism of protection is not yet clear, and hepato-protective effects aside from depleting Kupffer cells also appear to play a role (*40*).

In contrast to gadolinium chloride, liposome/clodronate treatment has been clearly shown to deplete Kupffer cells in the liver in a number of immunological studies (*31*, *41*, *42*). We have confirmed these findings by showing that this reagent causes both the loss of F4/80 immunochemical staining (Figure 1) and the inhibition of carbon particle uptake in the liver (*24*). Moreover, liposome/clodronate treatment leads to decreased mRNA expression levels of Kupffer cell receptors and Kupffer cell-related molecules under varied time and treatment conditions (Table 1). To ensure that cytokines and soluble factors return to baseline levels while Kupffer cells remain absent, mice were dosed with APAP 2 days after liposome/clodronate pretreatment (*25*, *31*). Interestingly, we found that Kupffer cell depletion with liposome/clodronate caused exacerbated liver injury at both 8 and 24 h following APAP treatment (Figures 1 and 2 and Table 2), while liposome/clodronate treatment alone did not cause any liver damage (data not shown). These results allow us to directly link the increased susceptibility to APAP-induced liver injury with the depletion of Kupffer cells. An earlier study showed that pretreatment of mice with liposome/clodronate caused a decrease of serum ALT at 2 h after APAP dosing, but this protective effect was not reported at later times (*43*). The implication of this study, therefore, is not clear since the peak of toxicity occurs much later than 2 h following APAP treatment (*2*, *17*). Nonetheless, our data are consistent with reports that hepatic damage after partial hepatectomy was more severe in mice depleted of Kupffer cells with liposome/clodronate compared with empty liposome-treated controls (*18*, *44*). The disparate results obtained from pretreatment of animals with gadolinium chloride versus liposome/clodronate may reflect mechanistic differences of the two reagents in their effects on Kupffer cells. For example, it has been shown that liposome/clodronate nonselectively depletes ED1- or ED2-positive rat Kupffer cells (*31*), whereas gadolinium chloride eliminates only ED2-positive cells (*45*). Moreover, the discrepancy may also be related to the distinct experimental systems being evaluated. In this regard, it has been shown that Kupffer cell activation is sensitive to many factors, including sex (*46*), age (*47*), and genetic variation (*48*, *49*) of the animals, as well as the levels of endogenous molecules such as glutathione (*50*) and hormones (*51*).

One possible way Kupffer cells may protect the liver from APAP-induced hepatotoxicity is through the synthesis of hepato-protective factors. Indeed, the APAP-induced increase of IL-10 expression was significantly hindered in Kupffer cell-depleted mice for at least 24 h following APAP treatment (Figure 6, panels A and B). The major cellular source of IL-10 in cases of acute liver injury had previously been unclear as IL-10 production was linked to numerous cell types including hepatocytes, Kupffer cells, monocytes, T- and B-lymphocytes, and dendritic cells (*52*, *53*). However, abrogation of IL-10 expression in Kupffer cell-depleted mice in this study suggests that Kupffer cells play an important role in IL-10 production not only in APAP-induced liver injury, but potentially in other forms of liver injury as well. Because IL-10 is an important antiinflammatory and a key immunosuppressive cytokine (*53*, *54*) and was recently reported to be hepato-protective based upon the profoundly increased susceptibility of IL-$10^{-/-}$ mice to APAP hepatotoxicity (*2*), it is possible that Kupffer cell-derived IL-10 has a role in protecting the liver against APAP hepatotoxicity.

Nevertheless, the increased susceptibility to APAP hepatotoxicity of Kupffer cell-depleted mice may not be explained solely by the lack of IL-10 production. Comparable to wild-type mice, we found that IL-$10^{-/-}$ mice were also made more susceptible to APAP-induced liver injury by depletion of Kupffer cells (Figure 6C). This finding suggests that other regulatory cytokines or mediators, which are also released by Kupffer cells, may play a protective and possibly compensatory role against APAP-induced liver injury, particularly when IL-10 synthesis is compromised. For example, we found that IL-6 mRNA expression levels were markedly decreased in Kupffer cell-depleted wild-type mice (Figure 7) and IL-$10^{-/-}$ mice (data not shown). In line with our results, it has been shown that Kupffer cell depletion by liposome/clodronate treatment causes an inhibition of IL-6 mRNA expression induced by partial hepatectomy, lipopolysaccharides, or Listeria monocytogenes challenge in mice (*18*, *55*, *56*). Moreover, a hepato-protective role for IL-6 is supported by recent studies showing that IL-$6^{-/-}$ mice are more susceptible to liver injury, including CCl_4-induced acute liver toxicity, which has been attributed to defective regeneration and increased hepatocyte apoptosis in IL-$6^{-/-}$ mice (*57*, *58*). Data from previous studies using COX-2 deficient mice suggested that COX-derived prostaglandins, including prostaglandin E2, have an important hepato-protective role against APAP-induced acute liver injury (*3*). In vitro studies have shown that prostaglandin E2 is released from Kupffer cells (*5*, *59*), and the expression of inducible COX-2 has been detected in isolated Kupffer cells in various models of liver injury (*5*, *60*). However, we found that Kupffer cell depletion by liposome/clodronate led to significantly increased levels of COX-2 mRNA expression in the liver at 24 h following APAP treatment, in contrast to the decreased levels of COX-1 mRNA expression during 24 h post-APAP treatment (data not shown). These results suggest that although Kupffer cells contribute to prostaglandin production in normal physiological states of the liver, other cells, including hepatocytes (*61*), bile duct epithelial cells (*61*), endothelial cells (*62*), and stellate cells (*63*), may also be an important source of prostaglandins during liver injuries.

Our results also suggested additional Kupffer cell-derived factors, including IL-18BP and C1q, which might partially account for the increased susceptibility to APAP-induced liver injury of the Kupffer cell-depleted mice (Table 1 and Figure 7). IL-18BP is a naturally occurring protein that binds to IL-18 with high specificity and affinity and blocks its biological activity (*64*). It has been shown that exogenous IL-18BP blocks lipopolysaccharides- and concanavalin A-induced liver injury (*65*). Evidence also suggests that C1q may be an important factor in antiinflammatory events due to its role in processing immune complexes and in clearance of apoptotic cells (*66*, *67*). Our finding of significantly decreased IL-18BP and C1q mRNA expression in Kupffer cell-depleted mice following APAP treatment suggests that Kupffer cell-derived IL-18BP and C1q may have a protective role in APAP-induced liver injury, although further study is required.

Using a method by which Kupffer cells were unequivocally depleted in mice, the present study provides the first direct evidence that Kupffer cells may have a protective role in addition to its protoxicant role in drug-induced acute liver injury. Our findings suggest that the hepatoprotective function of Kupffer cells is likely mediated by several cytokines and other mediators that are important in counteracting inflammatory responses and/or stimulating liver regeneration. Because Kupffer cells also appear to have a role in the immunological tolerogenicity of the liver (*7*, *11*, *33*), they may be important in protecting the liver against both acute and chronic drug-induced injury caused by immune- and nonimmune-mediated mechanisms. Our findings also suggest that endogenous and exogenous factors, including drugs (*68*, *69*), alcohol (*70*), aflatoxins (*71*), anti-oxidant levels (*72*), and genetic polymorphisms (*73*), which modulate Kupffer cell activities may affect the risk of individuals in developing liver injuries.

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TX0255976