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Limited role for CXC chemokines in the pathogenesis of α -naphthylisothiocyanate-induced liver injury

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Abstract

α -Naphthylisothiocyanate (ANIT) is a hepatotoxin that causes severe neutrophilic inflammation around portal tracts and bile ducts. The chemotactic signals that provoke this inflammatory response are unknown. In this study, we addressed the possibility that ANIT upregulates CXC chemokines in the liver and that these compounds mediate hepatic inflammation and tissue injury after ANIT treatment. Mice treated with a single dose of ANIT (50 mg/kg) exhibited rapid hepatic induction of the CXC chemokine macrophage inflammatory protein-2 (MIP-2). MIP-2 derived primarily from hepatocytes, with no apparent contribution by biliary cells. In ANIT-treated mice, the induction of MIP-2 coincided with an influx of neutrophils to portal zones; this hepatic neutrophil recruitment was suppressed by 50% in mice that lack the receptor for MIP-2 (CXCR2^{-/-}). Interestingly, despite their markedly reduced degree of hepatic inflammation, CXCR2^{-/-} mice displayed just as much hepatocellular injury and cholestasis after ANIT treatment as wild-type mice. Moreover, after long-term exposure, ANIT CXCR2^{-/-} mice developed liver fibrosis that was indistinguishable from that in wild-type mice. In summary, our data show that CXC chemokines are responsible for some of the hepatic inflammation that occurs in response to ANIT but that these compounds are not essential to the pathogenesis of either acute or chronic ANIT hepatotoxicity.

Keywords

hepatotoxicity; cholangiocyte; neutrophil; fibrosis; macrophage inflammatory protein-2

α -NAPHTHYLSIOTHIOCYANATE (ANIT) is a hepatotoxicant that damages biliary cells and hepatocytes. The drug is used experimentally in rodents as a model of intrahepatic cholestasis (10, 23, 27, 47). A single dose of ANIT induces acute cholangitis (10); prolonged exposure causes bile duct hyperplasia and biliary fibrosis (27, 47). Although the biochemical and histological features of ANIT toxicity are well documented, the mechanism by which ANIT causes liver injury remains uncertain. Cell culture studies (16, 17, 36) indicate that high doses of the drug are directly cytotoxic to hepatocytes and biliary cells. In vivo, however, ANIT does not cause liver damage until it appears in bile (11). The drug is initially detoxified in hepatocytes by conjugation with glutathione (7). ANIT-glutathione complexes are secreted into bile, but they are unstable and rapidly dissociate (7). This exposes biliary cells to high concentrations of the parent compound, which presumably causes direct cytotoxicity. The reappearance of ANIT in bile also leads to reuptake of the drug by hepatocytes and recycling

through repetitive rounds of glutathione conjugation and secretion (11, 20). This not only delays elimination of the drug, but also depletes glutathione progressively from hepatocytes and leads to hepatocellular toxicity.

Although the toxicity of ANIT in vivo can be explained, in part, by its concentration and recycling in bile, the drug also induces an intense hepatic inflammatory response (9, 10). Inflammation is particularly evident in the early phases of ANIT-induced liver injury, in which leukocytes invade portal areas and often completely surround damaged bile ducts. Some investigators (9) have shown that these infiltrating neutrophils play an active role in ANIT-induced cholestasis. Because neutrophils are active participants in the pathophysiology of ANIT-induced liver injury, it is important to identify how ANIT triggers neutrophil recruitment to the liver. Under disease conditions, liver cells are capable of synthesizing a variety of chemoattractants. Some are lipid mediators, such as platelet-activating factor (PAF) (12), 4-hydroxynonenal (38), and leukotriene B₄ (15), and others are peptides belonging to the chemokine superfamily. PAF and leukotrienes have already been investigated as putative inflammatory mediators in ANIT-treated liver. These compounds do not appear to be involved, because their blockade with specific inhibitors has no effect on ANIT-induced inflammation or cholestasis in vivo (2, 3). This raises the possibility that ANIT-induced neutrophilic inflammation is governed by chemokines.

