

Cytokine and Chemokine Expression Associated with Steatohepatitis and Hepatocyte Proliferation in Rats Fed Ethanol via Total Enteral Nutrition

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To determine the temporal relationship between alcohol-induced changes in cytokines and chemokines, development of liver pathology and stimulation of hepatocyte proliferation, male Sprague-Dawley rats were intragastrically fed low carbohydrate-containing ethanol (EtOH) diets via total enteral nutrition (TEN) for up to 49 d. Induction of EtOH metabolism and appearance of steatosis preceded development of oxidative stress, inflammation, and cell death. A transitory peak of tumor necrosis factor (TNF α) and interferon gamma (IFN γ) was observed at 14 d followed by reduced expression of TNF α , IFN γ and another Th1 cytokine IL-12 accompanied by reduced expression of the Th1 regulators T-bet and STAT4. After 35–49 d of EtOH, at a time when hepatocyte proliferation was stimulated, IL-12 returned to control values and a second peak of TNF α occurred. The Th2 cytokine IL-4 remained suppressed throughout the study and was accompanied by reductions in the Th2 regulator GATA3. There was no temporal effect of EtOH on expression of IL-6 or TGF β . IL-5 and IL-13 mRNA were

undetectable. Chemokine CXCL-2 expression increased progressively up to 35 d and preceded the appearance of inflammatory infiltrates. These data suggest that steatosis, increased ethanol metabolism, a transient induction of the innate immune response and suppression of Th2 responses were acute consequences of ethanol treatment and were followed by suppression of Th1 responses. However, the majority of necrosis, apoptosis and a late peak of TNF α only occurred after 6–7 weeks of ethanol, coincided with the appearance of inflammatory infiltrates and were associated with stimulation of hepatocyte proliferation. *Exp Biol Med* 233:344–355, 2008

Key words: alcohol; cytokine; TNF α ; IL-4; proliferation

Introduction

In human drinkers, alcoholic liver disease (ALD) is characterized by the acute appearance of fatty liver (steatosis) which is often a reversible condition, followed in some cases by more chronic irreversible pathologies including inflammation, apoptosis, necrosis (steatohepatitis), and fibrosis (1). Autoimmune responses recognizing self-proteins modified by alcohol metabolites or lipid peroxidation products appear to contribute significantly to the pathogenesis of ALD (2). In addition, significant changes in the pattern of expression of pro- and anti-inflammatory cytokines by parenchymal and nonparenchymal cells exposed to ethanol may also play an important part in regulating this process (3–8). It has been suggested that an acute innate immune response to EtOH by resident

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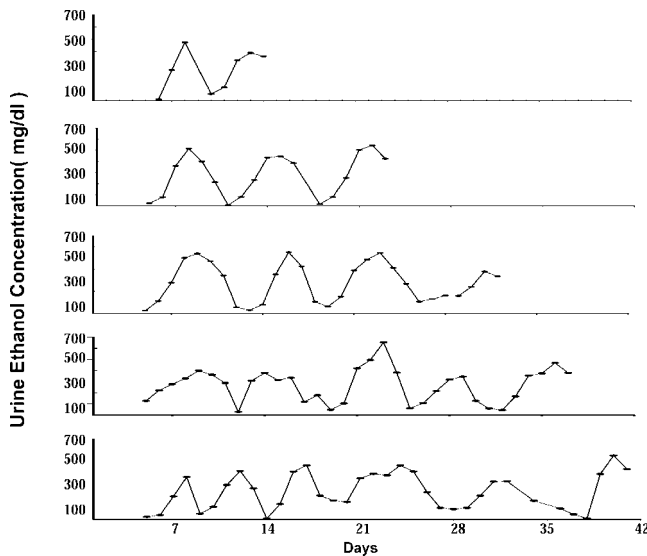


Figure 1. Urine EtOH pulses during continuous infusion of ethanol-containing TEN diets. UECs are plotted against duration of study for individual animals which are representative of the study.

macrophages associated with the development of steatosis may contribute to the progression of liver pathology and result in a shift in expression of T helper cytokines towards a Th1 response characterized by elevations in pro-inflammatory cytokines such as $\text{IFN}\gamma$, IL-12 and $\text{TNF}\alpha$ (8–10). In addition to a role in development of inflammation, elevations of $\text{TNF}\alpha$ have also been implicated in the regulation of hepatocyte proliferation in response to EtOH and other toxicant-induced liver injury (11–13).

Suppression of Th2 responses by EtOH characterized by reductions of IL-4 could also contribute to development of steatohepatitis and autoimmune responses (5, 9). Recent studies in mice by Kremer et al. (10) have demonstrated that steatosis produced by choline deficiency in mice increased expression of $\text{TNF}\alpha$, $\text{IFN}\gamma$ and IL-12 and of the IL-12 regulated transcription factors T-bet and STAT4 which are crucial for Th1 commitment. These authors also observed suppressed IL-4 and the Th2 specific transcription factor GATA-3 in choline-deficient steatotic mice following challenge with concanavalin A. This steatosis-related shift from Th2 to Th1 response resulted in enhanced development of hepatitis. However, data on the effects of EtOH and EtOH-associated steatosis on Th1 responses are mixed and other studies have shown impairment of *in vivo* Th1 responses, suppression of IL-12 and decreased dendritic cell differentiation associated with increased sensitivity to infection (14). It is possible that EtOH-stimulation of hepatic Th1 responses are transient. Declines in IL-12 and Th1 responses following longer term EtOH exposure have been proposed to result in a switch to a sustained hepatic Th2 response associated with development of fibrosis (8).

Elevation in liver expression and serum levels of chemokines such as cytokine-induced neutrophil chemo-attractant (CINC-1/GRO), MCP-1 and chemokine C-X-C

motif ligand (CXCL-2/MIP-2, IL-8) have also been reported following ethanol treatment in rats and in patients with alcoholic hepatitis (15–17). These chemokines could mediate the recruitment of neutrophils and monocytes associated with chronic ethanol-induced liver injury (15, 17–19).

We have established a rat model for the development of ALD in which ethanol is administered chronically for up to 50 d as part of an intragastric (IG) total enteral nutrition (TEN) system (5, 24–25). Progression from steatosis to steatohepatitis in this model has been shown to require diets high in polyunsaturated fats and low in carbohydrate (22, 23). Moreover, unlike other rodent IG models, ALD in the TEN model is not associated with elevated serum endotoxin perhaps as a result of diet-mediated protection against ethanol-induced gut permeability (23, 24). Although many studies have examined the effects of chronic ethanol-feeding on hepatic cytokine and chemokine expression, little attempt has been made to link temporal expression patterns of these factors or alteration of Th1/2 cytokine responses with development of alcoholic pathology or hepatocyte proliferation. The current study was designed to examine this question and also to determine if changes in cytokine or chemokine expression were also affected by the high and low BECs attained during ethanol pulses produced in this model.

Materials and Methods

Animals. All animal studies described below were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas for Medical Sciences. An intragastric cannula was surgically implanted into male Sprague-Dawley rats (300 g). Rats were infused with liquid diets using the low carbohydrate TEN system as described previously (21–25). Diets met NRC recommendations for the growing rat and produced growth rates comparable to *ad libitum* chow-fed animals (22). Ethanol (EtOH) was substituted isocalorically for carbohydrate calories to provide a dose of 13g/kg/d. 24 h urine ethanol concentrations (UECs) were measured daily using an Analox Instruments GL5 analyzer (Fig. 1). Groups of EtOH-infused rats were sacrificed at the 2nd, 3rd, 4th, and 5th UEC pulses at d 14, d 21, d 28, and d 35 of infusion; additional groups were sacrificed at UEC nadirs after 42–49 d of infusion and at high UECs after 42–49 d of infusion. Members of the control group were sacrificed at each time point.

