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TLR2- and TLR4-Mediated Signals Determine Attenuation or Augmentation of Inflammation by Acute Alcohol in Monocytes[1]

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Most pathogens express ligands for multiple TLRs that share common downstream signaling. In this study, we investigated the effects of acute alcohol on inflammatory pathways induced by TLR2 or TLR4 ligands and their combination. In human monocytes, alcohol attenuated TLR4- but not TLR2-induced TNF-α protein and mRNA levels and NF-κB activation. In contrast, acute alcohol augmented TNF-α production when both TLR2 and TLR4 ligands were present. IL-1R-associated kinase (IRAK)-1 activity was reduced by alcohol in TLR4, but it was augmented in TLR2- plus TLR4-stimulated cells. IRAK-monocyte, an inhibitor of IRAK-1, was induced in TLR4, but it was reduced in TLR2- plus TLR4-stimulated monocytes by alcohol. This was supported by decreased IRAK-1:TRAF6 association in TLR4 induced but sustained presence of IRAK-1:TRAF6 complexes in TLR2- plus TLR4-stimulated monocytes after alcohol treatment. Phosphorylation of MAPKs such as ERK1/2 was selectively inhibited by acute alcohol in TLR4-stimulated cells. In contrast, JNK phosphorylation as well as AP-1 nuclear binding were augmented by acute alcohol in the presence of combined TLR4 and TLR2 stimulation. Consistent with this result, the JNK inhibitor prevented alcohol-induced augmentation of TNF-α production. These results suggest that acute alcohol attenuates TLR4-induced inflammation via inhibition of IRAK-1 and ERK1/2 kinases and increases in IRAK-monocyte levels in monocytes. Conversely, in the presence of TLR2 and TLR4 ligands, acute alcohol augments inflammatory responses via IRAK-1 activation and JNK phosphorylation. Thus, the complexity of TLR-mediated signals may determine attenuation or augmentation of inflammatory responses by acute alcohol. *The Journal of Immunology,* **2006, 176: 7628–7635.**

Alcohol is the most commonly used and abused substance worldwide. Although clinical data suggest that moderate alcohol consumption (1–2 drinks/day in men and <1 drink in women) have protective effects on cardiovascular diseases, prolonged and excessive alcohol intake results in liver damage, increased susceptibility to infections, and immunosuppression after trauma (1–4). Alcoholic hepatitis is characterized by activation of the inflammatory cascade and elevated levels of proinflammatory cytokines including TNF-α (5, 6). It has been shown that bacterial LPS entering the portal and systemic circulation of chronic alcoholics contributes to activation of Kupffer cells and macrophages and to induction of inflammatory mediators (7).

Pathogen-derived danger signals are recognized by TLRs expressed on innate immune cells. Although individual TLRs recognize specific ligands, most pathogens contain sequences that trigger more than one TLRs (8, 9). LPS, a component of Gram-negative bacterial wall, is recognized by TLR4, whereas peptidoglycan (PGN),[4] another bacterial wall component, stimulates TLR2. Among the 10 different human TLRs, both TLR4 and TLR2 are expressed on the extracellular membrane as opposed to the intracellular localization of TLRs 3, 7, and 9 (8–10). TLRs have a short intracellular Toll/IL-1R (TIR) domain that, upon ligand binding, results in recruitment of adapter molecules and induction of downstream activation (11). Both TLR2 and TLR4 interact with the adapter molecule MyD88 that recruits members of the IL-1R-associated kinase (IRAK) family (12). IRAK1 and IRAK4 are serine threonine kinases involved in the phosphorylation and activation of the TNF receptor-associated factor 6 (TRAF6). IRAK-1/TRAF6 activation is negatively regulated by IRAK- monocyte (IRAK-M) in macrophages (13). TRAF6 activates MAPK kinase (MKK) kinase, called TGF-β-activated kinase-1, that in turn can phosphorylate MKK3 and MKK6, the kinases upstream of p38 MAPK, ERK, and JNK. In addition, TGF-β-activated kinase-1 activates the IκB kinase complex that results in NF-κB activation. In chronic alcoholics, there is increased NF-κB activation in monocytes (6). In contrast, acute alcohol was found to inhibit LPS-induced NF-κB activation in human monocytes and mononuclear cells (14–16). It remains to be determined whether these diverse effects of alcohol are related to the acute and chronic alcohol administration or to the activation state of the target cells. Here, we hypothesized that acute alcohol may affect inflammatory pathways differently depending on the complexity of TLR-mediated signals. In this study, we examined the effect of acute alcohol treatment on human monocytes in response to stimulation with selective TLR4 ligands (phenol-purified LPS), TLR2 (PGN) ligands, or their combination. We found that depending on the complexity of TLR-activating signals, acute alcohol can inhibit, not change, or augment proinflammatory cytokine production. Our study also