The chemokines represent a large family of chemotactic peptides with a broad range of cellular targets. They are produced by all resident liver cells, including hepatocytes (28, 39, 40, 46, 48), sinusoidal endothelia (5, 43), cholangiocytes (32, 41, 49), Kupffer cells (4, 5, 26, 28), and stellate cells (30, 31, 37, 44, 45). Chemokines that attract neutrophils typically contain a CXC motif near their NH₂ terminus (1); they exert their activity by binding to specific G protein-coupled receptors (CXCR) on target cells (34). In mice, the two major CXC chemokines that attract neutrophils are macrophage inflammatory protein-2 (MIP-2) and KC. MIP-2 and KC both interact with a single receptor, designated CXCR2 (34). CXC chemokines have recently been implicated as mediators of hepatic inflammation in a model of obstructive cholestasis induced by bile duct ligation (41); this makes them attractive candidates as mediators of inflammation in drug-induced cholestasis.

This study was designed to determine whether ANIT stimulates hepatic production of CXC chemokines and, if so, to determine the impact of these compounds on ANIT-induced inflammation and cholestasis. The data indicate that ANIT induces MIP-2 in vivo, primarily in periportal hepatocytes. CXC chemokines appear to play a role in ANIT-induced neutrophilic inflammation of the liver, because mice with targeted disruption of CXCR2 (CXCR2^{-/-}) display markedly reduced hepatic inflammatory responses to ANIT. Interestingly, however, CXC blockade has no effect on ANIT-induced cholestasis or biliary fibrosis. Overall, our findings suggest that acute and chronic ANIT toxicity can both occur in the absence of chemokine-dependent neutrophilic inflammation.

MATERIALS AND METHODS

Breeding and maintenance of CXCR2^{+/+} and CXCR2^{-/-} mice

CXCR2^{+/+} and CXCR2^{-/-} mice (BALB/c background) were bred from heterozygotes purchased from Jackson Laboratories (Bar Harbor, ME). Offspring were screened by PCR amplification of genomic DNA. Experiments were initiated when mice reached 8–12 wk of age. In all studies, CXCR2^{+/+} and CXCR2^{-/-} mice were assigned to age- and gender-matched treatment groups. All experimental protocols were approved by the University of California Committee on Animal Research.

Induction of ANIT toxicity in vivo

ANIT was purchased from Sigma (St. Louis, MO). For acute studies, the drug was dissolved in olive oil (10 mg/ml) and administered to mice by gavage at a dose of 50 mg/kg following an overnight fast. Control mice received olive oil alone (5 ml/kg). Two hours after gavage, mice were returned to solid food ad libitum. At various intervals (3–48 h) after ANIT treatment, mice were killed for collection of blood and liver tissue. Chronic ANIT toxicity was induced by adding the drug in powdered form to ground chow (1 g/kg). Mice were offered either ANIT-supplemented or control chow ad libitum for 8 wk. At the end of the experiment, mice were killed for collection of blood and liver tissue.

ANIT treatment of liver cells in culture

Hepatocytes were isolated from CXCR2^{+/+} mice by collagenase perfusion (29). Crude cell suspensions were centrifuged through Percoll (Amersham Biosciences, Piscataway, NJ) and plated on collagen-coated plastic at a density of 1×10^5 cells/cm² in medium containing 5% fetal bovine serum. After 2 h, the cultures were washed and replenished with serum-free medium containing ANIT. Intrahepatic biliary epithelial cells were isolated from BALB/c mice and immortalized with SV40 as described (18). Cells were maintained in hormonally defined medium containing 10% serum and used at *passage 19*. For ANIT treatment, biliary cells were plated on uncoated plastic at a density of 1×10^5 cells/cm². Cultures were washed free of serum before ANIT addition.

ANIT was added to hepatocyte and biliary cell culture medium (10–1,000 μ M) from 100 $\times$ stock solutions prepared in cell culture-grade DMSO (Sigma). Control cultures were treated with 1% (vol/vol) DMSO as vehicle. As a positive control for chemokine stimulation, parallel plates from each culture were treated with TNF (10 ng/ml). All cell cultures were incubated with test substances for 24 h. At the end of each experiment, culture medium was harvested and cell viability was assessed by methylthiazolyldiphenyl-tetrazolium bromide assay (Sigma).

ELISA for chemokines

MIP-2 and KC were measured in liver homogenates or cell culture supernatants by ELISA (Quantikine, R&D Systems, Minneapolis, MN). Livers were prepared for assay by homogenization in a neutral salt solution. Cell culture medium was clarified by centrifugation and stored at –20°C before assay. Raw data for liver homogenates were normalized to total protein (Bio-Rad Protein Assay, Richmond, CA). Cell culture data from parallel plates were recorded as picograms per milliliter of medium and expressed as a percent control.