Biochemical Analysis. Plasma ALT, lipid peroxidation, microsomal p-nitrophenol hydroxylase and ADH class I activity were assayed as described previously (22–26). CYP2E1 and STAT4 apoprotein expression were measured in liver microsomes and nuclear extracts, respectively, by Western immunoblot analysis (10, 22). Equal loading of protein was verified by Coomassie Blue

Table 1. Real Time PCR Primers Designed as Described in Materials and Methods

	Sequence 5'–3'	Product size, bp
IFN- γ forward	CAC GCC GCG TCT TGG T	82
IFN- γ reverse	TCT AGG CTT TCA ATG AGT GTG CC	82
IL 4 forward	GCA ACA AGG AAC ACC ACG G	91
IL 4 reverse	AAG CAC GGA GGT ACA TCA CGT	91
IL 5 forward	GTG TCT GGG CCA TTG CTA T	137
IL 5 reverse	GCT GGT GAT TTT TAT GAG TAG GAA	137
IL 6 forward	TGA TGG ATG CTT CCA AAC TG	230
IL 6 reverse	GAG CAT TGG AAG TTG GGG TA	230
IL 13 forward	ATC ACA CAA GAC CAG AAG ACT TC	218
IL 13 reverse	AAC TGG GCT ACT TCG ATT TTG G	218
TNF α forward	ACT GAA CTT CGG GGT GAT TG	153
TNF α reverse	GCT TGG TGG TTT GCT ACG AC	153
TGF β forward	CTT CAG CTC CAC AGA GAA GAA CTG C	298
TGF β reverse	CAC GAT CAT GTT GGA CAA CTG CTC C	298
CINC 1 forward	GCA CCC AAA CCG AAG TCA TA	146
CINC 1 reverse	GCC ATC GGT GCA ATC TAT CT	146
CXCL 2 forward	ATC CAG AGC TTG ACG GTG AC	248
CXCL 2 reverse	AGG TAC GAT CCA GGC TTC CT	248
Cyclophilin A forward	GCA TAC AGG TCC TGG CAT CT	192
Cyclophilin A reverse	TTC TTG CTG GTC TTG CCA TT	192

staining of duplicate gels. STAT4 antibodies were obtained from Santa Cruz Biotechnology (sc486) (Santa Cruz, CA).

Pathological Evaluation. Liver pathology was assessed in hematoxylin-eosin–stained liver sections and scored as described previously (22) by a board certified pathologist (S.K.). In addition, apoptotic hepatocytes were detected by the TUNEL procedure using a TdT-FragEL DNA fragmentation detection kit (EMD Biosciences, San Diego, CA).

Real-Time RT-PCR Analysis of Cytokine, Chemokine and Transcription Factor Expression. Expression of mRNAs coding for the cytokines IL-4, IL-5, IL-6, IL-12, IL-13, TNF α and TGF β and INF γ ; the chemokines CINC-1 and CXCL-2; and the transcription factors T-bet and GATA-3 were quantified by real time RT-PCR using the SybrGreen method (25). PCR was performed using the Applied Biosystems ABI PRISM[®] 7700 Sequence Detection System. Primers were designed using Primer3 software (Cambridge, MA) and synthesized by InVitrogen (Stockholm, Sweden). Primer sequences are listed in Table 1. All samples were run blind, and reactions were performed in at least duplicates from separate cDNA reactions. All primers were designed to span at least one intron.

Multiplex Immunoassay of Cytokine Proteins.

Analysis of TNF α , IFN γ , IL-12 and IL-4 protein expression in liver homogenates from control, 14, 28 and 42–49 d EtOH-treated rats was conducted using a 96-well plate rat cytokine/chemokine Linco_{plex} kit from Linco research (St. Charles, MO) and quantitated using a Bio-Rad Bio-Plex[™] 2000 system (Bio-Rad, Hercules, CA).

Proliferating Cell Nuclear Antigen Assay. Cell cycle progression was evaluated in liver slices taken from control rats and rats infused with ethanol for 14 d or 42–49 d. Hepatocyte proliferation was measured by immunohis-

tochemical analysis of PCNA expression as described by Greenwell et al. (27). For each rat, 1000 hepatocytes were counted.

Statistics. No changes associated with length of feeding control diets were observed in values of any parameter during the course of the study; therefore, for comparison with temporal EtOH effects, these data were combined into a single control group. For analysis of time course data and for cytokine and chemokine mRNA expression at peaks and nadirs of the UEC pulse, data are presented as mean $\pm$ SEM. However, since these data represent continuous variables and are not normally distributed populations, statistical comparisons to controls and across time intervals were performed using a Kruskal Wallis non-parametric ANOVA and non-parametric Mann Whitney tests to compare two points of time. Significance was set at $P < 0.05$. Liver pathology data from all animals sacrificed at the end of the study irrespective of urine EtOH concentration were expressed as mean $\pm$ SEM and were compared by one way analysis of variance (ANOVA) followed by Student Neuman-Keuls post-hoc analysis, and significance was set at $P < 0.05$. Pearson tests were performed to examine correlations. Statistical analysis was performed using SPSS (SPSS for Windows 11.0.1).

Results

Body weight gains in both control and EtOH groups were comparable to rats fed chow diets *ad libitum* as described previously (21–25) (data not shown). Mean UECs demonstrated a characteristic pulsatile pattern of high peaks around 500 mg/dl and nadirs of almost zero (Fig. 1) with a periodicity of 6–7 d as described previously utilizing IG infusion of EtOH-containing liquid diets (26, 28–30). 24 h UECs are an accurate reflection of blood ethanol concen-

Table 2. ADH and CYP2E1 Expression and Activity During the Time Course of EtOH Infusion in the Rat TEN Model

Days of Infusion ^b	CYP2E1			ADH ^a	
	PNP ^c	mRNA	Protein	ADH Activity	ADH mRNA
C	151 ± 29 ^d	1.0 ± 0.1	102 ± 19	3.9 ± 0.3	1.0 ± 0.2
Day 14	325 ± 140	2.5 ± 0.3*	496 ± 66*	7.5 ± 0.9*	2.1 ± 0.4*
Day 21	995 ± 78*	2.7 ± 0.2*	547 ± 65*	5.8 ± 0.4*	2.8 ± 0.2*
Day 28	727 ± 214*	3.2 ± 0.5*	428 ± 71*	6.4 ± 0.8*	2.7 ± 0.2*
Day 35	803 ± 209*	2.6 ± 0.2*	461 ± 87*	6.2 ± 0.8*	3.9 ± 0.7*
Day 42–49	784 ± 157*	not assayed	538 ± 99*	5.3 ± 0.8*	not assayed

^a ADH: Alcohol dehydrogenase.

^b Control group ($n = 11$), EtOH groups at day 14–day 35 ($n = 6$), group at day 42–49 ($n = 13$).

^c PNP: p-nitrophenol oxidation.

^d Data are presented as mean ± standard error. Groups all differed ($P < 0.05$) over time based on Kruskal Wallis non-parametric ANOVA.