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[4] Abbreviations used in this paper: PGN, peptidoglycan; TIR, Toll/IL-1R; IRAK, IL-1R-associated kinase; TRAF6, TNF receptor-associated factor 6; IRAK-M, IRAK-monocyte; MKK, MAPK kinase; CHO, Chinese hamster ovary; MBP, myelin basic protein; Ct, comparative method.

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identified signaling molecules that were specifically associated with the diverse effects of acute alcohol on inflammation.

Materials and Methods

Reagents

Phenol-purified LPS was obtained from List Biological Laboratories. *Staphylococcus aureus* PGN was obtained from Fluka, FCS was obtained from HyClone, and cell culture media DMEM and RPMI 1640 were obtained from Invitrogen Life Technologies. Anti-p50 and anti-p65 Abs were obtained from Santa Cruz Biotechnology; anti-IRAK-M was obtained from Chemicon International; anti-IRAK-1 Ab was obtained from Upstate Biotechnology; anti-phospho-p38, anti-total p38, anti-phospho-ERK, anti-total ERK, anti-phospho-JNK, and anti-total JNK Ab were obtained from Cell Signaling. FACE cell-based ELISA kit for MAPKs were obtained from Active Motif. MAPK inhibitors, SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole), PD98059 (2′-amino-3′-methoxyflavone), and JNK inhibitor II or stress-activated protein kinase inhibitor II or SP600125 (Anthra(1,9-cd)pyrazol-6(2H)-one 1,9-Pyrazoloanthrone) were obtained from Calbiochem. cDNA reverse transcription kit was obtained from Promega.

Isolation and stimulation of monocytes, cytokine measurements

PBMC were isolated from healthy donors by density gradient centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences), and monocytes were selected by adherence as described before (14, 17). After overnight culture in RPMI 1640 supplemented with 10% FBS, monocytes were stimulated with purified LPS (1 μg/ml) and/or PGN (1 μg/ml) in the presence or absence of 25 mM alcohol for 16 h, and TNF-α was measured in the supernatants by ELISA (BD Pharmingen). The study was approved by the Committee for Protection of Human Subjects in Research at the University of Massachusetts Medical School.

Maintenance of Chinese hamster ovary (CHO) cell line

The CHO cells stably transfected with cDNA for human CD14 and TLR2 were provided by Dr. D. Golenbock (University of Massachusetts Medical School, Worcester, MA). These cells express endogenously TLR4 receptors on the surface. CHO/CD14/TLR2 cells were maintained in Ham's F-12 medium containing 10% FBS, 400 U/ml hygromycin, and 0.5 mg/ml G418, and were passaged by detaching with trypsin-EDTA twice a week.

Electromobility gel shift assays

Cells were stimulated with purified LPS (1 μg/ml) and/or PGN (1 μg/ml) in the presence or absence of 25 mM alcohol for 1 h at 37°C. Preparation of cytoplasmic and nuclear extracts and EMSA was performed as described previously (14, 17, 18). Briefly, 5 μg of nuclear protein was incubated with buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 20% glycerol, 20 μg/ml BSA, 2 μg poly(dI:dC), and 30,000 cpm of $^{32}\gamma$P-labeled NF-κB or AP-1 oligonucleotide. Samples were incubated at room temperature for 30 min. Competition experiment was performed by adding 20-fold excess of specific unlabeled oligonucleotide to the reaction mixture. For supershift assay, 2 μl of anti-p65 or anti-p50 Ab was added to the reaction mixture and incubated for 30 min at room temperature before addition of $^{32}\gamma$P-labeled NF-κB oligonucleotide, and then the reaction was further incubated with radioactive nucleotide for an additional 30 min. Protein-DNA complexes were subsequently resolved on a 6% native polyacrylamide gel, and dried gels were exposed to autoradiographic films at −80°C.

Western blots

Fifty micrograms of cytoplasmic protein or whole cell lysates were separated on 10% SDS-PAGE and electroblotted onto nitrocellulose membrane. Membranes were then blocked in 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 2 h, incubated with the specific primary Ab, followed by incubation with secondary HRP-conjugated Ab. Protein bands were visualized by using the ECL method (Cell Signaling). Densitometry analysis was performed using GDS-800 system and Labwork 4.0 program (UVP BioImaging Systems).