Biochemical markers of liver injury

Alanine aminotransferase (ALT), alkaline phosphatase, and bilirubin were measured using a BAX autoanalyzer (Bayer, Tokyo, Japan).

Histology and immunohistochemistry

Formalin-fixed liver sections were stained with hematoxylin and eosin for routine histological analysis. In experiments evaluating the long-term effects of ANIT, serial sections of liver were stained with picosirius red to highlight connective tissue. Immunohistochemical staining was performed on frozen tissue. Neutrophils were identified using rat anti-mouse Ly-6G antibody (BD Pharmingen, San Diego, CA; 1:500), and MIP-2 was identified with rabbit anti-mouse MIP-2 (Abcam, London, UK; 1:40). The proteins of interest were detected with biotinylated secondary antibodies, followed by incubation with avidin-biotin-peroxidase (Vectastain Elite, Vector Laboratories, Burlingame, CA) and 3,3'

diaminobenzidine as a colorimetric substrate. Biliary cells were highlighted by immunofluorescence with a rat monoclonal antibody against cytokeratin-19 followed by a fluorescent anti-rat IgG (Alexa Fluor 488; Molecular Probes, Eugene OR). All histological sections were viewed and photographed with a Nikon Microphot-FXA epifluorescence microscope (Nikon, Tokyo, Japan). Bile duct area was determined by morphometry (Compix, Cranberry Township, PA)

Quantitation of neutrophils in liver sections

Neutrophils were quantitated by direct cell counting in liver sections stained with Ly-6G. Because the inflammatory response to ANIT is predominantly periportal, microscopic fields were centered on portal tracts. Data were reported as the mean number of neutrophils in eight separate $\times 20$ portal fields.

Measurement of liver hydroxyproline content

Hepatic hydroxyproline content was determined by the method of Jamall and colleagues (19). Tissue samples (200 mg) were homogenized in saline and hydrolyzed in 6N HCl for 20 h. Fifty-microliter aliquots of each hydrolysate were dried and assayed for hydroxyproline using a purified standard (4-hydroxy-L-proline, Sigma) and *para*-dimethylaminobenzaldehyde (Sigma) as a colorimetric substrate. Raw data were normalized to liver wet weight.

Statistical analysis

Experiments performed *in vivo* included at least four mice per study group. Cell culture experiments were performed in triplicate. Mean data from each study group were compared by analysis of variance. *P* values < 0.05 were considered statistically significant.

RESULTS

ANIT induces CXC chemokines in mouse liver

Mice were treated with ANIT (50 mg/kg) and followed for various intervals ranging from 6 to 48 h to determine whether the drug stimulates hepatic chemokine production. Within 12 h of ANIT administration, MIP-2 was markedly increased in the livers of both CXCR2^{+/+} and CXCR2^{-/-} mice (Fig. 1). In wild-type mice, hepatic MIP-2 levels peaked at 24 h and declined sharply by 48 h. In CXCR2^{-/-} mice, MIP-2 levels peaked at 24 h but declined more slowly. The CXC chemokine KC was also induced in mouse liver after ANIT treatment (data not shown). Because ANIT regulated MIP-2 and KC in a similar fashion, we elected to focus the remainder of the study on MIP-2 alone.

Experiments were performed to determine the cellular source of MIP-2 in ANIT-treated liver. Immunohistochemical staining conducted at 24 h after ANIT treatment, when hepatic MIP-2 concentrations were highest, showed that the chemokine was most abundant in periportal hepatocytes (Fig. 2A). In addition to the strong MIP-2 staining in periportal hepatocytes, faint MIP-2 immunoreactivity was noted throughout the hepatic parenchyma. Biliary epithelial cells did not show MIP-2 staining (Fig. 2B).

