Acute Hepatotoxic Exposure Induces TNFR-Mediated Hepatic Injury and Cytokine/Apoptotic Gene Expression

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Tumor necrosis factor receptor knockout (TNFR KO) mice were used to examine the role of tumor necrosis factor-α (TNFα) signaling during acute hepatotoxicant exposure. Mice were exposed intraperitoneally (ip) to either vehicle, phosphate-buffered saline (PBS), or dimethylnitrosamine (DMN, 100 mg/kg) for 24 h. Histological evaluation showed that DMN-treated TNFR-2 KO mice had increased liver damage compared to wild type (WT), TNFR-1 KO, or TNFR double KO (DKO) mice. Also, 3 of 8 TNFR-2 KO mice died following DMN treatment, suggesting that hepatic TNFR-2 signaling produces protective responses that counteract TNFR-1-mediated damage. DMN-induced cellular infiltration was absent in TNFR-1-deficient mice, indicating that infiltrating cells do not exacerbate acute hepatotoxic events. In separate experiments, mice were exposed ip to either DMN (5.0 or 100 mg/kg), carbon tetrachloride (CCl4, 0.3 or 1.0 ml/kg), or corresponding PBS/corn oil controls for 6 or 24 h to compare the hepatic mRNA expression of cytokine- and apoptotic-associated genes. Following 24 h of DMN (100 mg/kg) or 6–24 h of CCl4 treatment, hepatic transcripts for TNFα, interferon (IFN)-γ, IL (interleukin)-1RI, and transforming growth factor (TGF)-BRII were induced. Hepatotoxicant-treated WT and TNFR-2 KO mice induced hepatic transcripts for the pro- and anti-apoptotic genes, Bax and Bcl-XL, respectively, indicating TNF-independent gene activation. The anti-apoptotic gene, Bfl-1, was highly expressed in CCl4-treated, TNFR-positive strains, but minimally expressed in TNFR DKO mice, suggesting that hepatic Bfl-1 is TNF-regulated. Taken together, these data show that acute hepatotoxicant exposure is followed by upregulation of liver cytokine, cytokine receptor, and apoptotic transcripts, and that TNFα regulates various aspects of liver inflammation and injury in a TNFR-specific fashion.

Key Words: apoptosis; Bfl-1; carbon tetrachloride (CCl4); cytokines; dimethylnitrosamine (DMN); hepatotoxicity; inflammation; necrosis; TNF.

Tumor necrosis factor (TNF) α is a pleiotropic cytokine critically involved in inflammation and immunity. TNFα, in conjunction with interleukin (IL)-6, regulates the acute-phase response, adhesion molecule activation, and antioxidant gene expression. TNFα is perhaps the most critical and powerful mediator of inflammation, cellular injury, cell death/apoptosis, and wound healing (reviewed in Edwards et al., 1994).

There appears to be a critical role for TNFα in various models of hepatotoxicity. For example, carbon tetrachloride (CCl4)-induced hepatotoxicity is blocked by the administration of soluble TNFα receptors (TNFRs) (Czajka et al., 1995). Liver repair following CCl4 is mediated by TNFα (Bruccoleri et al., 1997; Yamada and Fausto, 1998). In cadmium-induced hepatotoxicity, pretreatment with anti-TNFα antibodies prevented focal inflammation as well as secretion of the acute-phase reactant, serum amyloid A (Kayama et al., 1995), indicating that these processes are cytokine-dependent. Similarly, neutralizing antibodies to TNFα delayed increases in serum levels of IL-1α and liver enzymes as well as shortened the recovery time following acetaminophen treatment (Blazka et al. 1995; 1996).

Acute inflammatory responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin were mimicked by the administration of exogenous IL-1β and TNFα (Moos et al., 1994). Taken together, these data suggest that TNFα contributes to pathological manifestations of chemical-induced liver damage. However, the mechanism by which TNFα controls hepatotoxicity remains undefined.

There are 2 cell surface receptors for TNFα of approximately 55 kDa (TNFR-1) and 75 kDa (TNFR-2), each with distinct functions (Tartaglia and Goeddel, 1992). TNFR-1 and TNFR-2 lack significant homology in the intracellular domains, further suggesting that they are involved in differential signaling pathways. For example, TNFR-1 is responsible for the upregulation of c-fos, IL-6 and manganese superoxide dismutase mRNA, prostaglandin E2 synthesis, surface expression of IL-2 receptor and MHC class I and II molecules, growth inhibition and cell death (Brakebusch et al., 1992; Engelmann et al., 1990; Espevik et al., 1990; Hohmann et al., 1990; Kruppa et al., 1992; Naume et al., 1991; Shalaby et al., 1990; Tartaglia et al., 1991; Thoma et al., 1990). TNFR-2 signaling seems to be restricted to T-cell proliferation (Grell et al., 1998; Tartaglia et al., 1991); however, recent evidence...
suggests that TNFR-2 mediates migration of Langerhans’ cells (Wang et al., 1996; 1997).