MAPK determinations

Cells were stimulated with purified LPS (1 μg/ml) and/or PGN (1 μg/ml) in the presence or absence of 25 mM alcohol for 15 min at 37°C. Quantification of phospho- and total p38-, ERK-, and JNK-kinase in monocytes was performed using specific ELISAs (FACE cell-based ELISA kit; Active Motif). For the MAPK inhibitor studies, monocytes were preincubated with p38 inhibitor (SB 202190, 10 μM), ERK inhibitor (PD98059, 50 μM), or JNK inhibitor (JNK inhibitor II, 40 μM) for 4 h before purified LPS or PGN stimulation overnight at 37°C, followed by TNF-α detection (ELISA) in culture supernatants. MAPKs in CHO/CD14/TLR2 cells were detected by Western blots using phospho-specific or total p38-, ERK-, or JNK-kinase Abs (Cell Signaling).

In vitro kinase assay

IRAK-1 kinase activity was determined in CHO/CD14/TLR2 cells or human monocytes after stimulation with purified LPS (1 μg/ml) and/or PGN (1 μg/ml) in the presence or absence of 25 mM alcohol. After stimulation for 15 min at 37°C, cells were washed with ice-cold PBS and lysed in 500 μl of buffer containing 50 mM HEPES (pH7.4), 250 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 2 mM DTT, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, and Complete Protease Inhibitor Cocktail (Roche Applied Science). Five hundred micrograms of lysate was incubated with 5 μg of anti-IRAK-1 Ab (Upstate Biotechnology) overnight at 4°C on the rotor and then precipitated for 2 h with 50 μl of 50% slurry of Protein A-agarose (Santa Cruz Biotechnology). The precipitated agarose beads were then washed four times with lysis buffer and subjected to in vitro kinase assay using MAPK assay kit (Upstate Biotechnology) using myelin basic protein (MBP) as a substrate. The amount of [γ-^{32}P] ATP-labeled substrate was determined using a scintillation counter. The background cpm of control samples that contained no enzyme was subtracted from each sample. Twenty microliters of each immunoprecipitated sample was separated onto a 15% SDS-PAGE gel, and phosphorylated MBP was visualized by autoradiography.

RNA extraction and real-time PCR

RNA was from normal human monocytes stimulated with purified LPS and/or PGN for 4 h at 37°C using RNeasy kit (Qiagen). Reverse transcription was performed with 1 μg of total RNA using Reverse transcription system (Promega) in a final volume of 20 μl. Real-time PCR were performed on an iCycler (Bio-Rad). For each PCR, a master mix was prepared on ice with 1× SYBR Green I PCR Master mix (Eurogentec), 500 nM of each primer, and 5 μl of diluted (1/5, v/v) reverse-transcribed cDNA. PCR conditions comprised of an initial denaturation step for 10 min at 95°C, and 50 cycles of 15 s at 95°C, 9 s at gene-specific annealing temperature, and 15 min primer extension at 72°C. All results were normalized with respect to the expression of housekeeping 18S gene. To confirm the specificity of the reaction product in each run, the melting profile of each sample was analyzed. Gene expression was quantified using the comparative method (Ct). Briefly, values are expressed relative to the value for a reference sample, called the calibrator (unstimulated monocytes). First, the Ct for the target amplicon and the Ct for the internal control (18S) were determined for each sample. Differences in the Ct for the target (TNF-α, IRAK-M) and the Ct for the internal control (18S) were calculated to normalize for the differences in the amounts of total nucleic acid added to the reaction mixtures and the efficiency of the reverse transcriptase step. The ΔCt for each experimental sample was subtracted from the ΔCt for the calibrator. This difference was called the $\Delta\Delta$Ct value. Finally, the arithmetic calibrator ($2^{-\Delta\Delta Ct}$) was used to calculate the amount of target normalized to the amount 18S control and relative to the amount of the calibrator. Thus, all the values for experimental samples were expressed as fold differences between the sample mRNA and the calibrator mRNA.

Immunoprecipitation experiments

One hundred micrograms of whole cell lysates were incubated with 5 μg of anti-IRAK-1 Ab (Santa Cruz Biotechnology) for 16 h at 4°C rocking gently. The immunocomplex was precipitated with 50 μl of 50% slurry True Blot anti-rabbit Ig IP beads (eBioscience). The beads were washed twice with 1 ml of lysis buffer, and 20 μl of each immunoprecipitated sample was separated onto a 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane. TRAF6 was detected using anti-TRAF6 by the Western blotting method. An equal amount of protein in the input sample was confirmed by β-actin Abs.