To determine whether MIP-2 production by hepatocytes in ANIT-treated livers represents a direct response to the toxin, we added ANIT to hepatocytes in primary culture and monitored the effect of the drug on MIP-2 secretion. At a dose of 100 μ M, ANIT significantly increased MIP-2 production by primary hepatocytes (Table 1). MIP-2 secretion was not directly linked to cell death; the chemokine appeared in cell culture medium before any loss of hepatocyte viability. Although ANIT appeared to be a much weaker inducer of MIP-2 in cultured hepatocytes than it was *in vivo*, this was likely due to high basal

expression of chemokines in hepatocyte cultures. High background expression of chemokines, which is a recognized phenomenon in cultured hepatocytes (48), limits the ability of these cells to exhibit large incremental responses to exogenous agents. This was evident in our experiments, in which even a positive control stimulus (10 ng/ml TNF) boosted hepatocyte MIP-2 production only 1.9-fold over control levels (Table 1). Thus the response of cultured hepatocytes to ANIT, albeit small, is consistent with the notion that the drug has a direct effect on hepatocyte chemokine production. In contrast to hepatocytes, but in keeping with their behavior in vivo, biliary epithelial cells displayed no response to ANIT in cell culture. Indeed, ANIT suppressed chemokine secretion by biliary cells in vitro (Table 1). The poor response of biliary cells to ANIT was not due to a global malfunction of the cultures, because the cells displayed a robust chemokine response to TNF.

ANIT-induced hepatic inflammation, but not cholestasis, is attenuated in CXCR2^{-/-} mice

To determine whether CXC chemokines play a role in ANIT-induced hepatic inflammation, we treated wild-type and CXCR2^{-/-} mice with ANIT and monitored the influx of hepatic neutrophils in each group. In wild-type mice, neutrophils invaded the liver within 12 h of ANIT treatment and remained abundant for at least 24 h (Fig. 3). In CXCR2^{-/-} mice, hepatic neutrophil infiltration was not as robust after ANIT treatment as it was in wild-type mice (Fig. 3); neutrophil counts rose somewhat over basal levels, but the change did not reach statistical significance. The blunted neutrophil response of CXCR2^{-/-} mice was noteworthy because these mice have larger numbers of circulating and tissue neutrophils than wild-type mice at baseline (6). When viewed as a proportional change from control levels, hepatic neutrophils rose only 2.5-fold in CXCR2^{-/-} mice after ANIT treatment, whereas they increased 9–17 times in wild-type mice (Fig. 3). Although the number of hepatic neutrophils was lower in CXCR2^{-/-} mice than wild-type mice from 12–24 h after ANIT, the pattern of hepatic neutrophil infiltration was similar in both groups. Neutrophils localized primarily to bile ducts and areas of hepatocellular destruction (Fig. 4).

In conjunction with the assessment of hepatic inflammation, we measured biochemical parameters of liver injury in wild-type and CXCR2^{-/-} mice at various intervals after acute ANIT treatment. Unlike hepatic neutrophil counts, which varied between wild-type and knockout mice, markers of liver injury and cholestasis were identical in the two groups (Fig. 5). ANIT provoked a rapid rise in serum ALT in both wild-type and CXCR2^{-/-} mice that was sustained for at least 48 h. Alkaline phosphatase also increased significantly in both groups but not until 48 h after ANIT administration. Bilirubin increased exponentially after ANIT treatment in wild-type and CXCR2^{-/-} mice, exceeding values of 10 mg/dl by 48 h.

ANIT-induced liver fibrosis is not regulated by CXC chemokines

Although the disparity in hepatic inflammation between wild-type and CXCR2^{-/-} mice after acute ANIT treatment had no impact on acute liver injury or cholestasis, we considered that over time it might influence ANIT-induced hepatic fibrosis. To test this hypothesis, we fed CXCR2^{+/+} and CXCR2^{-/-} mice a diet containing 0.1% (wt/wt) ANIT continuously for 8 wk. At the end of the experiment, livers from both groups of animals were examined for neutrophilic inflammation and fibrosis (Fig. 6). Wild-type mice continued to have more hepatic neutrophils than CXCR2^{-/-} mice after 8 wk of ANIT feeding (53.5 ± 8.1 vs. 20.7 ± 4.4 cells/field; $P < 0.01$). Hepatic fibrosis, however, was no different between the two groups. Both types of mice showed comparable increases in hepatic collagen after ANIT treatment compared with the levels measured in chow-fed controls (Table 2). Bile duct area and alkaline phosphatase also rose similarly in both groups of mice (Table 2), indicating equivalent degrees of chronic cholestasis.

