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TRIF and IRF-3 Binding to the TNF Promoter Results in Macrophage TNF Dysregulation and Steatosis Induced by Chronic Ethanol

Xue-Jun Zhao,* Qing Dong,* Julie Bindas,† Jon D. Piganelli,‡ Amy Magill,* Jakob Reiser,‡ and Jay K. Kolls2∗

Chronic ethanol (EtOH) abuse results in the development of steatosis, alcoholic hepatitis, and cirrhosis. Augmented TNF-α production by macrophages and Kupffer cells and signaling via the p55 TNF receptor have been shown to be critical for these effects of chronic EtOH; however, the molecular mechanisms leading to augmented TNF-α production remain unclear. Using cell culture models and in vivo studies we demonstrate that chronic EtOH results in increased TNF-α transcription, which is independent of NF-κB. Using reporter assays and chromatin immunoprecipitation we found that this increased transcription is due to increased IRF-3 binding to and transactivation of the TNF promoter. As IRF-3 is downstream from the TLR4 adaptor TIR-domain-containing adapter-inducing IFN-β (Trif), we demonstrate that macrophages from Trif−/− mice are resistant to this dysregulation of TNF-α transcription by EtOH in vitro as well as EtOH-induced steatosis and TNF dysregulation in vivo. These data demonstrate that the Trif/IRF-3 pathway is a target to ameliorate liver dysfunction associated with chronic EtOH. 


E thanol (EtOH) abuse significantly alters the host immune system in both patients and animal models (1). A number of critical immune responses including cytokine elaboration by macrophages are altered by EtOH. Among these cytokines, TNF-α production has been extensively studied in EtOH-intoxicated human subjects and animals (2–5). Our laboratory and others have demonstrated that acute EtOH exposure suppresses TNF-α production from monocytes and macrophages (2–5). However, chronic EtOH abuse results in increased macrophage production of TNF-α from both human PBMCs as well as Kupffer cells from experimental animal models of chronic EtOH abuse (6). Moreover, mice deficient in TLR4 or TNFRI signaling are remarkably protected against liver dysfunction induced by chronic EtOH (7, 8). Despite this, the cellular mechanisms involved in augmented TNF-α production by macrophages induced by chronic EtOH have not been fully elucidated. Studies by Nagy et al. (9) have shown that the transcription factor Egr-1 plays a key role in increased TNF production by Raw264.7 cells and in rat Kupffer cells (10) after exposure to chronic EtOH. However, Egr-1 nuclear translocation is not increased in human macrophages by chronic EtOH (11) (and unpublished observations), suggesting that other mechanisms are involved in increasing TNF-α production in macrophages by chronic EtOH. To further understand mechanisms by which chronic EtOH leads to aberrant TNF-α production, we developed an in vitro model using human Mono Mac 6 cells (12). These cells as well as primary murine bone marrow (BM)-derived macrophages cultured in chronic EtOH (for up to 6 days) show a redox-dependent increase in stimulated TNF-α production as well as increased TNF-α mRNA (12). Using these in vitro models we show that the increased TNF-α transcription observed in chronic EtOH is independent of NF-κB. Using a series of reporter assays and studies with deletion mutants of the human TNF promoter we observed increased IRF-3 reporter activity and increased IRF-3 binding to the human TNF-α promoter in cells exposed to chronic EtOH. To investigate the role of IRF-3 in liver dysfunction and TNF-α dysregulation in vivo, we examined female wild-type (WT) mice or mice lacking the critical upstream regulator of IRF-3 activation in response to LPS, TIR-domain-containing adapter-inducing IFN-β (Trif). WT or Trif−/− mice fed a Lieber-DeCarli diet where 36% of the calories are derived from EtOH (13, 14). To induce hepatitis, at the end of 4 wk of the diet, mice were administered 1.0 μg/g weight of LPS immunoprecipitation (IP) as previously described by McClain et al. (15). Trif−/− mice were significantly protected from liver injury in this model and failed to show EtOH-induced augmented TNF-α responses that were observed in WT mice fed chronic EtOH. Moreover, Trif mice showed significantly less steatosis. Taken together, these data show that the Trif/IRF-3 pathway is critical for EtOH-induced TNF-α dysregulation in macrophages and EtOH-induced liver dysfunction and suggest that this pathway represents a novel target to prevent or treat liver dysfunction induced by EtOH.