Statistical analysis

Statistical values were determined using Wilcoxon signed-rank nonparametric data analysis for human monocytes, and Student's *t* test (two-tailed, paired) was used for experiments with CHO/CD14/TLR2 cells. Data are presented as mean ± SD. A p value of <0.05 was considered significant.

Results

Distinct regulation of TLR2- and/or TLR4-induced TNF-α production by acute alcohol in human monocytes

Previous data from our and other laboratories have demonstrated that acute alcohol treatment can attenuate TNF-α production in monocytes and macrophages in response to stimulation with LPS, a TLR4 ligand (1, 14, 19). Complex pathogens carry ligands for multiple TLRs and, thus, may activate multiple TLRs. In this study, we investigated the effects of acute alcohol on selective cell activation through TLR4, TLR2, or their combination. Human monocytes expressed both TLR2 and TLR4 on their surface (data not shown). Stimulation with a selective TLR4 ligand (phenol-purified LPS), a TLR2 ligand (PGN), or combination of these TLR4 and TLR2 ligands, resulted in significant induction of TNF-α mRNA and protein in monocytes (Fig. 1). Acute alcohol treatment significantly inhibited TLR4- but not TLR2-stimulated monocyte TNF-α mRNA ($p < 0.02$; Fig. 1*A*) and protein production ($p < 0.05$; Fig. 1*A*). In contrast, alcohol augmented TNF-α mRNA and protein production when monocytes were stimulated simultaneously with a TLR4 (LPS) and a TLR2 ligand (PGN) (Fig. 1). These results suggested that depending on the complexity of the stimulatory signals, acute alcohol could inhibit, not change, or augment monocyte TNF-α production.

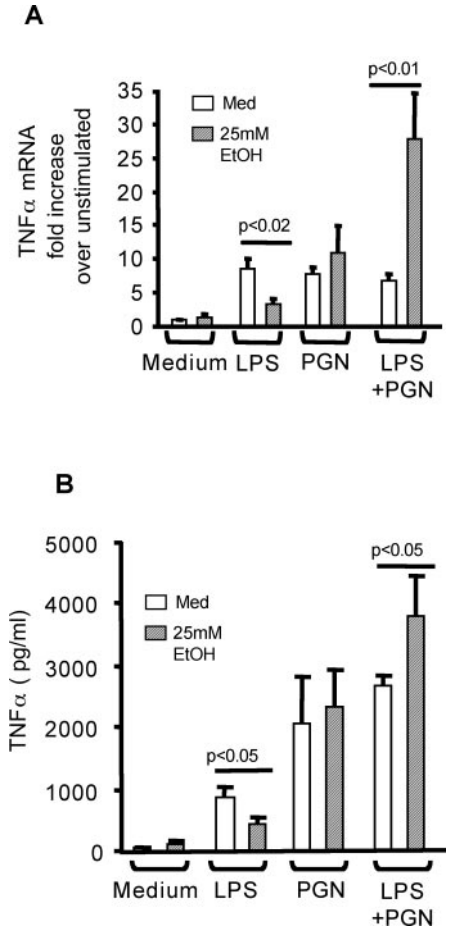

FIGURE 1. Acute alcohol decreases TLR4-induced TNF-α but augments TLR2- plus TLR4-stimulated TNF-α production in monocytes at the mRNA and protein levels. *A*, Normal human monocytes were stimulated with purified LPS (1 μg/ml), PGN (1 μg/ml), or their combination in the presence or absence of 25 mM alcohol for 4 h. Total RNA was isolated, and semiquantitative real-time PCR was performed using specific primers as described in *Materials and Methods*. The data are represented as mean ± SE of TNF-α mRNA corrected for the 18S housekeeping gene ($n = 7$). *B*, Adherence-isolated normal human monocytes were stimulated with purified LPS (1 μg/ml), PGN (1 μg/ml), or their combination in the presence or absence of 25 mM alcohol for 16 h, and TNF-α was measured in the supernatants by ELISA. The data are represented as mean ± SE ($n = 9$).