* indicates $P < 0.05$ vs. control group using non-parametric Mann Whitney analysis.

trations (23, 24). Expression of mRNA and activity of the major hepatic alcohol metabolizing enzyme ADH class I and mRNA, apoprotein and activity of CYP2E1 are given in Table 2. After 14 d of ethanol infusion 2–6-fold increases in mRNA and activity were observed for both enzymes ($P < 0.05$).

The temporal pattern of development of liver pathology, serum ALT values and lipid peroxidation are shown in Table 3. Using Kruskal Wallis non-parametric ANOVA, there was a significant increase in all these parameters over time ($P < 0.05$). Using a non-parametric Mann Whitney test to compare two points of time, total pathology score after 14 d infusion increased compared to the control rats as the result of development of steatosis ($P < 0.05$). Further increases in the severity of steatosis occurred with longer periods of infusion ($P < 0.05$). Increased lipid peroxidation and serum ALT values in tail vein bleeds were observed at periods of infusion greater than 21 d ($P < 0.05$). Increases in inflammation; and necrosis scores were not apparent until 35 d. However, there were significant correlations between ALT values and lipid peroxidation (Pearson Correlation = 0.48, $P < 0.01$); between ALTs and CYP2E1 activity (Pearson Correlation = 0.42, $P < 0.01$) and between CYP2E1 activity and lipid peroxidation (Pearson Correlation = 0.49, $P < 0.01$).

Pathology in rats killed after 42–49 d of EtOH infusion was compared with pathology in the control group (Table 3, Fig. 2). Controls had normal hepatic morphology. Ethanol treatment resulted in extensive centrilobular macro- and microvesicular steatosis accompanied by inflammatory infiltrates, foci of necrosis and also exhibited significantly increased levels of apoptosis (Fig. 3) ($P < 0.05$). Serum ALT levels in trunk blood at sacrifice were also higher than controls (Table 3) ($P < 0.05$).

Cytokine expression levels were measured at the peaks of the EtOH pulses and assessed at the peak and nadir of UECs on d 42–49. Temporal profiles of cytokine mRNA expression are shown in Figure 4. Protein values for TNF α and IFN γ are shown in Figure 5. Expression of mRNA for TNF α showed a difference over time of ethanol treatment ($P < 0.013$) and occurred as two distinct peaks at 14 d ($P < 0.001$), and from 35 d onwards ($P < 0.013$). A similar temporal pattern was observed in the hepatic concentrations of TNF α protein ($P < 0.05$) (Fig. 5). There was a transient peak in expression of mRNA encoding the Th1 cytokine IFN γ at 14 d ($P < 0.05$) (Fig. 4) which was mirrored by an increase in mean protein concentration at that time point (Fig. 5) after which levels fell to at or below control for the remainder of the study. mRNA encoding another Th1

Table 3. Oxidative Stress and Liver Pathology in Relation to the Length of EtOH Infusion in the TEN Rat Model

Days of Infusion ^a	Oxidative Stress (TBARS) ^b	ALT ^c	Steatosis ^c	Inflammation ^c	Necrosis ^c	Total ^c
C	0.21 ± 0.03 ^d	78 ± 9	1.0 ± 0.0	1.25 ± 0.1	1.0 ± 0.0	3.25 ± 0.1
Day 14	0.23 ± 0.07	92 ± 14	2.9 ± 0.5*	1.75 ± 0.2	1.3 ± 0.1	5.95 ± 0.4*
Day 21	0.67 ± 0.18*	146 ± 23*	2.7 ± 0.4*	1.83 ± 0.3	1.3 ± 0.1	5.75 ± 0.7*
Day 28	1.20 ± 0.30*	148 ± 10*	3.8 ± 0.4*	1.83 ± 0.2	1.3 ± 0.2	7.00 ± 0.6*
Day 35	0.88 ± 0.24*	198 ± 14*	3.1 ± 0.4*	2.07 ± 0.5*	1.6 ± 0.3*	6.79 ± 0.9*
Day 42–49	0.82 ± 0.14*	137 ± 18*	4.0 ± 0.3*	2.00 ± 0.4*	1.7 ± 0.2*	7.69 ± 0.9*

^a Control group ($n = 11$), EtOH groups at day 14–day 35 ($n = 6$), group at day 42–49 ($n = 13$).

^b Oxidative stress assessed by measurement of lipid peroxidation (μmol thiobarbituric acid reactive material/g protein, TBARS).

^c Liver necrosis biochemically assessed using serum alanine amino transferase (ALT) activity (S.F. Units/ml). Pathology scores calculated as described in Materials and Methods. No pathology = 1 for individual categories, $n = 3$ for total score.

^d Data are presented as mean ± SEM. Groups all differed ($P < 0.05$) over time based on Kruskal Wallis non-parametric ANOVA.

* indicates $P < 0.05$ vs. control TEN animals, using non-parametric Mann-Whitney U analysis.

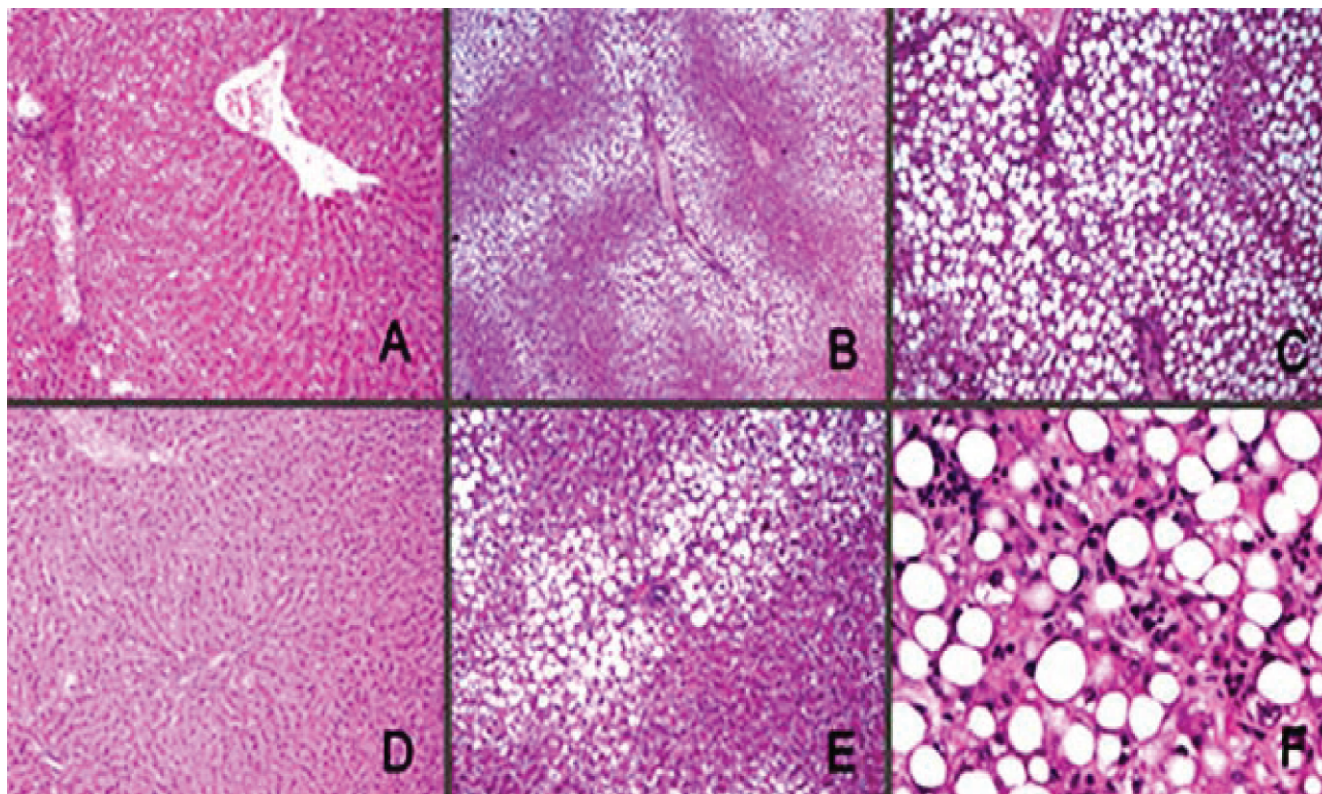


Figure 2. Liver pathology produced by chronic ethanol exposure in the TEN model. Panels A and D: controls; Panels B and E: perivenous macro/microsteatosis in EtOH-treated animals; Panels C and F: inflammatory foci and necrosis in EtOH-treated animals, low and high power images.