Dimethylnitrosamine (N-nitrosodimethylamine, DMN) is a member of the family of N-nitroso compounds that has potent hepatotoxicant activity in mammals (Cradock, 1983) and is a suspected carcinogen in humans. Lifetime exposure to nitrosamines occurs via dietary sources (cheeses, and cured meats and fish), alcoholic beverages, and cigarette smoke, as well as in industrial situations. The primary site for DMN metabolism and toxicity is the liver. DMN bioactivation is thought to occur either through the liver mixed-function oxidases (cytochrome P450 2E1) or through hepatic monoamine oxidase (reviewed in Myers and Schook, 1996). The end result is the formation of toxic intermediates such as hydroxyl radicals, reactive oxygen intermediates, and formaldehyde, with the consequence being lipid peroxidation and/or DNA alkylation (Montesano and Bartsch, 1976). Hepatic centrilobular necrosis has been observed in both mice and humans exposed to DMN.

Molecular mechanisms accounting for DMN-induced hepatotoxicity have been studied. Previously, we have demonstrated that exposure to DMN in vivo induces an acute-phase response for serum amyloids A and P and albumin (Lockwood et al., 1994). DMN exposure also results in a transient increase in serum cytokine levels for IL-1α, TNFα, and IL-6 (Schook et al., 1994). These data show that DMN exposure is associated with hepatic proinflammatory gene expression. However, previous studies may have suffered from incomplete neutralization of TNFα activity, especially membrane-associated TNFα. Further, there has been no distinction between activities mediated by TNFR-1 versus TNFR-2.

To determine the signaling mechanism for TNFα during acute liver injury, strains of TNFR knockout (KO) mice, lacking either TNFR-1, TNFR-2, or both receptors were exposed to vehicle (phosphate-buffered saline [PBS]) or DMN (100 mg/kg) for 24 h. Hepatic histopathological and cellular changes during DMN exposure were examined using TNFR KO mice compared against each TNFR KO strain as well as to TNFR intact wild type (WT) mice. In addition, multi-probe RNase protection assays (RPAs) were used to examine the hepatic cytokine, cytokine receptor and apoptotic gene expression patterns following low- and high-dose acute DMN exposure in WT and TNFR KO mice. Because both DMN and CCl4 are bioactivated to reactive intermediates by cytochrome P450 2E1 (Guengerich, 1987, 1991; Haggerty and Holsapple, 1990; Koop, 1992) and both cause hepatic centrilobular necrosis (Butler and Hard, 1971; Rechnagel and Glende, 1973; Reynolds and Ree, 1971), WT and TNFR KO mice were injected with either DMN or CCl4 in order to further investigate the molecular events associated with TNFα during different xenobiotic exposure regimens. Results suggest that, overall, similar mechanisms exist and TNFα shares a common role during different xenobiotic exposure regimens.

**MATERIALS AND METHODS**

**Mice and dosing regimens.** Male and female WT (C57BL/6) mice (Harlan Sprague Dawley, Indianapolis, IN) and male and female TNFR KO mice (a gift from Immunix Corp., Seattle, WA) were housed in cages on a 12-h light/dark cycle. TNFR-1 KO (p55–/– p75–/–) mice have a C57BL6 genetic background while TNFR-2 KO (p55–/– p75–/–) and TNFR double KO [DKO (p55–/– p75–/–)] mice have a C57BL6 × 129 background (Peschon et al., 1998). DMN (Sigma Chemical Co., St. Louis, MO) was diluted from a 10 mg/kg stock solution in PBS (pH 7) and stored at 4°C for less than 3 weeks. Solutions were tested for endotoxin by the limulus amebocyte assay (Associates of Cape Cod, Woods Hole, MA) prior to use. All mice (20 g) were between 6–8 weeks old and were rested 1 week prior to dosing. Mice received 0.2 ml of either vehicle (PBS) or DMN (at a final dose of 5.0 or 100 mg/kg) given as a single daily injection intraperitoneally (ip) between 8–10 am. CCl4 (Sigma) was diluted 0.3:10 or 1:10 in corn oil and mice were ip injected with 0.2 ml of either corn oil or CCl4, (0.3 or 1.0 ml/kg, respectively). All mice were sacrificed 6 or 24 h post-injection and livers removed. Endotoxin [lipopolysaccharide (LPS), 50 μg E. coli 055:B5, Difco Laboratories, Detroit, MI] diluted in PBS was administered ip for 6 h. All animal procedures followed standard protocols approved by the University of Minnesota Animal Welfare Committee.

**Histopathology.** A portion of the liver was fixed in 4% buffered paraformaldehyde for 18–24 h at 4°C and paraffin embedded. Serial sections (4 μm) were mounted on poly-L-lysine-coated slides, deparaffinized and stained with hematoxylin and eosin for separate analyses of histopathology and cellular infiltration (below).

**Histopathological and cellular infiltration analyses.** Blinded liver samples were submitted for histological evaluation and at least 2 sections from each liver were examined. Histopathological evaluation for necrosis included, but was not limited to, assessing the distribution and extent of cell death (hepatocytes and non-parenchymal cells). Lesions were graded on a semiquantitative scale: 0, normal; 1, rare foci of necrotic cells in centrilobular zones (no more than 1–2 sites per section); 2, few necrotic foci (less than half of centrilobular zones had sites of necrosis); 3, many/diffuse centrilobular zones with necrosis (confined to centrilobular zone only); 4, diffuse centrilobular to midzonal necrosis; and 5, diffuse submassive to massive necrosis (most or almost all of lobule was necrotic).