Acute alcohol augments TLR2- plus TLR4-induced NF-κB activation

Engagement of TLR2 or TLR4 with their specific ligands results in downstream activation of NF-κB, a critical transcription factor in regulation of proinflammatory cytokine genes including TNF-α (8, 9, 18). Our previous studies demonstrated that attenuation of LPS-induced TNF-α production by acute alcohol treatment correlated with decreased NF-κB binding in monocytes (14, 15). In this study, we sought to investigate the signaling cascade involved in up-regulation of TNF-α production by alcohol in monocytes stimulated with the combination of TLR2 and TLR4 ligands. In the presence of alcohol, TLR4-induced NF-κB activation was markedly decreased ($p < 0.05$; $n = 7$), whereas PGN (TLR2)-induced NF-κB binding was not affected in monocytes (Fig. 2*A*). Consistent with up-regulation of TNF-α production, acute alcohol resulted in a significant ($p < 0.05$) increase in NF-κB binding in monocyte stimulation with both TLR2 and TLR4 ligands (Fig. 2*A*).

To circumvent limitations related to low monocyte counts from blood donors, CHO cells were used in some of the signaling experiments. These CHO cells were stably transfected with hTLR2, and CD14 providing an optimal model for investigation of TLR2- and TLR4-induced downstream pathways. These CHO/CD14/TLR2 cells expressed endogenous TLR4 (18, 20) and showed the same pattern of NF-κB activation with acute alcohol and TLR4 and/or TLR2 stimulation (Fig. 2*B*) as found in human monocytes (Fig. 2*A*), where alcohol inhibited TLR4- but augmented combined TLR2- and TLR4-induced NF-κB activation.

NF-κB/Rel family of proteins translocate into the nucleus and bind DNA as dimers of which the p65/p50 heterodimer is transcriptionally active and regulates TLR4-induced TNF-α production (14, 21). To evaluate the composition of NF-κB complexes regulated by alcohol, supershift analysis was performed using Abs against the p65 and p50 subunits of NF-κB/Rel. Fig. 2*C* shows the supershifted bands of p50-containing NF-κB complexes in all cell stimulation groups in the presence of an anti-p50 Ab. The presence of p65 was evident in all stimulation groups based on the reduced NF-κB binding in the presence of the anti-p65 Ab (Fig. 2*C*). These results imply that the p65/p50 NF-κB/Rel heterodimer is induced in TLR2- and TLR4-stimulated cells, and acute alcohol differently regulates this heterodimer depending on the complexity of TLR stimulation.

Opposite regulation of IRAK-1 kinase and IRAK-M by acute alcohol in monocytes

TLR2 and TLR4 trigger a common intracellular signaling pathway originating from the cytoplasmic TIR domains (11). The adaptor molecule, MyD88, associates with the TIR domain of TLR2 and TLR4 and recruits IRAK-1, which upon phosphorylation leads to activation of MAPKs and NF-κB (8–12). We hypothesized that diverse alcohol-induced regulation of NF-κB binding after TLR2 and/or TLR4 stimulation is related to changes in IRAK-1 kinase activity. We found rapid activation of IRAK-1 kinase activity in CHO/CD14/TLR2 cells after stimulation with TLR4 ligands, TLR2 ligands, or their combination (Fig. 3*A*). Acute alcohol treatment significantly inhibited IRAK-1 kinase activity ($p < 0.03$) in TLR4- but not in TLR2-stimulated cells. In contrast, IRAK-1 kinase activity was up-regulated by acute alcohol treatment in the presence of combined TLR2 and TLR4 ligand stimulation ($p <$