DISCUSSION

The hepatotoxicant ANIT causes severe cholestatic liver injury that is reportedly neutrophil dependent (9). The mechanism by which neutrophils are recruited to ANIT-treated livers, however, is unknown. We undertook this study to determine whether CXC chemokines function as neutrophil chemoattractants in liver after ANIT treatment. If so, we reasoned that these compounds would represent important intermediates in the pathogenesis of ANIT toxicity. We found that ANIT stimulated the production of CXC chemokines in the liver in a time frame and location consistent with their involvement in ANIT toxicity. Moreover, we found that CXC chemokines promoted neutrophil recruitment to the liver after ANIT treatment. Despite this, blockade of CXC chemokine activity had little impact on the development of ANIT-induced hepatocellular injury or cholestasis. On the basis of these observations, we conclude that CXC chemokines are relevant chemoattractants in ANIT-treated livers but are not essential mediators of ANIT toxicity.

Our initial studies placed CXC chemokines in a logical position to contribute to ANIT toxicity. MIP-2 rose 10-fold in the liver within 12 h of toxin administration; moreover, the chemokine localized preferentially to periportal zones, where neutrophil infiltration was most intense. In addition, hepatic neutrophil counts rose and fell over roughly the same interval as MIP-2 following ANIT treatment, suggesting that chemokine induction and hepatic inflammation were causally related. Studies using CXCR2^{-/-} mice confirmed that inhibition of CXC chemokine activity reduced ANIT-related hepatic inflammation by 50%. Overall, the data indicated that CXC chemokines are important mediators of hepatic inflammation in the setting of ANIT treatment.

Given these results, we were surprised to find that inhibition of CXC chemokine-dependent neutrophil infiltration had virtually no impact on ANIT-induced hepatocellular injury or cholestasis. Why a 50% reduction in hepatic neutrophils would not cause any improvement in other parameters of ANIT toxicity is not immediately apparent; the data clearly indicate, however, that in ANIT-treated mice, the relationship between hepatic neutrophil infiltration and liver injury is not linear. Our findings differ from those of Dahm et al. (9), who reported that ANIT toxicity could be successfully averted in experimental animals when neutrophils were depleted by 90%. Together, the two studies suggest that neutrophils are important to ANIT toxicity but that only a fraction of the total number that typically invades the liver is actually required to produce the full spectrum of ANIT-related hepatocellular injury and cholestasis. Our experience with CXCR2^{-/-} mice suggests that ANIT induces more than one inflammatory mediator in the liver, with similar or even overlapping effects. If these inflammatory mediators are redundant, then inhibition of CXC chemokines alone would not be sufficient to prevent ANIT toxicity.

The fact that CXC chemokines controlled only 50% of the neutrophils invading ANIT-treated livers lends independent support to the notion that chemokines are not exclusive mediators of ANIT toxicity. The remaining neutrophils, which reached the liver despite CXC chemokine blockade, must be under the influence of other, as yet unidentified, chemoattractant(s). One possibility is that ANIT itself promotes neutrophil recruitment to the liver; the drug has been reported (20) to have chemotactic activity toward neutrophils in vitro and appears to be effective in concentrations that are relevant to ANIT toxicity in vivo. Another possibility is that lipid peroxides act as neutrophil chemoattractants. These compounds are present in ANIT-treated livers (22), where they can induce neutrophil migration either directly (8, 33) or indirectly by stimulating chemokine production (35). Yet another option is that neutrophils respond to chemotactic lipids released by dying cells within ANIT-treated livers. A recent report (25) indicated that apoptotic cells elaborate lysophosphatidylcholine and that this phospholipid is important for provoking an

inflammatory response. Whether any or all of these compounds contributes to ANIT toxicity will require further study.

Noteworthy is that CXC chemokines did not emerge as dominant mediators of ANIT-induced liver injury even though these compounds were substantially upregulated by ANIT treatment. This disparity between chemokine concentration and biological activity may be related to the distribution of CXC chemokines in ANIT-treated livers. Much of the MIP-2 in ANIT-treated livers was spread diffusely throughout the hepatic parenchyma (Fig. 2); this may have limited the formation of chemokine gradients, which are essential for recruiting neutrophils from the circulation. Similar diffuse patterns of CXC chemokine expression have been observed previously (41) in acute liver injury without resulting in hepatic inflammation. In contrast, some of the MIP-2 in ANIT-treated livers concentrated around portal tracts. This periportal MIP-2, which derived from hepatocytes and possibly also from nearby nonparenchymal cells, likely promoted neutrophil recruitment (21).