Materials and Methods

Cells and reagents

The human Mono Mac 6 cell line was obtained from DSMZ. Escherichia coli LPS 0111:B4 was purchased from List Biological Laboratories, and
PMA from Sigma-Aldrich, pIFN-stimulated response element (pISRE)-Luc and pAP-1-Luc plasmids were from Clontech Laboratories. pTNF(−528)Luc, pTNF(−387)Luc, and pTNF(−120)Luc were generously provided by Dr. J. Economou (University of California-Los Angeles, Los Angeles, CA). Mouse and human TNF-α were measured by ELISA kits purchased from R&D Systems.

Mono Mac 6 cells were cultured in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 1× nonessential amino acid, and 1× penicillin-streptomycin (Invitrogen). The cells were incubated in 5% CO2 at 37°C and treated with 0, 50, or 100 mM EtOH for up to 6 days in a “master flask,” with monocytes/cm2 density of 1×106/ml. Mouse BM cells were harvested using the TransFactor Extraction Kit (BD Biosciences). Total protein was determined and equal amounts of nuclear protein were assayed in a chronic EtOH incubator system which has been previously described.

At various time intervals, cells were stimulated with LPS/PMA and TNF was measured 3 h later by ELISA (n = 4–5 per group; *p < 0.05 compared with 0 mM control). At day 9, the cell media with 12 ng/ml murine GM-CSF was added. On day 4, the BM cells were treated with 0 or 50 mM EtOH for another 5 days. On day 9, the cell media and floating cells were discarded; the fresh media with 12 ng/ml murine GM-CSF was added. On day 4, the BM cells were incubated with 0 or 100 ng/ml LPS (E. coli 0111:B4; List Biological Laboratories). The culture supernatants were collected and stored at −20°C for late analysis of TNF-α by ELISA. Macrophage preparations were also harvested and stained with 10 μg/ml anti-CD-11b (BD Biosciences). Cells were analyzed on a Becton Dickinson FACSAria flow cytometer. CD11b positivity in macrophage cultures was over 90%.

Real-time PCR analysis for TNF-α

Total RNA was isolated from Mono Mac 6 cells treated with and without EtOH by a single step method using TRIzol reagent as per manufacturer’s instructions. Thereafter, cDNA was transcribed to cDNA by real-time PCR was performed according to the TaqMan Two-Step Real-Time PCR Master Mix Reagents Kit protocol supplied by the manufacturer (Applied Biosystems). Human TNF-α primers and probe were obtained from Maxim Biotech. This assay has a correlation coefficient of 0.98 over 6-logs of TNF-α RNA concentration.

TNF-α mRNA half-life assay

A total of 1 μg/ml actinomycin was added to the culture at 0, 30, and 60 min after stimulation with LPS/PMA. Mono Mac 6 cells were collected at each time point and TNF-α mRNA was measured by real-time PCR described previously.

Measurement of nuclear NF-κB, p65, p50, FoxB, and Egr-1

Mono Mac 6 cells culture in control or EtOH conditions were harvested 1 h after no stimulation or stimulation with LPS/PMA and nuclei were harvested using the TransFactor Extraction Kit (BD Biosciences). Total proteins was determined and equal amounts of nuclear protein were assayed for NF-κB p65, p50, FoxB, and Egr-1 using specific Abs to these proteins in a sandwich ELISA (BD TransFactor assay).
FIGURE 2. Chronic EtOH inhibits NF-κB signaling in Mono Mac 6 cells. Nuclear NF-κB p65 (A) or p50 (B) were measured 1 h after LPS/PMA stimulation as described in Materials and Methods (n = 5–6 per group; * denotes p < 0.05 compared with negative LPS/PMA control). C, NF-κB luciferase activity in Mono Mac 6 NF-κB reporter cells cultured in 0, 25, or 50 mM EtOH for 6 days and stimulated with LPS/PMA for 6 h (n = 5–6 per group; * denotes p < 0.05 compared with negative LPS/PMA control). D, LPS/PMA-stimulated TNF-α responses at 3 h in Mono Mac 6 cells expressing a IkBBSR or control vector (n = 4–6 per group; * denotes p < 0.05 compared with 0 mM EtOH control, ** denotes p < 0.05 compared with 0 mM IkB control vector, *** denotes p < 0.05 compared with respective 0 mM EtOH control with IkB overexpression).