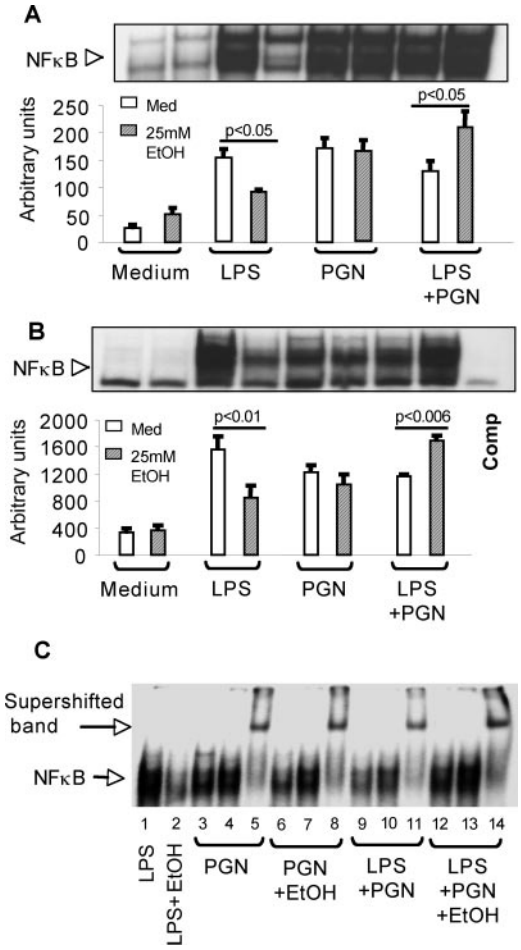

FIGURE 2. Acute alcohol treatment augments NF-κB binding in TLR2- plus TLR4-stimulated monocytes and CHO/CD14/TLR2 cells. *A*, Normal human monocytes or CHO/CD14/TLR2 cells (*B*) were stimulated with purified LPS (1 μg/ml), PGN (1 μg/ml), or their combination for 1 h in the presence or absence of 25 mM alcohol. NF-κB activity was detected in the nuclear extracts by EMSA. A 20-fold excess of the unlabeled NF-κB fragment was included as a cold competitor (Comp). *Bottom panel* shows densitometric analysis of the NF-κB bands presented as mean ± SD of seven different experiments. *C*, Supershift analysis of NF-κB. Nuclear extracts from CHO/CD14/TLR2 cells stimulated as described in *Materials and Methods* were incubated with either anti-p65 or anti-p50 Ab for 20 min before addition of the γ^{32}P- labeled NF-κB oligonucleotide, and then EMSA was performed. The supershifted band with the anti-p50 Ab is indicated by an arrow on the *left side*. Incubation of the nuclear extract with anti-p65 Ab shows reduction in the intensity of the NF-κB bands. *Lanes 3*, *6*, *9*, and *12*: anti-p65 Ab; *lanes 5*, *8*, *11*, and *14*: anti-p50 Ab.

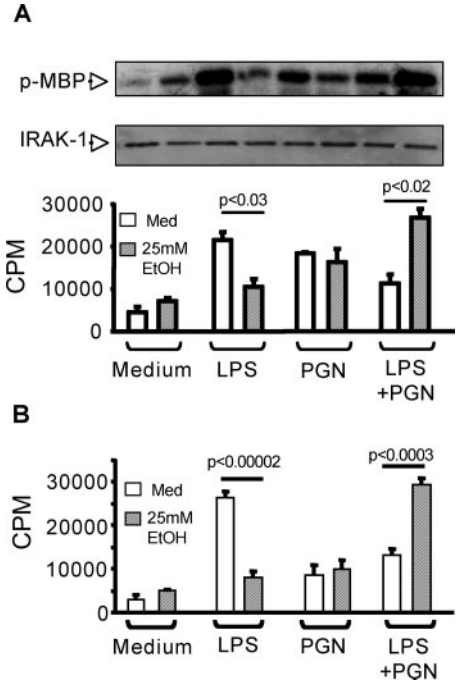

FIGURE 3. The effect of alcohol on IRAK-1 kinase activity in the presence of TLR2 and/or TLR4 stimulation. Cells were stimulated with LPS (1 μg/ml), PGN (1 μg/ml), or their combination in the presence or absence of 25 mM ethanol for 15 min. IRAK-1 protein was immunoprecipitated from equal amounts of total cellular protein of CHO/CD14/TLR2 cells (*A*) or normal human monocytes (*B*). The immunoprecipitated protein was subjected to an in vitro kinase reaction using MBP-based assay as described in *Materials and Methods*. *A*, Twenty microliters of the kinase reaction from CHO/CD14/TLR2 cells was electrophoresed and subjected to autoradiography. A representative gel of three different experiments is shown. Similar amounts of immunoprecipitated IRAK-1 is shown following various treatments. The phosphorylation of MBP was also determined using a scintillation counter. The data are represented as mean cpm ± SE ($n = 4$). *B*, IRAK-1 kinase activity in normal human monocytes is depicted. The graph demonstrates the amount of phosphorylated MBP using a scintillation counter representing IRAK-1 kinase activity ($n = 3$) as mean cpm ± SE.

0.02) (Fig. 3*A*). Similar results were observed in human monocytes, where we found down-regulation of LPS-induced ($p < 0.00002$; $n = 4$) and significant up-regulation of combined LPS and PGN-induced ($p < 0.0003$; $n = 4$) IRAK-1 kinase activity (Fig. 3*B*).