The extent of inflammatory cellular infiltration into the liver was examined using the same liver samples as for histopathology. Cellular infiltration was graded visually on a semiquantitative scale: 0, no influx; 1, rare inflammatory cells (no more than 1–3 cells in 1 or 2 centrilobular zones per section); 2, few inflammatory cells (1–5 cells in less than 50% of centrilobular zones); 3, moderate inflammatory cell infiltration (5–15 cells in most centrilobular zones); 4, marked inflammatory cell infiltration (greater than 15 cells in most centrilobular to midzonal areas); and 5, severe inflammatory cell infiltration (too numerous to count and infiltrating most of lobule).

**RNA extraction.** Liver sections (≤200 mg) were quickly thawed and homogenized in 2 ml of TRIZOL (Life Technologies, Grand Island, NY) and total RNA was isolated according to the manufacturer’s directions. RNA was resuspended in RNase-free water, quantitated using UV spectrophotometry, and stored at −80°C.

**Multi-probe RPAs.** The DNA templates (mCK3, mCR4, mAPO2, and mAPO3), and In Vitro Transcription and RPA kits were purchased from PharMingen (San Diego, CA). Briefly, RNA probes were generated using 1 μg of template DNA and in vitro transcribed using T7 RNA polymerase. Yeast tRNA (10 μg) and PharMingen-supplied mouse control RNA (2 μg) were used as negative and positive controls, respectively. Total liver RNA (10–20 μg) was dried and resuspended in 2.4–4 × 105 cpn/μg and hybridized at 56°C for 16–18 h. Following hybridization, RNA samples were RNase A/T1-digested for 45 min at 30°C, followed by incubation with proteinase K for 15 min at 37°C and then ethanol precipitated. Protected RNAs were separated on a 6% polyacrylamide/7 M urea denaturing gel. Following electrophoresis, the gels were dried and exposed to X-ray film or phosphorimager screens and bands
were quantitated using densitometry or phosphorimager, respectively (Molecular Dynamics, Sunnyvale, CA).

**Statistical analyses.** All necrosis and cellular infiltration experiments were performed with at least 5 mice and the results are represented as an average ± SE for each mouse strain within each dosing group. The results were analyzed using the Kruskal-Wallis H test, a nonparametric equivalent to a 1-way analysis of variance. A 95% confidence level was used to define statistical significance. The hepatic gene expression data shown is from one group of mice and results are representative for 2 independent, identically treated groups (n = 2).

**RESULTS**

**Histological Analyses Following Acute Exposure to DMN**

Previous studies using various hepatotoxicants such as cadmium (Kayama et al., 1995) and acetaminophen (Blazka et al., 1995; 1996) have focused on exposure to large acute doses in which TNFα has been shown to play a role in hepatotoxicity. Therefore, WT and TNFR KO mice received either an acute vehicle (PBS) or DMN (100 mg/kg) dose ip and were sacrificed 24 h post-exposure. This DMN dose has been previously shown to produce both liver necrosis and hepatic DNA fragmentation (Ray et al., 1992).

All vehicle-treated mice had normal liver histology irrespective of TNFR genotype (Fig. 1). Centrilobular and midzonal necrosis was evident in all WT and TNFR KO mice exposed to DMN. Hepatic cords in affected areas were often disrupted and exhibited individualization of hepatocytes. Hepatocytes in affected centrilobular zones were characterized by clumped chromatin, karyorrhexis, or nuclear pyknosis, and by hypereosinophilic cytoplasm. In more severely affected areas, there was loss of hepatocytes with centrilobular to midzonal hemorrhage (Fig. 1).

**Necrosis and Cellular Infiltration following DMN Exposure**

To further characterize the observed changes following an acute dose of DMN (100 mg/kg), liver lesions for each treatment group were evaluated using a semiquantitative scale, as described in the Materials and Methods. However, since we observed that WT mice exposed to DMN contained inflammatory cell foci, which was reduced up to 50% in the TNFR KO strains (data not shown), it was necessary to develop a scoring procedure to separately analyze the degree of liver necrosis independently from hepatic cellular infiltration.

All vehicle-treated WT and TNFR KO mice had necrosis and cellular infiltration scores of 0 (data not shown). Necrosis and cellular infiltration scores for mice exposed to DMN (100 mg/kg) are shown in Figure 2. No significant differences in necrosis were observed between WT, TNFR-1 KO, and TNFR DKO mice exposed to DMN. However, TNFR-2 KO mice had significantly (p < 0.05) higher necrosis scores (3.4 ± 0.2) compared to WT (2.6 ± 0.2), TNFR-1 KO (2.4 ± 0.2) and TNFR DKO (2.9 ± 0.1) mice (Fig. 2A). In addition, 37.5% (3/8) of TNFR-2 KO mice succumbed to DMN-induced death prior to tissue harvest at 24 h post-exposure (dead TNFR-2 KO mice were not included in the scoring procedure). We also observed an almost complete absence of inflammatory cells in TNFR-1 KO and TNFR DKO mice (Fig. 2B), indicating that TNFR-1 mediates DMN-induced cellular influx into the liver. These infiltrating cells were observed to be mainly neutrophils, macrophages, and T cells (data not shown).

**Hepatotoxicant-Induced Cytokine and Cytokine Receptor mRNA Expression in WT Mice**

Because TNFR signals appear to differentially regulate inflammatory-cell influx during DMN exposure, we determined hepatic proinflammatory cytokine gene expression in the same livers as above. Multi-probe RPA mCK3 and mCR4 template DNAs were used to quantitate hepatic cytokine and cytokine receptor mRNA expression, respectively following acute hepatotoxicant exposure. In addition, to compare hepatic gene expression for different hepatotoxicants, mice were acutely exposed to either DMN (5.0 or 100 mg/kg) or CCl4 (0.3 or 1.0 ml/kg) for up to 24 h. For comparison, LPS (50 μg) was administered to stimulate hepatic cytokine/cytokine receptor gene expression.