Chromatin IP (ChIP) assay for human IRF-3

Mono Mac 6 cells culture in control or EtOH conditions were plated to 24-well plate at a cell density of 2 × 10^5 of cells/ml/well, and stimulated with LPS and PMA for 1 h as stated above. Then the cells were crosslinked with 1% (v/v) formaldehyde at 37°C for 20 min. Following crosslinking, the cells were resuspended in SDS lysis buffer with protease inhibitors (Upstate Cell Signaling Solutions). Total cell lysate was isolated, and the genomic DNA was sheared to sizes between 200 bp and 1000 bp by sonication on ice. The sample was precleared with Salmon Sperm DNA/protein A-agarose beads for 30 min at 4°C to reduce nonspecific background. ChIP assay was performed with a rabbit polyclonal Ab to human IRF-3 (Santa Cruz Biotechnology) or with normal rabbit IgG (Santa Cruz Biotechnology) as a negative control according to the protocol of Upstate Cell Signaling Solutions. The immunocomplex was heated at 65°C for 4 h to reverse the crosslinking between DNA and proteins. DNA was purified by repeated phenol/chloroform extraction and EtOH precipitation. The purified DNA (designated as bound) was dissolved in 20 μl of TE buffer. The DNA purified using the same procedure with omission of the IP step was designated as the input DNA. Both bound and the input DNA were analyzed by PCR (35 cycles) with primers that amplify a 310-bp fragment of the human TNF promoter region, circumventing the IRF sequence. The PCR condition was 95°C for 5 min, 95°C for 45 s, 58°C for 45 s, 72°C for 45 s, and 72°C for 10 min. The resulting product of 310 bp in length was separated by 1.6% agarose gel electrophoresis. The primer pairs used for human TNF promoter were: forward 5'-ACTACCGGCCTTCCTCCAGAT GAG-3'; reverse 5'-TCATGGTGTCCTTTCCAGGG-3'. ChIP assays were also validated by real-time PCR using the same primers and SYBR green and expressed as ΔΔCT in relation to a no template control.

Generation of lentiviral reporter vectors

To generate lentiviral reporters, the firefly luciferase gene was amplified by PCR from pIRES-Luc (BD Clontech) and cloned into pENTR/D-TOPO (Invitrogen). ISRE, AP-1, NF-κB, TNF-528, −387, and −120 were then each cloned into pENTR 5′-TOPO. To construct the lentivirus destination vector with luciferase and the appropriate reporter element, a LR recombination reaction was performed between pENTR/D-TOPO luciferase vector, pENTR 5′TOPO reporter vector, and pLenti6/R4R2/V5-DEST (Invitrogen). The resulting plasmids were then cotransfected using CaPO4, with ViraPower Packaging Mix (Invitrogen) in 293T cells. The virus was purified 60–65 h after transfection, concentrated, and quantified by real-time PCR for luciferase. To investigate the putative IRF-3 site within the TNF promoter, nucleotides AAGAAACCGAGACAGAAGG were deleted from the TNF-528 promoter using PCR as described by Higuchi (16).