Although the inflammatory response is critical to control the growth of pathogenic microorganisms, excessive cytokine production is harmful to the host and can lead to acute lethality or to chronic inflammation. Several mechanisms protect the host from TLR-induced overactivation of proinflammatory cascades that involve down-regulation of TLR4, decreased NF-κB activation (22–24), and molecules that interfere with TLR signaling (13). IRAK-M, expressed in monocytes, is a negative regulator of IRAK activation that increases after stimulation with TLR ligands but lacks kinase activity (13). IRAK-M prevents the dissociation of the IRAK-1 and IRAK-4 complex from MyD88, thereby preventing the formation of the IRAK1-TRAF6 complex (13, 25). We found reciprocal regulation of IRAK-M levels and IRAK-1 kinase activity by acute alcohol treatment in monocytes. IRAK-M levels were increased in monocytes after stimulation through TLR4 and/or TLR2 compared with unstimulated monocytes reflective of induction of natural counterregulatory pathways at the protein and mRNA level (Fig. 4). More importantly, TLR4-induced IRAK-M protein (Fig. 4*A*) and mRNA (Fig. 4*B*) levels were further increased in the presence of acute alcohol treatment, suggesting that IRAK-M contributes to persistent attenuation of TLR4-induced inflammatory activation by alcohol ($p < 0.05$; $n = 5$). IRAK-M levels were not affected by alcohol in TLR2-stimulated cells (Fig. 4*A*), where we found no change in IRAK-1 kinase activity (Fig. 3). However, IRAK-M protein (Fig 4*A*) and mRNA (Fig. 4*B*) levels were significantly reduced in monocytes stimulated with both TLR2 and TLR4 ligands ($p < 0.05$; $n = 5$) (Fig. 4). This reduction in IRAK-M levels was complementary to alcohol-induced increase in IRAK-1 activity (Fig. 3). These novel results suggest that alcohol-induced changes in the levels of IRAK-M may persistently regulate TLR signaling via inhibition of IRAK-1 and IRAK-4 dissociation.

Because IRAK-M prevents the dissociation of the IRAK-1 and IRAK-4 complex from MyD88, thereby preventing the formation

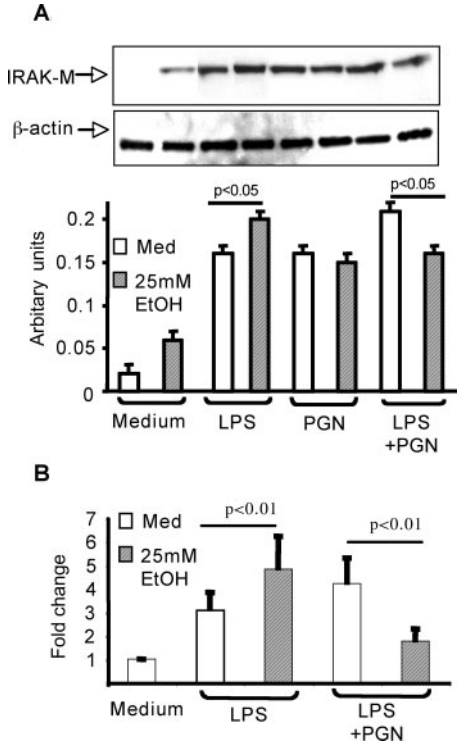

FIGURE 4. Effect of alcohol on IRAK-M protein and mRNA levels in TLR2- and/or TLR4-stimulated monocytes. *A*, Normal human monocytes were stimulated for 12 h as described in *Materials and Methods*, and total protein extracts (30 μg) were subjected to Western blot analysis with an IRAK-M Ab. β-Actin was used as a loading control. Densitometric analysis of six different experiments normalized to β-actin is shown. The data are represented as mean $\pm$ SE ($n = 6$). *B*, Normal human monocytes were stimulated for as indicated in the graph for 6 h, and total RNA was extracted as described in *Materials and Methods*. The ratio of IRAK-M:18S mRNA expression was determined by real-time quantitative PCR using the iCycler system. The graph represents the fold change in IRAK-M:18S ratios as compared to the unstimulated cells. Mean $\pm$ SE of a total of three experiments performed are depicted.