We originally anticipated that biliary cells would be important sources of CXC chemokines in ANIT-treated livers, because neutrophils localize preferentially around bile ducts after ANIT administration. We also considered biliary cells attractive candidates because Hill et al. (16) demonstrated that ANIT stimulates cholangiocytes in cell culture to produce a factor with chemotactic activity toward neutrophils. We theorized that this factor was MIP-2, because biliary cells are known to produce CXC chemokines (13, 24, 32, 41); contrary to our hypothesis, however, we found no evidence that biliary cells secrete CXC chemokines in response to ANIT either in culture or in vivo. Our findings do not exclude cholangiocytes as key participants in the pathogenesis of ANIT-induced inflammation. Additional experiments are necessary, though, to establish the identity of the chemoattractant(s) secreted by biliary cells after ANIT treatment and to determine whether these compounds are also induced in vivo.

Although most of our experiments focused on the role of CXC chemokines in acute ANIT toxicity, we also found that long-term suppression of chemokine-related neutrophil recruitment in ANIT-treated mice had absolutely no effect on chronic cholestasis or liver fibrosis. This outcome, although paradoxical, is reminiscent of that in other models of liver disease in which neutrophilic inflammation has had little influence on hepatic fibrogenesis (42). The fact that neutrophil counts can vary widely in the liver with no impact on either acute tissue injury or fibrosis suggests that a substantial proportion of these inflammatory cells migrate into the liver for a nondestructive purpose. This theory was recently proposed by Gujral et al. (14), who showed that neutrophils accumulate in the portal tracts of bile duct-ligated mice without demonstrating any evidence of activation. It will be of great interest to determine whether these cells are responding to unique chemotactic signals and whether their recruitment is critical to tissue reorganization or repair.

In summary, CXC chemokines are induced in the liver after ANIT treatment, where they stimulate hepatic neutrophil recruitment. CXC chemokine-dependent inflammation, however, is not a prerequisite to ANIT-induced liver injury and cholestasis. If neutrophils are vital to the pathogenesis of ANIT toxicity, as has been suggested previously (9), then other neutrophil chemoattractant(s) must be capable of inducing ANIT-related inflammation and liver injury in the setting of CXC chemokine blockade. The identity of these inflammatory intermediate(s) and their specific roles in ANIT toxicity remain to be established.

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Fig. 1. Induction of macrophage inflammatory protein-2 (MIP-2) in mouse liver after α -naphthylisothiocyanate (ANIT) treatment. Histogram shows the amount of MIP-2 in liver homogenates from wild-type (WT) and CXCR2^{-/-} [knockout (KO)] mice at various intervals after a single dose of ANIT (50 mg/kg). Chemokine levels rose abruptly at 12 h and declined toward normal at 48 h. Values represent means $\pm$ SE for $n = 4$. * $P < 0.05$ vs. 6-h value of similar genotype. # $P < 0.05$ vs. 48-h WT mice.

Fig. 2. Localization of MIP-2 in mouse liver after ANIT treatment. MIP-2 was identified in mouse liver sections by immunohistochemistry (see MATERIALS AND METHODS). Twenty-four hours after ANIT, MIP-2 was visible in periportal hepatocytes (*a*; arrows) but not in biliary epithelial cells (*b*; arrows). Control sections of ANIT-treated liver, which were incubated with nonimmune IgG in place of anti-MIP-2, showed no staining (*c*). The livers of control mice that received vehicle instead of ANIT displayed no MIP-2 at 24 h (*d*).

Fig. 3. Hepatic neutrophil counts in WT and KO mice after acute ANIT treatment. Histogram illustrates the number of neutrophils present in the livers of WT and KO mice at various intervals after a single dose of ANIT (50 mg/kg). Neutrophils were quantitated by direct counting of tissue sections stained with anti-neutrophil antibody (Ly-6G; see MATERIALS AND METHODS). Values represent means $\pm$ SE for $n = 4$. * $P < 0.05$ vs. 6-h value of similar genotype. # $P < 0.05$ vs. WT mice at the same interval.

Fig. 4.