The proinflammatory cytokines TNFα, lymphotoxin (LT)β, TNFα, IL-6, interferon (IFN)-γ, IFN-β, transforming growth factor (TGF)-β1 and TGFβ2 were analyzed by RPA (Fig. 3). As expected, LPS increased hepatic transcripts for TNFα and IL-6. At 6-h post-exposure, vehicle-treated animals showed enhanced transcripts for TNFβ and TNFα compared to naive and DMN (5.0 mg/kg)-treated mice, and increased IFN-γ transcripts as compared to DMN-treated mice. Increased cytokine expression observed following 6 h of vehicle treatment was consistent with a transient increase in inflammatory gene expression that subsides by 24 h (Lockwood et al., 1991; 1994; Schook et al., 1992). Following 24 h of high-dose DMN (100 mg/kg), hepatic expression of TNFβ, LTβ, TNFα, and IFN-γ was induced 3- to 10-fold compared to vehicle controls, with little change in IL-6, IFN-β, TGFβ1, or TGFβ2 (Fig. 3). However, compared to naive mice, only transcripts for TNFα were greatly increased following DMN (100 mg/kg).

In contrast to what we observed for 6-h DMN (5.0 mg/kg), hepatic transcripts for TNFβ, TNFα, IFN-γ, and TGFβ1 were higher following 6-h CCl4 (0.3 or 1.0 ml/kg) as compared to corn oil controls, whereas LTβ was induced only following 6-h CCl4 (1.0 ml/kg) (Fig. 4). Hepatic cytokine expression following CCl4 exposure showed similar results as compared to 24-h DMN (100 mg/kg). Following 24-h CCl4 (0.3 or 1.0 ml/kg), hepatic cytokine mRNA expression for TNFα, IFN-γ, LTβ, and TGFβ1 was still upregulated compared to corn oil controls. In addition, however, we also observed that hepatic TGFβ2 transcripts were induced following 24-h CCl4 (0.3 or 1.0 ml/kg) compared to 6-h CCl4 treatment and corn oil controls. In contrast to DMN exposure, CCl4-treated mice had upregulated TGFβ1 and TGFβ2 transcript levels, demonstrat-
ing that these hepatotoxicants differentially regulate inflammation.

Importantly, we observed differences in hepatic cytokine expression following PBS and corn oil treatments (Figs. 3 and 4). Whereas PBS induced a transient increase in hepatic transcripts for TNFα and TNFβ (compared to naive), corn oil exposure had little effect on these cytokines. Compared to naive mice, 24-h corn oil treatment suppressed transcripts for TNFβ, LTβ, and TGFβ1, similar to what was observed for 24-h PBS exposure. These data further demonstrate that vehicle effects must be considered during interpretation of molecular data and that both naive and vehicle controls must be considered.

Because cytokines must signal through specific receptors in order to elicit biological effects, proinflammatory cytokine receptor transcript levels were analyzed (Fig. 5). Differences in hepatic cytokine receptor expression in WT mice were not observed between vehicle and DMN (5.0 mg/kg) following a 6-h exposure. In contrast, a high-dose 24-h DMN (100-mg/kg) exposure induced hepatic expression of IL-1RI and IL-1RII, but suppressed gp130 transcripts compared to both naive and vehicle controls (Fig. 5A). IL-6Rα transcripts were not significantly altered during any vehicle or DMN exposure. In contrast to DMN, CCl4 (0.3 or 1.0 ml/kg) was a potent inducer of hepatic cytokine receptor expression in WT mice. Mice treated with CCl4 (0.3 or 1.0 ml/kg) showed upregulated transcripts for IL-1RI, TNFR-2, TNFR-1, IL-6Rα, gp130, TGFβRI and TGFβRII at both 6 and 24 h. Interestingly, and in contrast to DMN, CCl4 did not alter expression of IL-1RII (Fig. 5B). These data further confirm differential effects of cytokine production and signaling in the liver for these 2 hepatotoxicants.

Apoptotic Liver mRNA Expression following Hepatotoxicant Exposure in WT and TNFR KO Mice

High-dose DMN (100-mg/kg) exposure initiates apoptosis in the liver (Ray et al., 1992). A link between TNFα and apoptosis within the liver has been established (Leist et al., 1995; 1997; Rolfe et al., 1997; Takehara et al., 1998), and apoptosis is one proposed contributor to xenobiotic-induced liver injury. Thus, the regulation of apoptotic promoting genes, Bcl-W, Bak, Bax, and Bad, and anti-apoptotic genes, Bcl-XL, Bcl-2, and Bak were measured by RPA. Data showed that CCl4 exposure upregulates Bcl-W expression in both WT and TNFR KO mice compared to naive mice (Fig. 5A).

Comparing vehicle and naive controls, exposure of WT mice to DMN (5.0 mg/kg) for 6 h did not alter hepatic Bcl-W transcripts. In contrast, TNFR DKO mice exposed to DMN (5.0 mg/kg) exhibited a 2.5-fold increase in Bcl-W expression compared to vehicle controls (Fig. 6A). Exposure to high-dose DMN (100 mg/kg) for 24 h increased (1.5- to 1.7-fold) hepatic Bcl-W expression in both WT and TNFR DKO mice compared to vehicle controls.