Cell transduction with lentivirus and luciferase assay

Mono Mac 6 cells were transduced by lentivirus with ISRE, AP-1, and TNF-α promoters driving luciferase as a reporter. Cells (5 × 10^5/ml) mixed with viral supernatants in the presence of 8 μg/ml polybrene in a 5-ml tube were centrifuged at 1800 g for 3 h at 32°C. After overnight culture with viral supernatants, cells were washed and transferred to a 24-well plate and incubated in fresh culture medium. To generate a stable cell line, 3 μg/ml blastidicin (Invitrogen) was added to the cells, and medium was changed every 3 days with blastidicin until drug-resistant clones appeared. BM cells were transduced overnight by lentivirus with ISRE, AP-1, driving luciferase as a reporter on day 3 before treating EtOH, and followed with EtOH and LPS as described above. After stimulation by LPS/PMA (for Mono Mac 6 cells) or LPS (for BM cells) for 6 h, the cells were harvested and ISRE, AP-1, or TNF-α promoters activities were analysis using the Luciferase Reporter Assay System (Promega). The luciferase activities were normalized by total cellular proteins.

FACS analysis

BM-derived macrophages profile was determined by flow cytometry. After blocking with 1% BSA in PBS, the macrophages were labeled with a rat anti-mouse CD11b Ab (10 μg/ml) and then fixed with 1% formaldehyde. The CD11b-positive cells in BM-derived macrophages were over 90%.

Experimental model of alcoholic hepatitis

Female 6- to 8-wk-old WT or Trif−/− mice (The Jackson Laboratory) were acclimated to isocaloric to EtOH containing Lieber-DeCarli diet (Bio-Serv) for 2 wk followed by 4 wk of 36% EtOH-derived calories in the EtOH group. After 4 wk, to induce hepatitis, mice were administered 1.0 μg/g weight E. Coli LPS IP. Mice were euthanized at 2 h for serum TNF and liver histology or at 6 h for serum alanine aminotransferase (ALT).
Measurement of mouse serum TNF-α and ALT levels

TNF-α and ALT level from mouse serum were determined by ELISA (R&D Systems) and ALT kit (Biotron Diagnostics), respectively, according to the manufacturer’s instructions.

Liver histology

Formalin-fixed tissues were paraffin embedded, sectioned, and stained with H&E at Histo-Scientific Research Laboratories. Steatosis was scored on 10 random low powered fields using a scoring system of 0–3 as described by Brunt and colleagues (17).

Statistical methods

All data are presented as mean ± SEM. Significance was estimated using ANOVA followed by Tukey’s Multiple Comparison Procedure with \( p < 0.05 \) being considered significant.

Results

NF-κB-independent activation of TNF-α transcription by chronic EtOH in vitro

Although mice with defective TLR4 and TNFRI signaling are protected against alcohol-induced liver injury in vivo (7, 8), the mechanisms of EtOH-induced augmentation of TNF-α production remains undefined. To better define molecular mechanisms behind this response, we developed an in vitro model (12) where macrophages are exposed to chronic EtOH. Using the Mono Mac 6 cell line (18), cultures of these cells in EtOH for 6 days (or beyond) results in augmented TNF-α production in response to stimulation with LPS/PMA (Fig. 1A) (12). We have examined TNF-α production as far out as 28 days in cells cultured in EtOH and TNF production remains elevated (unpublished observations); however for the purpose of these studies, we focused on the 6 day time point. Similar results have also been observed with U937 and THP-1 cells (data not shown) and with primary murine BM-derived macrophages (see below). To assess whether the
increased TNF-α production was due to increased TNF-α transcription, we performed in vitro analysis of transcripts for TNF-α from purified nuclei 1 h after stimulation using quantitative real-time PCR and normalized these data to 18 s rRNA-encoding DNA transcripts. Cells exposed to EtOH for 6 days followed by stimulation with LPS/PMA displayed significantly greater numbers of transcripts compared with cells not exposed to EtOH (Fig. 1B). Using actinomycin D to arrest transcription 1 h after LPS/PMA stimulation, we also observed a modest increase in TNF-α mRNA stability by EtOH (Fig. 1C), but this effect was modest compared with the increase in transcription (Fig. 1B). The increase in TNF-α mRNA stability was inhibited by the p38MAPK inhibitor SB203580 (Fig. 1D), a known regulator of TNF-α mRNA stability; however, this compound had a minimal effect on the augmented TNF-α production in cells exposed to chronic EtOH (Fig. 1E), again supporting a role for augmented TNF transcription in EtOH-induced TNF-α superinduction. Optimal TNF production from Mono Mac 6 cells requires LPS and PMA whereas, in primary macrophages, LPS is sufficient to induce TNF-α. To exclude an artifact of the dual stimulus used on the human cell line to induce TNF-α, we generated BM-derived macrophages in the presence of 0 or 50 mM EtOH. At the end of a 6-day culture period in control or EtOH, grown in chronic EtOH showed significantly augmented TNF-α production to LPS (Fig. 1F) similar to immortalized Mono Mac 6 cells. There were no differences in cell viability or cell cycle between control or EtOH cultures (data not shown).