Liver histology in WT and KO mice after acute ANIT treatment. Mice received a single dose of ANIT (50 mg/kg) by gavage and were killed 12 h later, the time point at which neutrophils were most numerous. WT mice are represented in *a*, *c*, and *e*; KO mice are represented in *b*, *d*, and *f*. WT mice show robust periductular inflammation in response to ANIT (*a*; arrowheads). The neutrophilic component of the inflammatory infiltrate is highlighted by Ly-6G immunohistochemistry (*c*, *e*). Compared with WT mice, KO mice display less overall biliary ductular inflammation after ANIT treatment (*b*; arrowheads) as well as a lesser degree of neutrophil infiltration (*d*, *f*).

Fig. 5.

Biochemical markers of liver injury in WT and KO mice after acute ANIT treatment.

Histograms show serum alanine aminotransferase (ALT), alkaline phosphatase, and total bilirubin in WT and KO mice at various intervals after a single dose of ANIT (50 mg/kg).

Values represent means $\pm$ SE for $n = 4$. * $P < 0.05$ vs. 6-h value of similar genotype.

Fig. 6.

Liver histology in WT and KO mice after chronic ANIT treatment. Photomicrographs illustrate neutrophilic inflammation (*a–d*) and fibrosis (*e, f*) in mouse liver after 8 wk of ANIT feeding. *a, c*, and *e* are from WT mice; *b, d*, and *f* are from KO. Neutrophils are much more prominent in the livers of WT mice than KO mice. WT livers contain clusters of neutrophils scattered throughout the hepatic parenchyma (*a*). High-power magnification shows that these cells are localized in expanded portal tracts (*c*). In the livers of KO mice, neutrophils are scarce (*b, d*). Despite the difference in hepatic inflammation, hepatic fibrosis is similar in both WT and knockout mice (*e, f*).

Table 1

MIP-2 production by cultured hepatocytes and biliary cells in response to ANIT treatment

Treatment	Hepatocytes		Biliary Cells	
	MIP-2, % control	Survival, % control	MIP-2, % control	Survival, % control
ANIT (10 μ M)	88 $\pm$ 3	86 $\pm$ 2	103 $\pm$ 10	91 $\pm$ 7
ANIT (20 μ M)	104 $\pm$ 6	86 $\pm$ 1	88 $\pm$ 12	101 $\pm$ 7
ANIT (50 μ M)	108 $\pm$ 2	86 $\pm$ 1	67 $\pm$ 23	94 $\pm$ 0
ANIT (100 μ M)	122 $\pm$ 7 *	90 $\pm$ 3	0 $\pm$ 0 ‡	88 $\pm$ 5
ANIT (1,000 μ M)	19 $\pm$ 5 ‡	19 $\pm$ 3 ‡	0 $\pm$ 0 ‡	47 $\pm$ 3 ‡
TNF (10 ng/ml)	188 $\pm$ 10 ‡	103 $\pm$ 3	377 $\pm$ 13 ‡	89 $\pm$ 8

Values represent means $\pm$ SE for $n = 3$. Mouse liver cells were treated with α -naphthylisothiocyanate (ANIT) or TNF for 24 h. At the end of the experiment, macrophage inflammatory protein-2 (MIP-2) was measured in cell culture medium by ELISA and expressed as a percentage of the amount in medium from cells treated with vehicle alone (control). Cell survival was measured by methylthiazolyldiphenyltetrazolium assay (see MATERIALS AND METHODS) and expressed as % control.

* $P < 0.05$,

‡ $P < 0.01$ vs. control.

Table 2

Parameters of liver injury in mice after 8 wk of ANIT feeding

	Control		ANIT	
	WT	CXCR2 ^{-/-}	WT	CXCR2 ^{-/-}
Hydroxyproline, µg/g liver	108.1 ± 7.7	141.6 ± 9.1	255.2 ± 30.0 [†]	266.4 ± 20.7 [†]
Bile duct area, %	3.5 ± 0.4	2.1 ± 0.2	8.0 ± 2.2 [*]	7.3 ± 2.1 [*]
ALT, IU/l	47 ± 13	48 ± 4	90 ± 13 [*]	103 ± 4 [†]
Alkaline				
Phosphatase, IU/l	139 ± 11	100 ± 0	793 ± 126 [†]	855 ± 131 [†]
Bilirubin, mg/dl	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0

Values represent means ± SE for 4–5 mice in each group.

^{*}*P* < 0.05,[†]*P* < 0.01 vs. control mice of the same genotype.

WT, wild type.