cytokine, IL-12 was suppressed to below control values from d 21 until 42–49 d of EtOH treatment ($P < 0.001$) but protein values were too low for accurate quantitation. Reductions in IL-12 mRNA expression were accompanied by significant reductions in expression of mRNA encoding the Th1 specific T-cell T-box transcription factor T-bet and STAT-4 protein in Western blots (Fig. 6). TGF β mRNA and IL-6 mRNA expression were unchanged by EtOH feeding (Fig. 4) ($P = 0.26$ and $P = 0.6$, respectively). Expression of

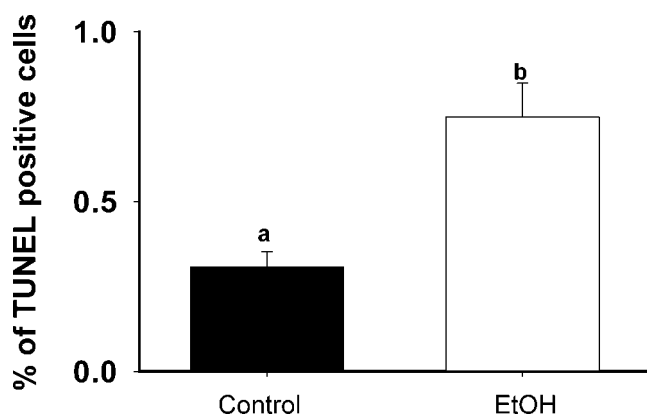


Figure 3. Apoptosis as determined using the TUNEL assay. Data are presented as mean $\pm$ SEM for $n = 11$ controls, and $n = 13$ rats treated with EtOH for 42–27 d. Means with different superscripts differ significantly ($P < 0.05$) where $b > a$, using one way ANOVA followed by Student's Neuman-Keuls post-hoc analysis.

mRNA encoding the Th2 cytokine IL-4 showed a dramatic and sustained decrease after EtOH administration ($P < 0.001$) (Fig. 4) and was accompanied by sustained reductions in mRNA expression of the Th2-specific transcription factor GATA-3 ($P < 0.05$) (Fig. 6). IL-4 protein and the mRNAs encoding other Th2 cytokines IL-5 and IL-13 were too low for accurate quantitation. There were significant negative correlations between the activities of ethanol metabolizing enzymes and expression of IL-4 mRNA (Pearson correlations of -0.43 and -0.48 , $P < 0.001$) and ADH and CYP2E1 activities. Cytokine mRNA expression in relation to peaks and nadirs of UEC pulses are shown in Figure 7. Expression of TGF β mRNA was increased and expression of IL-12 mRNA was decreased when blood EtOH levels were at their highest ($P < 0.05$). The expression of IL-4 was lower in both the EtOH-treated groups ($P < 0.01$).

Data for hepatic mRNA expression of chemokines CINC1 and CXCL2 are shown in Figure 8. Kruskal Wallis non-parametric ANOVA analysis demonstrated significant increases in expression over the time of EtOH infusion ($P < 0.05$); CXCL2 showed a steady increase in expression from the onset of EtOH administration up to day 35, after which it decreased back to levels approaching those seen in day 21 ($P < 0.05$). CINC1 mRNA levels were reduced from control at 14 d ($P < 0.01$) then increased and reached their highest levels at d 42–49 ($P < 0.007$). When examined in