As NF-κB is a key transcription factor regulating TNF-α transcription, we assessed nuclear levels of NF-κB p65 and p50 by ELISA, normalized for nuclear protein. LPS/PMA stimulation resulted in a significant increase in nuclear NF-κB p65 and p50 (Fig. 2, A and B); however, these levels were actually decreased in cells cultured in chronic EtOH despite augmentation of TNF-α transcription and protein production (Fig. 1). These data were confirmed by generating a permanent Mono Mac 6 cell line encoding a NF-κB luciferase reporter using lentiviral-mediated gene transfer (NF-κB Luc/Mono Mac 6 cells, Fig. 2C). When NF-κB Luc/Mono Mac 6 cells were stimulated with LPS/PMA, there was significant induction of NF-κB transcriptional activity. However, this activity was unaltered by culture in chronic EtOH (Fig. 2C). Furthermore, to exclude a role of NF-κB, we generated a Mono Mac 6 cell line encoding an IκBα super repressor (IκBαSR) (19). Overexpression of the IκBαSR resulted in nearly a 75% reduction in LPS/PMA-stimulated TNF response in these cells compared with a mutant IκBαSR (Fig. 2D); however, despite expression of the IκBαSR, there remained a significant augmentation of TNF production by chronic EtOH in cells expressing the IκBαSR (Fig. 2D). Taken together, these data demonstrate that transactivation of TNF production by EtOH is independent of NF-κB.

Critical role of Trif/IRF-3 in TNF-α transcription induced by chronic EtOH in vitro

To explore other potential transcription factors that may be involved in EtOH-induced TNF transactivation, we harvested nuclear proteins in Mono Mac 6 cells grown in control or EtOH conditions 60 min after LPS/PMA stimulation. Nuclear FosB and Egr-1 were measured by ELISA, and IRF-3 was assayed by Western blot (Fig. 3A). We observed significant increases in nuclear FosB, an AP-1 family member, and IRF-3 but not Egr-1 (Fig. 3A). To understand cis-regulatory elements in regulating the augmented TNF transcription by EtOH in vitro, we generated Mono Mac 6 cells stable transfectants with recombinant HIV-1-based vectors encoding deletion mutants of the TNF promoter driving firefly luciferase. In these stable transfectants, both the −528 and the −327 promoter showed significant transactivation by LPS/PMA (Fig. 3B) as previously described in transient transfection systems (20), and these promoters showed the greatest transactivation in the presence of 50 mM EtOH (Fig. 3B).