Because we observed differences in inflammatory cytokine and receptor gene expression for DMN and CCl4 treatment, we examined whether these would impact apoptotic gene expression. Exposure of WT mice to CCl4 (0.3 and 1.0 ml/kg) did not induce hepatic Fas expression (Fig. 6B). Hepatic Fas was decreased (1.9- to 3.5-fold) in TNFR DKO mice exposed to CCl4, both 6 and 24 h (Fig. 6B). The fact that Fas was expressed in the liver in both WT and TNFR DKO mice suggests that TNFα is not required for hepatic Fas expression, but may regulate Fas mRNA levels.

Hepatic expression of the Bcl-2 family of genes was measured in WT and TNFR DKO mice exposed to DMN (5.0 or 100 mg/kg) for 6 or 24 h, respectively (Table 1). We observed that naive TNFR DKO mice had 41% fewer relative Bcl-XL transcripts. Hepatic Bcl-XL and Bax expression were increased following low- and high-dose DMN in both WT and TNFR DKO mice, whereas a down-regulated expression (37–68%) of Bcl-W was observed at 24 h in both strains of mice. Only minor differences between vehicle and DMN in WT or TNFR DKO were observed for Bfl-1 and Bak (Table 1). Hepatic Bcl-2 was not detected following vehicle or DMN exposure in either strain (data not shown).

Hepatic expression of the Bcl-2 family of genes was also measured in WT and TNFR DKO mice exposed to CCl4 (Table 2). Similar to DMN exposure, hepatic mRNA expression of Bcl-XL and Bax was induced for all CCl4 exposures in both WT and TNFR DKO mice compared to corn oil controls. In contrast to DMN, WT and TNFR DKO mice exposed to CCl4 (0.3 or 1.0 ml/kg) showed increased (1.7- to 3.8-fold) liver expression of Bcl-W at 24 h compared to corn oil controls. Also, whereas DMN exposure did not alter hepatic Bfl-1 transcripts in either mouse strain, we observed significant levels of Bfl-1 transcripts following 24 h CCl4 exposure in WT, but not TNFR DKO mice (Table 2). This again demonstrates intricate differences for these two hepatotoxicants, and suggests that hepatic Bfl-1 expression may be TNF-mediated in certain conditions.

To further characterize how TNFα regulates hepatic Bfl-1 gene expression, WT, TNFR-1 KO, TNFR-2 KO and TNFR DKO mice were exposed to corn oil or CCl4 (0.3 or 1.0 ml/kg) for 6 or 24 h and hepatic Bfl-1 transcripts were then quantitated by RPA. Data showed that CCl4 exposure upregulates liver Bfl-1 in WT, TNFR-1 KO and TNFR-2 KO mice, with little alteration in TNFR DKO mice (Fig. 7). This suggests that hepatic Bfl-1 is TNF-mediated during CCl4 exposure, but that either TNFR can mediate its upregulation during acute CCl4 exposure.

DISCUSSION

The inflammatory response associated with chemical-induced hepatotoxicity, including DMN, is characterized by hepatic proinflammatory cytokine and acute-phase reactant gene expression (Bhattacharjee et al., 1998; Horn et al., 1998;
Lockwood et al., 1994; Schook et al., 1992). Aberrant TNFα production is associated with DMN exposure and is consistent with reports demonstrating a role for TNFα in DMN hepatotoxicity (Schook et al., 1992). These observations have led to the hypothesis that toxic intermediates, produced both as a result of xenobiotic metabolism and the inflammatory response, contribute to the hepatic centrilobular necrosis that occurs following toxicant exposure. While inflammation and hepatic TNFα are thought to be part of the healing process (Akerman et al., 1992; Bruccoleri et al., 1997; Kimura et al., 1997), an uncontrolled inflammatory response may be detrimental to the host. Accumulation of liver immunocytes (neutrophils, monocytes/macrophages, and/or T cells) may lead to additional hepatocyte death through production and release of reactive oxygen intermediates that can damage liver architecture. Conversely, increased immunocyte influx may also be needed to clear dead or dying hepatocytes in order for liver regeneration to occur.

In order to understand the cellular and molecular effects of TNFα in the liver during acute chemical-induced hepatotoxicity, WT, TNFR-1 KO, TNFR-2 KO and TNFR DKO mice were exposed to high-dose DMN (100 mg/kg). Moreover, acute exposure to another hepatotoxicant, CCl4, was compared at the level of hepatic gene expression. In order to focus on apoptotic genes with minimal necrosis, a dose of 0.3 ml/kg CCl4 was given for 6 h as previously discussed (Shi et al., 1998). Similarly, a low-dose exposure to DMN (5.0 mg/kg) (Bhattacharjee et al., 1998; Lockwood et al., 1994; Myers and Schook, 1996) was used because necrosis is not detected following 6 h of treatment (manuscript in preparation). A 24 h exposure to CCl4 (1.0 ml/kg) was used to induce hepatic necrosis (Yamada and Fausto, 1998) with levels comparable to our high-dose DMN (100 mg/kg) exposure regimen (T. D. O’Brien, unpublished results). Hence, we were able to compare both low- and high-dose acute exposures for these xenobiotics to reveal subtle differences in hepatic gene regulation and the role of TNFα.