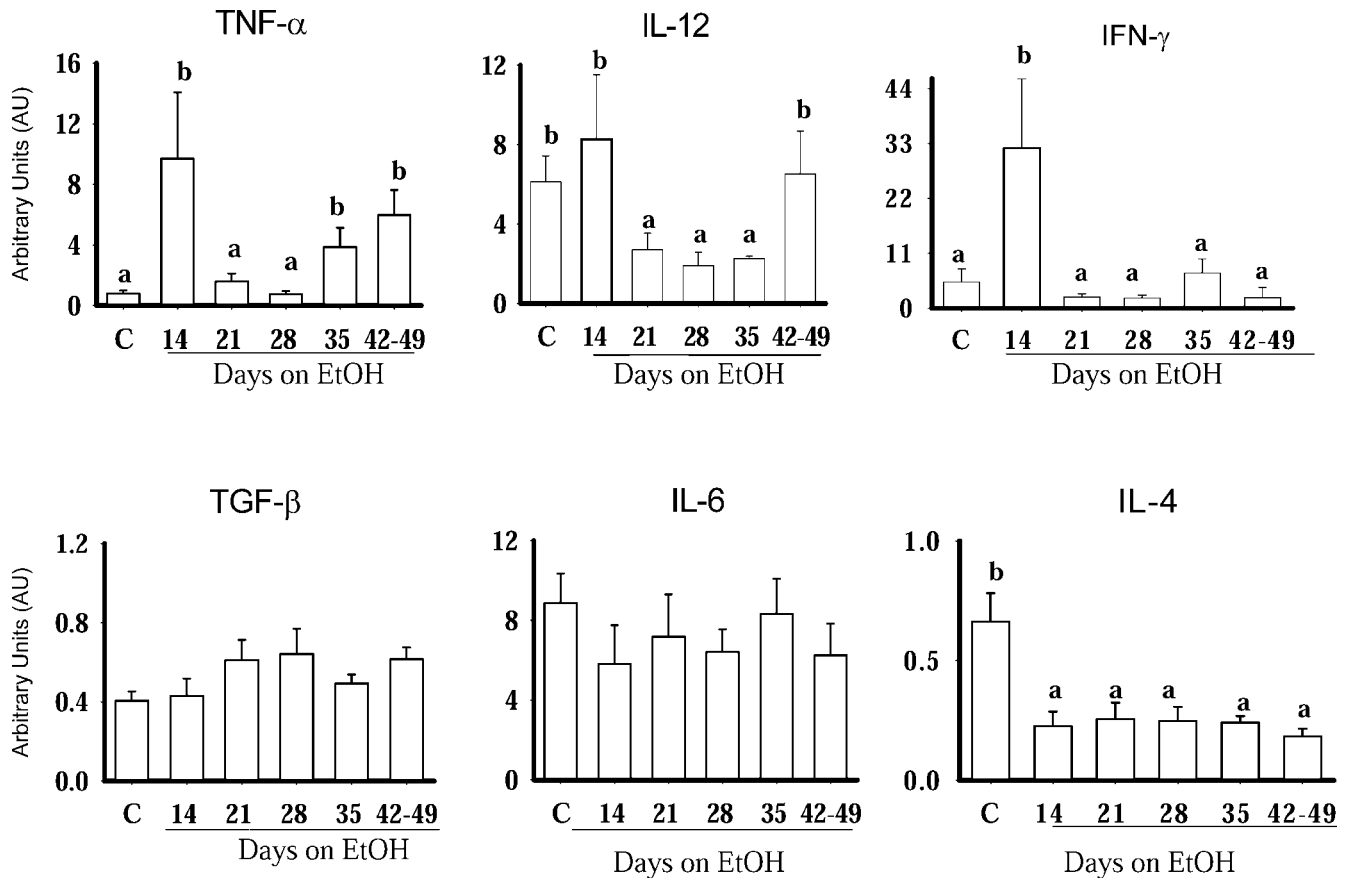


Figure 4. Expression of cytokines during the time course of EtOH treatment. mRNAs were quantified by real time RT-PCR, expressed in arbitrary units and normalized to cyclophilin A. Data are presented as mean $\pm$ SEM for $n = 11$ controls, $n = 6$ EtOH-treated rats sacrificed at UEC peaks after 14 d, 21 d, 28 d or 35 d, and $n = 13$ rats sacrificed after 42–49 d of EtOH treatment. Groups all differed ($P < 0.05$) over time based on Kruskal Wallis non-parametric ANOVA. Groups with different superscripts differ significantly ($P < 0.05$) where $b > a$, using non-parametric MannWhitney analysis to compare two points of time.

relation to high or low EtOH levels, there was a trend to increase CINC1 mRNA expression when EtOH levels were at their peak ($P = 0.12$). CINC1 expression after 42–49 d correlated well with inflammation score ($r^2 = 0.9$).

Proliferation of hepatocytes was assessed immunohistochemically by PCNA staining in rats sacrificed after 14 and 42–49 d corresponding to the first and second peaks of TNF α mRNA expression. The data are shown in Figure 9. A substantial increase in the % of cells in G1, S, G2 and M phases was observed only during the second TNF α peak and coincided with the appearance of inflammatory infiltrates, apoptosis and necrosis ($P < 0.05$).

Discussion

In this study we have described a detailed time course of the biochemical events and liver pathology produced by chronic intragastric feeding of ethanol to rats in the context of low carbohydrate, high polyunsaturated fat diets and related this to pulsatile patterns of urine ethanol concentration and to stimulation of tissue repair. To our knowledge, this is the first demonstration of the temporal relationship between cytokine and chemokine profiles and

the initial pathological stages of ALD. These data provide new insights into the mechanisms underlying the development of alcoholic steatohepatitis and alcohol-induced hepatocyte proliferation.

No increase in lipid peroxidation was observed at 14 d despite the development of steatosis, a 3-fold induction of CYP2E1 and a 2-fold increase in ADH. Although there was a significant overall correlation between activity of CYP2E1 and lipid peroxidation in liver homogenates, these data suggest that metabolism of ethanol may contribute less to the overall oxidative stress produced by chronic alcohol consumption than previously thought. Three weeks further ethanol exposure was required prior to the appearance of additional liver pathology including appearance of inflammatory infiltrates, necrosis, apoptosis and enhanced tissue repair. Increases in lipid peroxidation coincided with elevation of ALTs, suggesting that a significant amount of the oxidative stress may be secondary to liver injury. Many sources of oxidative stress have been suggested following EtOH exposure in addition to ethanol metabolism (31). These include reactive oxygen species produced by uncoupling of mitochondrial respiration in hepatocytes

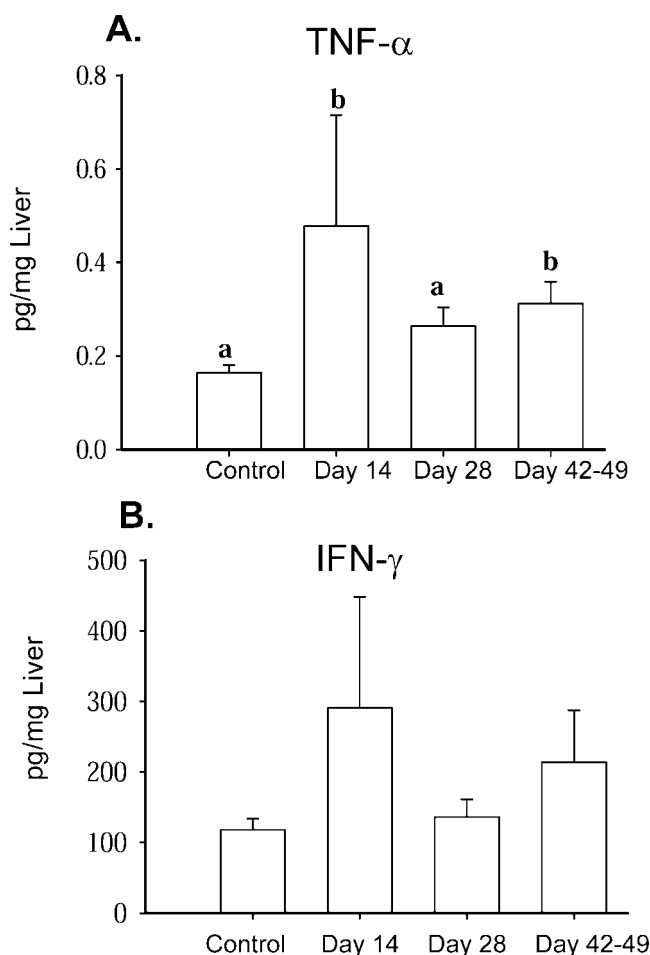


Figure 5. Expression of A: TNF α and B: IFN γ proteins in liver during the time course of EtOH treatment. Proteins were quantified by multiplex immunoassay and expressed as pg/mg liver protein. Data are presented as mean $\pm$ SEM for $n=6$ controls, $n=6$ EtOH-treated rats sacrificed at UEC peaks after 14 d, 28 d or 42–47 d of EtOH treatment. TNF α groups differed ($P < 0.05$) over time based on Kruskal Wallis non-parametric ANOVA. Groups with different superscripts differ significantly from control ($P < 0.05$) where $b > a$, using non-parametric Mann Whitney analysis to compare two points of time.

and species derived from Kupffer cell activation and inflammatory infiltrates. A major source of prooxidants in activated Kupffer cells, monocytes/macrophages are NAD(P)H oxidases (NOX). In addition inducible nitric oxide synthetase (NOS), xanthine oxidase (XO) and myeloperoxidase are present in monocytes and macrophages. Previous studies have shown protection against development of ALD by NOX, NOS and XO inhibitors and in p47^{phox} $-/-$ mice (31). In addition, mice deficient in expression of intercellular adhesion molecule-1 are also protected against oxidative stress and ALD which correlated with a decrease in neutrophil accumulation and myeloperoxidase activity in the liver (31).