Bio-informatic analysis of the human TNF-α promoter (www.genomatix.de) revealed a putative AP-1 sites at position −439 to −429 and an IRF-3 site at −319 to −301. To investigate whether AP-1 and IRF-3 activities were increased in these cells, we generated stable Mono Mac 6 cell transfectants with recombinant HIV-1-based vectors encoding AP-1 (Fig. 3C) or
an ISRE, a measure of IRF-3 activity (Fig. 3D) driving luciferase. LPS/PMA stimulation alone or in the presence of EtOH significantly increased AP-1 activation at 3, 6, and 24 h after stimulation (Fig. 3C). In contrast to AP-1, we observed a specific effect of the combination of LPS/PMA and EtOH (as opposed to LPS/PMA alone) on ISRE activity in stimulated cells at 3 and 6 h, suggesting that IRF-3 transcriptional activity was induced selectively by the combination of LPS/PMA and chronic EtOH (Fig. 3D). To confirm the requirement of the IRF-3 site in the TNF promoter for the transactivation observed with TNF-528 promoter, we generated a Mono Mac 6 cell line carrying the TNF-528 promoter with a deletion of the putative IRF-3 site (TNF-528delRIF3). Compared with the WT TNF-528 promoter, the TNF-528delRIF3 construct showed production by LPS/PMA stimulation (Fig. 3E) but no transactivation by chronic EtOH. To examine whether IRF-3 was binding specifically to the human TNF promoter, we performed ChIP in Mono Mac 6 cells (Fig. 4A). Control cells (Fig. 4A, lane 4) showed no IRF-3 binding. There was minimal enhancement of IRF-3 binding to the TNF promoter by EtOH (Fig. 4A, lane 3). LPS/PMA stimulation for 90 min resulted in increased IRF-3 binding (Fig. 4A, lane 2), whereas the combination of chronic EtOH and LPS/PMA stimulation significantly increased IRF-3 binding (Fig. 4A, lane 1). These data were also confirmed by real-time PCR (Fig. 4A). IRF-3 activation upon TLR4 stimulation with LPS is regulated by the MyD88-independent/Trif-dependent pathway (21–23). Therefore, we investigated whether EtOH-induced TNF transactivation and ISRE activation was dependent on Trif in mouse BM-derived macrophages. Macrophages cultured in 50 mM ETOH showed significant enhancement of TNF (Fig. 4B), whereas this transactivation was absent in macrophages form Trif−/− mice. Transduction of these cells with the lentiviral ISRE reporter showed a significant increase in ISRE activity in cells cultured in EtOH and stimulated with LPS that was completely absent in Trif−/− macrophages (Fig. 4C).

Critical role of Trif in TNF-α production induced by chronic EtOH and steatosis in vivo

To investigate whether the Trif pathway was involved in alcohol-induced liver injury and steatosis, which is dependent on TLR4 signaling and p55 TNFR signaling, we placed female mice on a Lieber-DeCarli diet where 36% of the calories are derived from EtOH or pair-fed control mice. To induce systemic TNF responses at the end of 4 wk, mice were administered vehicle or 1.0 g/g of body weight of LPS by i.p. injection. Mice injected with vehicle had no detectable TNF responses in serum (data not shown). Mice fed the EtOH-containing diet demonstrated significant increases in serum TNF obtained 2 h after LPS (n = 4–5 per group; * denotes p < 0.05 compared with isocaloric control animals).
mice demonstrating a critical role of Trif in EtOH-induced TNF transactivation. Examination of liver histology revealed significant EtOH-induced steatosis in WT mice (means steatosis score 2.6 ± 0.27), which was a significant decreased in Trif−/− mice (mean steatosis score 1.6 ± 0.26, Fig. 5, B–E). EtOH also resulted in sensitization to LPS-induced liver injury as measured by serum ALT levels, which is dependent on TNF (15). In contrast to WT mice (Fig. 5F), Trif−/− mice showed no elevation in ALT after 4 wk of EtOH and LPS sensitization, demonstrating the critical role of the Trif signaling pathway in EtOH-induced liver disease in mice.

Discussion
Alcohol abuse has a causal role in a wide range of physical, mental, and social harms, with practically no organ in the body spared its ill effects (1). The level of alcohol problems is related to both the per-capita alcohol consumption and the particular pattern of drinking. The World Health Organization tracks mortality and morbidity from 20 alcohol-related causes through The Global Alcohol Database (24). Among diseases attributed to alcohol abuse, age-adjusted death rates for alcohol dependence syndrome, chronic alcoholic liver disease including alcoholic hepatitis and cirrhosis are highest in the high-alcohol-consumption countries of Eastern and Central Europe (1). However, in the United States alone, it is estimated that alcohol abuse costs US $166 billion per year (of which more than US $30 billion is for the direct costs of medical care).