Previous reports have used antibodies or soluble receptors directed against TNFα in order to neutralize its effect (Blazka et al., 1995; 1996; Bruccoleri et al., 1997; Czaja et al., 1995; Kayama et al., 1995), but this approach can be problematic because the neutralization may not be 100%, particularly during chronic exposure regimens. Further, the reagents may not neutralize membrane bound TNFα. In addition, because each TNFα receptor has structurally dissimilar intracellular domains (Tartaglia and Goeddel, 1992), differential receptor signaling may regulate separate hepatotoxic or repair events. Indeed, TNF-1 appears to be required for the rapid influx of immunocytes into the liver during high-dose, acute DMN exposure. We have used this model to show that immunocyte influx was not required for liver pathology to occur during acute DMN exposures. Our results further suggested that TNFα-1 signals may be detrimental in the liver unless TNFR-2 signals also occur (Fig. 2). Thus, genetic TNFR KO mice permit the study of TNFα effects with respect to individual receptor signaling events.

Acute exposure to DMN or CCl4 induced a proinflammatory response accompanied by induction of hepatic TNFα and IFN-γ (Figs. 4 and 5). Thus, the liver is a potential source for TNFα detected in the serum following low-dose, multiple exposures to DMN (Schook et al., 1992). In addition, CCl4 rapidly upregulated hepatic expression of TNFR-1 and TNFR-2, whereas the effect of DMN on these transcripts was indistinguishable from vehicle controls (Fig. 5). Our data further substantiates that TNFα is a key cytokine involved in

FIG. 2. Necrosis and cellular infiltration scores following acute DMN exposure. WT and TNFR KO mice received an acute DMN (100-mg/kg) exposure ip and were sacrificed 24 h post-exposure. Livers were removed, processed for paraffin embedding, and liver sections graded using a semiquantitative scale as described in Materials and Methods. The individual necrosis (A) and cellular infiltration (B) scores from each mouse strain were determined (average ± SE). 0 = score of 0 for that mouse strain. All vehicle-treated animals had scores of 0 (data not shown). A minimum of 5 animals per group was used. (Significant difference [p < 0.05]: +, compared to WT; triangle, compared to TNFR-1 KO; #, compared to TNFR DKO).

FIG. 1. Hepatic histopathology following acute DMN exposure in vivo. WT and TNFR KO mice received an acute ip injection of either vehicle (PBS) or DMN (100 mg/kg) and were sacrificed 24 h post-injection. Livers were removed and processed for hematoxylin and eosin staining as described in Materials and Methods. Liver photomicrographs were from one group of mice exposed to either vehicle or DMN and are representative of 5 mice for each exposure group. Note disruption of hepatic cord architecture along with hepatocellular necrosis (black arrows) in all DMN-treated mice. Prominent hemorrhage was also visible in the sections from WT, TNFR-1 KO and TNFR-2 KO mice. Inflammatory cells are apparent in the DMN-treated WT and TNFR-2 KO liver sections (white arrows). Original magnification × 82.5; hepatic central vein, C.
DMN and CCl\textsubscript{4} hepatotoxicity. However, our results also showed that there are subtle, yet critical differences in the hepatic gene expression for these diverse xenobiotics despite a common bioprocessing pathway. This may result from the

FIG. 3. Hepatic proinflammatory cytokine expression following acute DMN exposure. WT mice were exposed to either vehicle (PBS) or DMN for 6 or 24 h. Liver RNA (20 µg) was isolated and used for RPA with mCK3 template DNA (PharMingen). Results shown are representative for 2 independent identically-treated groups. (A) All RNA samples were run on the same gel. However, the gel was exposed to X-ray film for 3 days for high-copy mRNAs (L32, GAPDH, diluted probes and positive control) or 10 days to detect low-copy transcripts (hepatic cytokines). DP, diluted undigested RPA probes (ladders); Y, yeast tRNA (negative control); P, PharMingen control RNA (2 µg); N, naive; L, LPS (50-µg dose for 6 h); V, vehicle; D, DMN (subscript numbers represent the dose in mg/kg). (B) Relative cytokine mRNA abundance was determined using densitometry. Results were normalized to the housekeeping gene, GAPDH, and were determined by dividing the cytokine densitometry value by the GAPDH densitometry value and multiplying by 100.

FIG. 4. Hepatic proinflammatory cytokine expression following acute CCl\textsubscript{4} exposure. WT mice were exposed to either corn oil or CCl\textsubscript{4}, for 6 or 24 h. Liver RNA (20 µg) was isolated and used for RPA with mCK3 template DNA (PharMingen). Results shown are representative for 2 independent, identically treated groups. (A) All RNA samples were run on the same gel. However, the gel was exposed to X-ray film for 1 day for high-copy mRNAs (L32, GAPDH, diluted probes and positive control) or 10 days to detect low-copy transcripts (hepatic cytokines). Abbreviations: DP, diluted undigested RPA probes (ladders); Y, yeast tRNA (negative control); P, PharMingen control RNA (2 µg); N, naive; L, LPS (50-µg dose for 6 h); O, corn oil; C, CCl\textsubscript{4} (subscript numbers represent the dose in ml/kg). (B) Relative cytokine mRNA abundance was determined using phosphorimaging. Results were normalized to the housekeeping gene, GAPDH, and were determined by dividing the cytokine phosphorimaging value by the GAPDH phosphorimaging value and multiplying by 100.