The increase in TNF α mRNA and protein and decrease in IL-4 mRNAs following EtOH treatment are consistent with previous studies in the TEN model (5, 23–25), while the lack of effects of EtOH on hepatic IL-6 mRNA

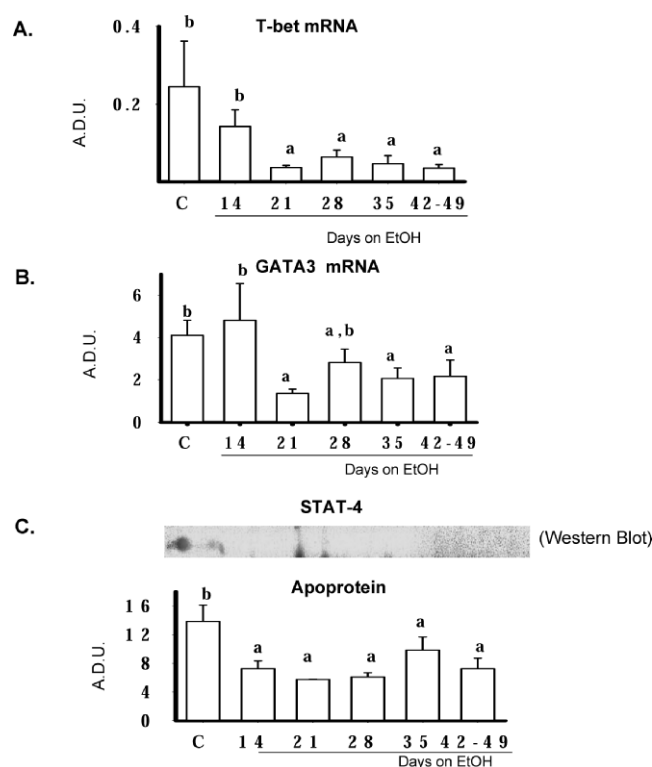


Figure 6. Hepatic expression of mRNA encoding the transcription factors T-bet and GATA3 and STAT4 apoprotein during the time course of EtOH treatment. mRNAs were quantified by real time RT-PCR, expressed in arbitrary units and normalized to cyclophilin A. STAT4 apoprotein expression was quantified by densitometric analysis of Western blots as described in Materials and Methods. Data are presented as mean $\pm$ SEM for $n=11$ controls, $n=6$ EtOH-treated rats sacrificed at UEC peaks after 14 d, 21 d, 28 d or 35 d, and $n=13$ rats sacrificed after 42–47 d of EtOH treatment. Groups all differed ($P < 0.05$) over time based on Kruskal Wallis non-parametric ANOVA. Groups with different superscripts differ significantly ($P < 0.05$) where $b > a$, using non-parametric Mann Whitney analysis to compare two points of time.

expression agree both with our previous studies and other studies of ethanol exposure in rats using the Lieber DeCarli model (5, 25, 32). Increases in expression of hepatic IFN γ following chronic ethanol have been reported following both Lieber DeCarli and intragastric ethanol feeding (9, 32). However, previous studies documenting changes in hepatic TGF β and IL-12 expression after ethanol treatment have been quite variable (5, 9, 14, 23–25). Part of this variability may relate to differences between liquid diet and intragastric infusion models of ethanol exposure. In addition to the length of exposure and stage of liver pathology, blood ethanol concentration and diet are also important variables which may influence cytokine and chemokine expression. In liquid feeding models such as Lieber DeCarli only low blood ethanol concentrations are attained and EtOH exposure always occurs in the context of underfeeding since rodents do not like to consume such diets and need to be pair-fed (23).

It has been suggested that the TNF α and IFN γ are derived from Kupffer cells and produced in response to

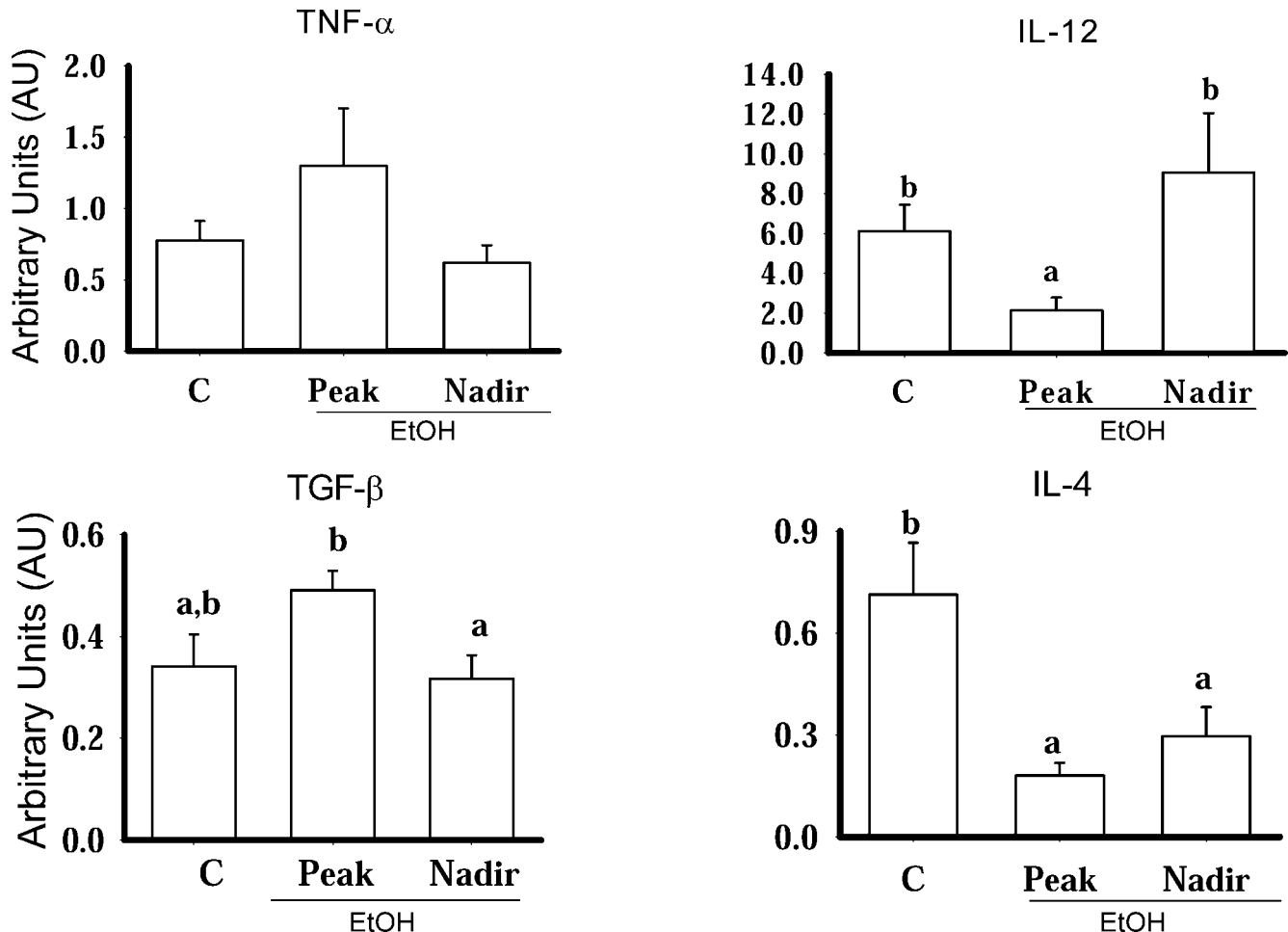


Figure 7. Expression of the cytokines at UEC peaks ($n = 7$) and nadirs in EtOH concentration ($n = 6$) after 42–49 d of EtOH treatment. mRNAs were quantified by real time RT-PCR, expressed in arbitrary units and normalized to cyclophilin A. Data are presented as mean $\pm$ SEM. Groups with different superscripts differ significantly ($P < 0.05$) where $b > a$, based on Kruskal Wallis non-parametric ANOVA.