Among alcohol-related end-organ damage, liver disease has been intensely studied in humans and experimental animal models. Macrophages from both rodents fed chronic ETOH and humans that suffer from alcoholic hepatitis show elevated TNF-α response to LPS (6, 15, 25). Using the Tsukamoto-French diet, Thurman and colleagues (7, 26) have shown that alcoholic liver disease is dependent on TLR4 signaling likely from translocation of bacterial endotoxin from the gastrointestinal tract. Moreover, mice with homozygous deletion of the p55TNF-R are protected against EtOH-induced liver disease (8). Anti-TNF has been considered as a potential treatment for patients with alcoholic liver disease (27); however, due to concerns with susceptibility to infection, this approach would need to be considered with much caution. Rather than neutralizing all TNF as a form of therapy, we undertook studies to define the mechanism by which EtOH resulted in dysregulated TNF synthesis. To accomplish this, we developed an in vitro model using human macrophages subjected to chronic low-dose alcohol that recapitulated the phenotype of EtOH-induced TNF transactivation in vitro (12). Other investigators have taken similar approaches using murine macrophages (9). Studies in the Raw264.7 cell line have implicated a role of Egr-1 in cells that have been exposed to relatively short-term EtOH (24–28 h) (9). Furthermore, Gobejishvili and colleagues (25) have recently shown a role for reduced levels of cAMP in rodent macrophages as another factor responsible for augmented TNF responses after chronic EtOH. Moreover, Thakur et al. (28) have shown in rat Kupffer cells that Erk1/2 phosphorylation is augmented and contributes to increases in TNF synthesis. Despite these data, the precise molecular mechanisms of increased TNF transcription have not been determined.

In the human monocytic cell line Mono Mac 6, we present data that demonstrates a dominant effect of EtOH on TNF biosynthesis at the level of TNF transcription rather than regulating mRNA stability. Moreover this increased TNF transcription is independent of NF-κB using multiple parallel strategies including determination of nuclear protein levels, reporter assays, and overexpression of an IκB-BSR. It is likely that the IκB-BSR was only 75% effective in reducing LPS/PMA-induced TNF in non-EtOH exposed cells due to the fact that we used a dual stimulus to optimize TNF production in these cells. Both deletion mutant studies and measurement of nuclear transcription factor at the protein level revealed a potential role for AP-1 and IRF-3. However, using reporter assays, the most specific EtOH effect on transcription was seen with an IRF-3-dependent reporter (29). Moreover, deletion of the IRF-3 site in the TNF-528 promoter markedly attenuated the transcription observed by chronic EtOH. As IRF-3-dependent gene transcription downstream from TLR4 is dependent on the Trif, we investigated whether Trif was required for alcohol-induced TNF transactivation in murine BM-derived macrophages in vitro as well as TNF sensitization and alcohol-induced liver injury in vivo. Using Trif−/− mice, these data show a critical role of the Trif-sensing pathway in EtOH-induced TNF transactivation. Based on these studies, one could envision rational approaches to target this TLR4 signaling pathway leaving NF-κB-dependent gene transcription intact. Thus, it may be possible to target this pathway without resulting in profound immunosuppression as the NF-κB pathway is more critical for host defense against extracellular and intracellular pathogens. One potential caveat of this approach is that the Trif pathway is critical for murine CMV infection (21), and, as many patients with EtOH abuse are coinfected with hepatitis C, it would be important to further determine the role of the Trif pathway against this pathogen. To this end, it has been reported that the hepatitis C virus NS3/4A protease can cleave Trif and reduce TLR3 signaling, which may represent an immune evasion strategy by the virus (30). Furthermore, it will be important to determine the role of Trif on nonalcohol chronic liver disease such as nonalcohol steatohepatitis where TNF and NF-κB signaling have also been implicated (31, 32).

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Disclosures
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References