DMN and CCl\textsubscript{4} hepatotoxicity. However, our results also showed that there are subtle, yet critical differences in the hepatic gene expression for these diverse xenobiotics despite a common bioprocessing pathway. This may result from the
different bioreactive intermediates that are produced from each compound. Our histological data show that TNFR-1-deficient mice exposed to high-dose DMN lack hepatic cellular infiltration, yet show no reduction in necrosis compared to DMN-treated WT mice (Fig. 2). However, a previous report observed decreases in both CCl₄-mediated hepatotoxicity and inflammation following pretreatment with soluble TNFR (Czaja et al., 1995). These differences could be related to the high dose (5.0 ml/kg) of CCl₄ used in that study, as was previously suggested (Bruccoleri et al., 1997; Louis et al., 1998). This high dose of CCl₄ could result in extensive hepatic necrosis and TNFα production. In addition, important differences in the metabolites generated from CCl₄ and DMN may also influence subsequent TNFα effects. We have also observed differences in TNFα dependence for DMN-mediated hepatotoxicity in acute high-dose DMN (100 mg/kg) versus subacute low-dose DMN (5.0 mg/kg) exposure regimens (manuscript in preparation). Finally, whereas genetic knockouts completely abrogate TNFα signals, it cannot be excluded that soluble TNFα receptors did not completely neutralize TNFα signals; small amounts of TNFα may signal protective responses that are masked in the presence of elevated TNFα signaling. Although we cannot as yet link Bfl-1 expression to hepatotoxicity, it is interesting that CCl₄ differed from DMN by upregulating TNFR-1, TNFR-2 and Bfl-1 transcripts, the latter in a TNFR-dependent manner. Thus, our data show differences exist between hepatic cyto-

**FIG. 5.** Hepatic proinflammatory cytokine receptor expression following acute hepatotoxicant exposure. WT mice were exposed to either (A) vehicle (PBS) or DMN or (B) corn oil or CCl₄ for 6 or 24 h. Liver RNA (10 μg) was isolated and used for RPA with mCR4 template DNA (PharMingen). Results are representative of 1 of 2 independent groups. Relative cytokine mRNA abundance was determined using (A) densitometry or (B) phosphorimagery. Results were normalized to the housekeeping gene, GAPDH, and were determined by dividing the cytokine receptor value by the GAPDH value and multiplying by 100.

**FIG. 6.** Hepatic Fas mRNA expression following acute hepatotoxicant exposure. WT and TNFR DKO mice were exposed to either (A) vehicle (PBS) or DMN (5.0 or 100 mg/kg) or (B) corn oil or CCl₄ (0.3 or 1.0 ml/kg) for 6 or 24 h. Liver RNA (10 μg) was isolated and used for RPA with mAPO3 template DNA (only Fas is shown). Hepatic Fas expression was normalized to GAPDH by dividing the Fas densitometry/phosphorimagery value by the GAPDH densitometry/phosphorimagery value and multiplying by 100. Representative results from one of two experiments are shown. Abbreviations: N, naive; L, LPS (50 μg dose for 6 h); V, vehicle; D₅ or D₁₀₀, DMN (5.0 or 100 mg/kg, respectively); O, corn oil; C₀.₃ or C₁₀ (CCl₄, 0.3 or 1.0 ml/kg, respectively).
kine, cytokine receptor, and apoptotic genes following acute DMN and CCl₄ exposures, and that the role of TNF-α may be more critical for mediating specific pathologic and molecular outcomes in each system.

TNF-α has been shown to down-regulate P450 activities by decreasing their expression (Ghezzi et al., 1986; Renton and Armstrong, 1994; Warren et al., 1999). Thus, the use of TNFR DKO mice may be problematic because the lack of TNF-α signaling could lead to uncontrolled P450-mediated bioactivation of DMN or CCl₄ leading to increased hepatotoxicity. However, Boess et al. (1998) have shown that P450 2E1 activity, which is the principle enzyme responsible for the conversion of acetaminophen to its hepatotoxic metabolite (Lee et al., 1996; Thomsen et al., 1995), was not altered following acute exposure to acetaminophen (400 mg/kg) in TNF-α/LT-α double-deficient mice. This would suggest that the lack of TNF signaling does not alter the metabolic machinery during DMN exposure. Our results support this hypothesis, because TNFR DKO mice did not show increased hepatotoxicity following a 24-h DMN (100 mg/kg) exposure compared to WT, TNFR-1 KO and TNFR-2 KO (Fig. 2A).

Following TNF-α administration, vascular cell adhesion molecule-1 and E-selectin up-regulation does not occur in TNFR-1 KO mice, as compared to WT mice (Neumann et al., 1999). TNF-α challenge caused mononuclear and neutrophil infiltration in the liver, lungs, and kidneys in WT mice, but not in TNFR-1 KO mice, suggesting that the TNFR-1 is responsible for mediating leukocyte infiltration in vivo. In agreement with this, during an acute DMN (100 mg/kg) exposure, cellular infiltration was almost completely absent in TNFR-1 KO mice, and was completely absent in TNFR DKO as compared to WT and TNFR-2 KO mice (Fig. 2B). Importantly, our results also show that high-dose DMN-induced hepatotoxicity can occur in the absence of liver inflammation. TNFR-1 KO and TNFR DKO mice had similar necrosis scores to WT mice despite the paucity of inflammatory cell accumulation (Fig. 2).