endotoxin (LPS) which enters the circulation as the result of ethanol-induced gut permeabilization (1, 6–9, 33, 34). However, in the TEN model, we have previously reported a lack of endotoxemia in response to ethanol feeding and little evidence of LPS-dependent Kupffer cell activation such as increased expression of CD14 (23, 24). It is possible that cellular stress resulting from alcohol metabolism or related to the development of steatosis can result in transient activation of Kupffer cells to produce TNF α and IFN γ independent of LPS stimulation. It is also possible that that cytokines measured in whole liver homogenates after ethanol treatment are derived at least in part from other cell types such as resident natural killer T cells (NKT cells) which are abundant in the liver and are part of the innate immune system (35) or other populations of liver associated lymphocytes (36). These cells are known to produce cytokines, are activated in liver following ethanol treatment and have been implicated in alcohol-induced liver injury (35–37). In addition, peripheral blood monocytes which can migrate to the liver are also a known source of TNF α and IL-12 in alcoholic patients (38). Although Kremer et al. (10)

have demonstrated that steatosis produced in mice by dietary manipulation can exacerbate development of inflammatory hepatitis as the result of stimulating a sustained shift towards a Th1 cytokine response, we have not observed this shift in the current rat model where steatosis results from alcohol treatment. Initial transient increases in TNF α and IFN γ expression at 14 d decreased to control values or below following more prolonged EtOH exposure and were accompanied by repression of the Th1 cytokine IL-12 until the appearance of inflammatory infiltrates after 6–7 weeks of treatment. IL-12 controls the expression of both T-bet and STAT4 which are considered crucial for Th1 commitment and reductions in both of these factors accompanied the down-regulation of IL-12 expression. These data are consistent with an acute innate immune response to EtOH followed by a subsequent repression of IL-12 and Th1 responses proposed by Wheeler (8) associated with the progression of ALD.

EtOH treatment substantially suppressed expression of IL-4, a pleiotropic cytokine which is a growth factor for preactivated B and T lymphocytes and a repressor of MHC-

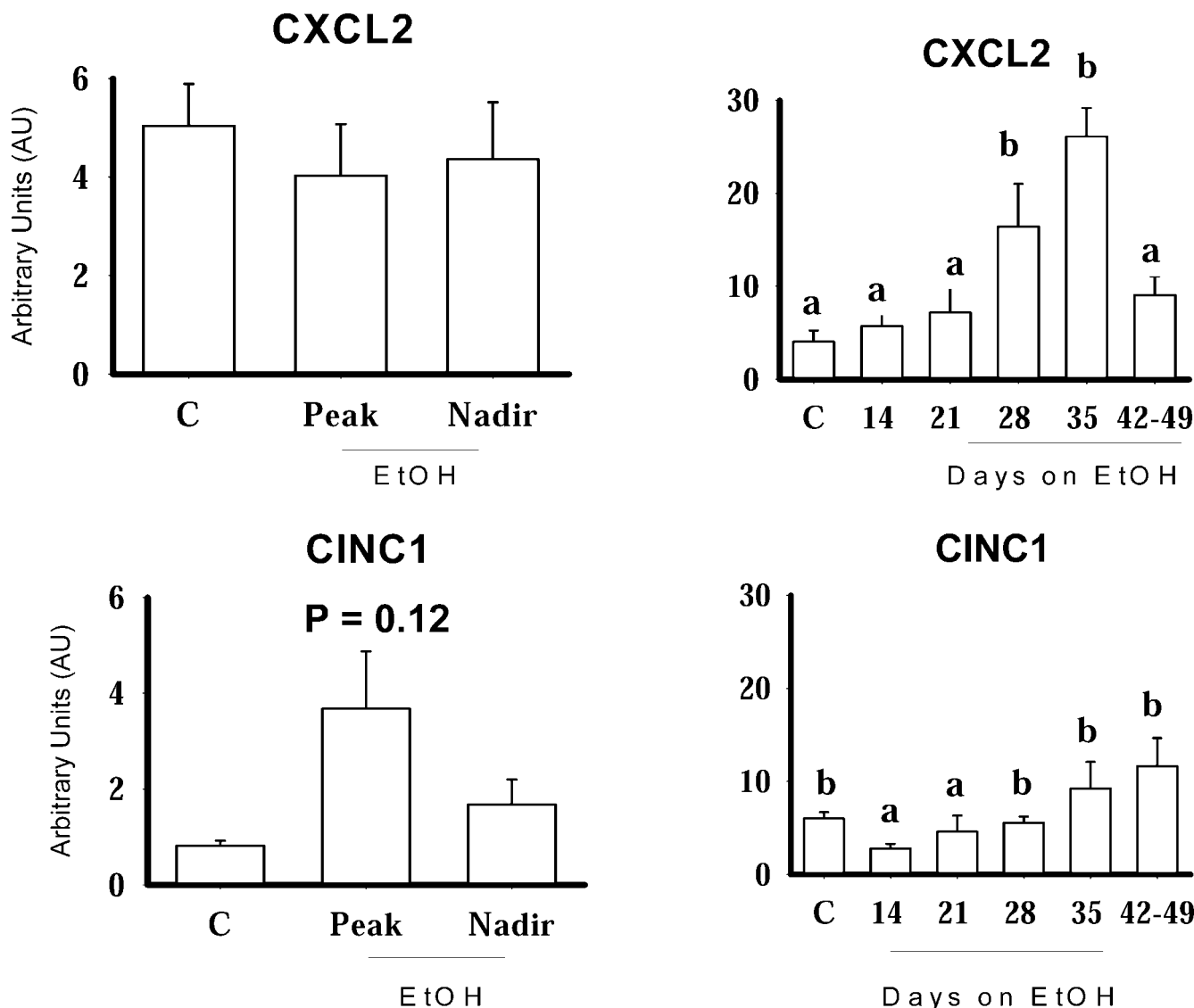


Figure 8. Expression of the chemokines CXCL2 and CINC1 in relation to time of EtOH infusion and in peak and nadirs of UEC pulses in the TEN model. For UEC peaks ($n=7$) and nadirs in EtOH concentration ($n=6$) after 42–49 d of EtOH treatment, mRNAs were quantified by real time RT-PCR, expressed in arbitrary units and normalized to cyclophilin A. Data are presented as mean $\pm$ SEM. Groups with different superscripts differ significantly ($P < 0.05$) where $b > a$, based on Kruskal Wallis non-parametric ANOVA. For time course, mRNAs were quantified by real time RT-PCR, expressed in arbitrary units and normalized to cyclophilin A. Data are presented as mean $\pm$ SEM for $n=11$ controls, $n=6$ at 14 d, 21 d, 28 d, 35 d, and $n=13$ at 42–47 d of EtOH treatment. Groups all differed ($P < 0.05$) over time based on Kruskal Wallis non-parametric ANOVA. Groups with different superscripts differ significantly ($P < 0.05$) where $b > a$, using non-parametric Mann Whitney analysis to compare two points of time.

mediated immune responses (37). These data and the reduction of GATA3 expression suggest a sustained suppression of Th2 responses. A suppression of the Th2 response may contribute to the autoimmune response mounted against protein adducts of lipid peroxidation products, acetaldehyde and the hydroxyethyl radical described in previous rodent and human studies of ALD (2, 25, 39). Reduced IL-4 has also been suggested to contribute to the induction of TNF α during ischemia/reperfusion injury (40) and to increase oxidative stress by increasing levels of reactive oxygen and nitrogen species (41). The current data suggest IL-4 suppression is an early

and sustained event in ALD and that it may be dependent on ethanol metabolism but the molecular mechanisms underlying this effect are currently unknown. Although it has been suggested that more chronic EtOH treatment may produce a switch from Th1 to a Th2 cytokine profile including increases in IL-4, IL-5 and IL-13 and increased TGF β production associated with the development of fibrosis (8) no evidence for such a response or for fibrotic lesions was observed in the current study following up to 49 d of EtOH treatment. It may be that detection of such increased Th2 responses would require a considerably longer period of EtOH treatment since fibrosis does not