Recently, Peschon et al. (1998) observed decreased pulmonary neutrophil influx in TNFR-1 KO and TNFR DKO mice and exacerbated neutrophil accumulation in the lungs of TNFR-2 KO mice following Micropolyspora faeni infection. They concluded that TNFR-1 is responsible for neutrophil influx during bacterial-induced pulmonary inflammation and that TNFR-2 may be important for down-regulating the inflammatory response. These data further support our acute, high-dose DMN experiments in which TNFR-1 KO and TNFR DKO mice had little cellular infiltrates, while TNFR-2 KO mice had levels comparable to WT (Fig. 2B). Hepatotoxicity following an acute DMN (100-mg/kg) dose is significantly higher in TNFR-2 KO mice compared to WT, TNFR-1 KO, and TNFR DKO. Interestingly, TNFR-2 KO mice were apparently more susceptible to acute DMN (100 mg/kg)-induced lethality, since 37.5% were found dead prior to sacrifice. Because these dead TNFR-2 KO mice were not included in the scoring procedure, the level of necrosis presented in Figure 2A may be underestimated. This further suggests that TNFR-2 signaling may be providing some protective responses because

### TABLE 1

<table>
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<tr>
<th>Apoptotic Gene</th>
<th>Naive</th>
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<th>PBS³</th>
<th>DMN (5.0)</th>
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**Note.** WT and TNFR DKO mice were exposed to either vehicle (PBS) or DMN ip for 6 or 24 h. In addition, naive (no treatment) or 6-h LPS (50 mg)-exposed mice were used as controls. Livers were removed and total liver RNA was isolated. RNA (10 μg) was then used with mAPO2 multi-probe RPA to detect hepatic Bcl-2 family of apoptotic genes.

³ Bcl-W, Bfl-1 and Bcl-X₂ are anti-apoptotic, while Bak and Bax promote apoptosis.

⁴ Mice were exposed ip to either vehicle (PBS) or DMN (5.0 mg/kg) for 6 h or exposed to vehicle or DMN (100 mg/kg) for 24 h.

⁵ Results from a single experiment are shown and are representative of 2 different identically treated mice. Results were normalized to the housekeeping gene, GAPDH, and were determined by dividing the apoptosis densitometry/phosphorimagery value by the GAPDH densitometry/phosphorimagery value and multiplying by 100.
TNFR-1 KO mice had lower necrosis compared to TNFR-2 KO mice. Taken together, these data suggest that TNFR-1 delivers detrimental signals that may be counteracted by TNFR-2-mediated signals. It has not been determined whether TNFR-2-deficient mice reflect a down-regulated inflammatory response at cytokine transcript levels.

TNFR-2-deficient mice (same strains as used in our experiments) express 5-fold higher TNFα protein following LPS exposure than do WT mice (Peschon et al., 1998), and this may explain the elevated damage in TNFR-2 KO mice following DMN (100 mg/kg) (Fig. 2A). Because TNFR-1 signaling is still intact in TNFR-2 KO mice, increased hepatic expression of TNFα following exposure to inflammatory mediators (LPS, DMN or CCl₄) can act on TNFR-1 to initiate destructive responses. However, it cannot be ruled out that the differences we observed in necrosis scores may be due to signaling through TNFR-2 that provides protective responses to counteract TNFR-1-mediated damage.

Recent evidence indicates that high dose DMN exposure (100 mg/kg) induces an apoptotic mechanism that may contribute to cytotoxic response at cytochrome oxidase activity levels.

<table>
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<tr>
<th>Apoptotic gene</th>
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<th>CCl₁ (1.0)</th>
<th>Corn oil</th>
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Note. WT and TNFR DKO mice were exposed to either corn oil or CCl₁, ip for 6 or 24 h. In addition, naive (no treatment) or 6 h LPS (50 μg)-exposed mice were used as controls. Livers were removed and total liver RNA was isolated. RNA (10 μg) was then used with mAPO2 multi-probe RPA to detect hepatic Bcl-2 family of apoptotic genes.

¹ Bcl-W, Bfl-1 and Bcl-X₁ are anti-apoptotic, while Bak and Bax promote apoptosis.

Results from a single experiment are shown and are representative of 2 different identically treated mice. Results were normalized to the housekeeping gene, GAPDH, and were determined by dividing the apoptosis densitometry/phosphorimagery value by the GAPDH densitometry/phosphorimagery value and multiplying by 100.

Not detected.
regulated in a TNFR-dependent manner during CCl₄, but not DMN exposure. High-dose DMN exposure slightly induced hepatic Bfl-1 expression compared to naive but not vehicle controls in WT mice (Table 1). In contrast, corn oil controls did not upregulate Bfl-1 transcript levels, and Bfl-1 transcripts were greatly elevated in CCl₄-treated livers from WT but not TNFR DKO mice (Fig. 7 and Table 2). Thus, TNFα mediates CCl₄, but not DMN-induced hepatic expression of Bfl-1, and either TNFR can provide the necessary signals.

In summary, through the utilization of TNFR KO mice, we have shown that TNFα is a key mediator in hepatic immunocyte infiltration in addition to regulating necrotic events during acute, high-dose chemical exposure. The outcome of hepatotoxicity and inflammation depends on specific TNFR signaling. Further, both the molecular response and the role of TNFα in regulating hepatic inflammatory cytokine, cytokine receptor, and apoptotic gene expression is unique for DMN and CCl₄. Thus, it may be necessary to individually evaluate the contribution of TNFα to individual hepatotoxic processes for various xenobiotics.

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REFERENCES