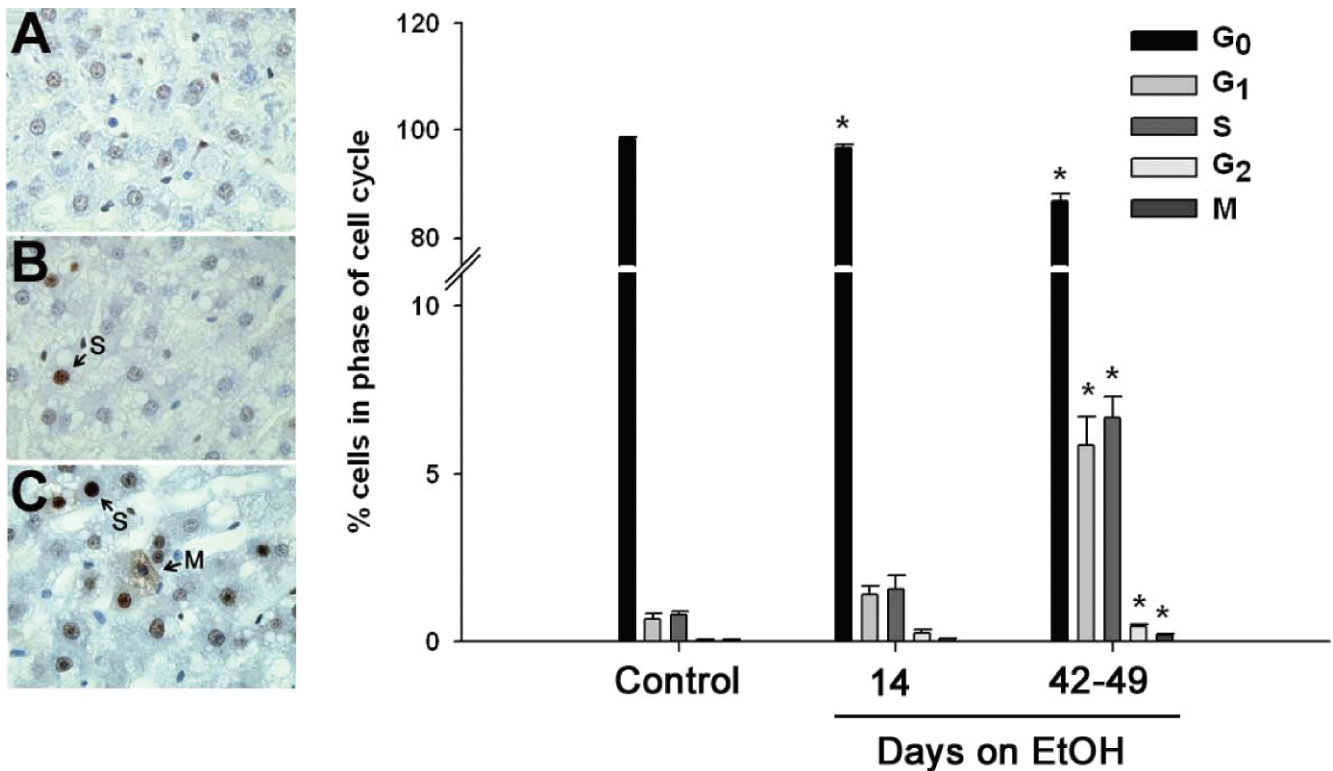


Figure 9. Hepatocyte proliferation in the livers of rats chronically treated with EtOH in the TEN model. PCNA analysis of cell cycle by immunohistochemistry from control ($n = 11$) (A) or EtOH-treated rat livers at 14 d ($n = 6$) (B) or 42–49 d ($n = 13$) (C). G0 cell with blue nuclear staining, G1 cell with light-brown nuclear staining, S-phase (S) cell with dark brown nuclear staining, G2 cell with cytoplasmic staining and with or without speckled nuclear staining, metaphase (M) cell with diffuse cytoplasmic and deep blue chromosomal staining. Original magnification $\times 20$. Data for % cells in each phase are presented as mean $\pm$ SEM. Means with different superscripts differ significantly ($P < 0.05$) where $b > a$, based on one way ANOVA followed by Student's Neuman-Keuls post-hoc analysis.

develop in rodent intragastric models of EtOH exposure until after 16–20 weeks (42).

It has been suggested that chemokines such as CINC1 and CXCL2 produced in the liver in response to EtOH could be responsible for subsequent neutrophil infiltration (33). Studies in human fetal hepatocytes and heptoma cells exposed to EtOH *in vitro* have demonstrated marked increases in expression of the chemokine IL-8 associated with inhibition of proteasome function and activation of JNK (43). In the current study, CXCL2, which is a rat analogue of human IL-8 (44), increased steadily following EtOH treatment and may contribute to the appearance of inflammatory infiltrates. However, expression of CINC1 was initially suppressed and only returned to control values after 35 d correlating with the degree of inflammation. Since CINC1 can be produced by macrophages and neutrophils (15–17), it is possible that increased expression of this chemokine after chronic EtOH exposure is actually a consequence rather than a cause of the appearance of inflammatory infiltrates.

In addition to pro-inflammatory effects and involvement in cell death, TNF α has also been proposed to play an important role in tissue repair following injury. Data from experiments with acute hepatotoxins such as acetaminophen and carbon tetrachloride in TNFR1 knockout mice

found increased rather than decreased injury as a result of impaired hepatocyte proliferation in response to tissue injury (11–13, 45). The current study demonstrated that the second, late peak of TNF α expression coincident with the appearance of inflammatory infiltrates and necrosis also coincided with significant increases in hepatocyte proliferation. This is consistent with the hypothesis that TNF α derived from inflammatory infiltrates plays a pleiotropic role, stimulating tissue repair in response to damage in addition to apoptosis and possibly contributing to an increased incidence of hepatocellular carcinoma (46). However, the data also show that elevated TNF α is insufficient in itself to produce hepatocyte proliferation. No increase in proliferation or shift in cell cycle was observed at the time of the first peak of TNF α and thus complex interactions of TNF α with other growth factors at different times following ethanol exposure and at different stages of tissue injury must ultimately be responsible for the enhanced proliferative responses. These data are consistent with a recent study by Isayama et al. (12) in mice fed EtOH intragastrically in which increases in hepatocyte proliferation following 4 weeks of EtOH treatment were associated with activation of the proto-oncogene Ras and were blunted in TNFR1 $-/-$ mice (12). Previous studies using the Lieber DeCarli model have reported a much more rapid increase in

hepatocyte proliferation following as little as one week of EtOH feeding (47). However, in this model, the aversion of rodents to EtOH results in reduced consumption of diet and a necessity for pair-feeding. Previous findings from Apte et al. (48) suggested that diet restriction protects against hepatotoxicity by enhancing promotogenic signaling. We have recently demonstrated that undernutrition can exacerbate EtOH-induced hepatocyte proliferation in the TEN model (46).

Taken together these data demonstrate complex temporal patterns of hepatic cytokine and chemokine synthesis in response to chronic ethanol exposure and further elucidate their relationship to the subsequent infiltration of inflammatory cells, development of liver pathology and proliferative repair responses.

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