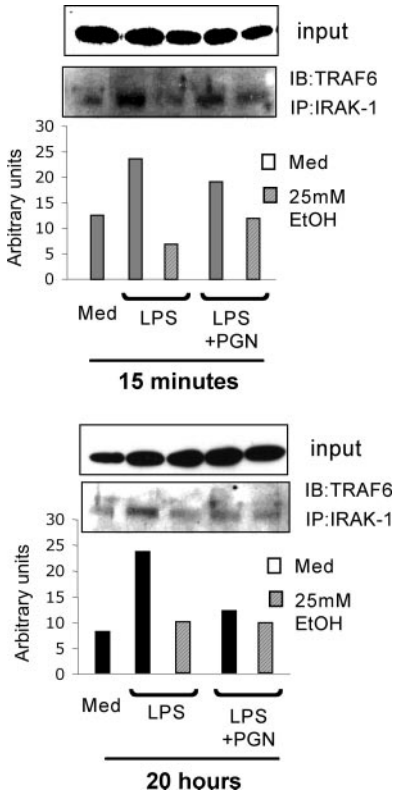

FIGURE 5. Alcohol treatment affects IRAK-1:TRAF6 complexes in TLR4- or TLR2- plus TLR2-treated monocytes. Normal human monocytes were stimulated with purified LPS (1 μg/ml) or the combination of purified LPS (1 μg/ml) and PGN (1 μg/ml) in the presence or absence of alcohol. Whole cell lysates prepared were immunoprecipitated (IP) with anti-IRAK-1 Ab and analyzed by Western blot (IB) using the anti-TRAF6 Ab. β-Actin of the input sample was performed to confirm equal protein used in IP. The bar graph and gel shown is representative of a total of three experiments.

of the IRAK1-TRAF6 complex (13, 25), we further confirmed the role of IRAK-M in alcohol-induced regulation of TLR4- and TLR2-induced signaling by performing immunoprecipitation experiments. Fig. 5 shows that immunoprecipitation using the anti-IRAK-1 Ab followed by detection of TRAF6 by Western blotting revealed decreased TRAF-6 in the immunoprecipitated complex of alcohol plus TLR4-treated cells as compared with TLR4 stimulation alone, indicating inhibition of TLR4-induced down-stream signaling. The reduced TRAF6 and IRAK-1 association occurred in the presence of acute alcohol both at 15 min and 20 h after stimulation. Conversely, sustained presence of TRAF6 in the immunoprecipitated complex of TLR2- plus TLR4-treated cells indicated ongoing activation of the combined TLR4- plus TLR2-induced down-stream pathway even in the presence of alcohol.

Acute alcohol differently regulates MAPKs depending on TLR2 and/or TLR4 costimulation

TLR ligand-induced IRAK-1 phosphorylation leads to a cascade of intracellular signaling events including phosphorylation and activation of the MAPK, p38-, ERK1/2-, and JNK-kinases (26, 27). In this study, we show that monocyte stimulation with TLR4 and/or TLR2 ligands resulted in increased ERK1/2 kinase activity (Fig. 6*A*). We found that acute alcohol treatment down-regulated TLR4- but not TLR2- or TLR4- plus TLR2-induced ERK1/2 kinase activity in monocytes (Fig. 6*A*). An identical pattern of ERK1/2 activation was found in CHO/CD14/TLR2 cells, where alcohol down-regulated TLR4-induced phospho-ERK1/2 levels but did not change TLR2- or combined TLR2- and TLR4-induced phospho-ERK1/2 levels (Fig. 6*B*). These results suggested that down-regulation of ERK1/2 was involved in alcohol-induced inhibition of TLR4-mediated inflammatory pathways.

Monocyte stimulation with a selective TLR4 or TLR2 ligand increased JNK kinase phosphorylation, and this was not affected by alcohol (Fig. 7*A*). However, acute alcohol treatment significantly augmented phosphorylation of the JNK kinase both in monocytes ($p < 0.05$; Fig. 7*A*) and CHO/CD14/TLR2 cells (Fig. 7*B*) in the presence of combined TLR2 and TLR4 ligand stimulation. The protein levels of total-JNK remained comparable between the different stimulation groups (Fig. 7*B*). JNK kinases phosphorylate the DNA binding protein c-Jun, a component of the AP-1 transcription complex (28). Investigation of AP-1 nuclear binding revealed that acute alcohol treatment increased AP-1 activation in combined TLR2 and TLR4-stimulated monocytes but not significantly in monocytes stimulated through TLR2 or TLR4 (Fig. 8).

The role of MAPKs in alcohol-induced regulation of proinflammatory cytokines was further investigated by using specific inhibitors. Monocyte pretreatment with PD98059, an ERK1/2 inhibitor, resulted in an overall decrease in LPS-, PGN-, or combined LPS- and PGN-induced TNF-α production (Fig. 9). The alcohol-induced

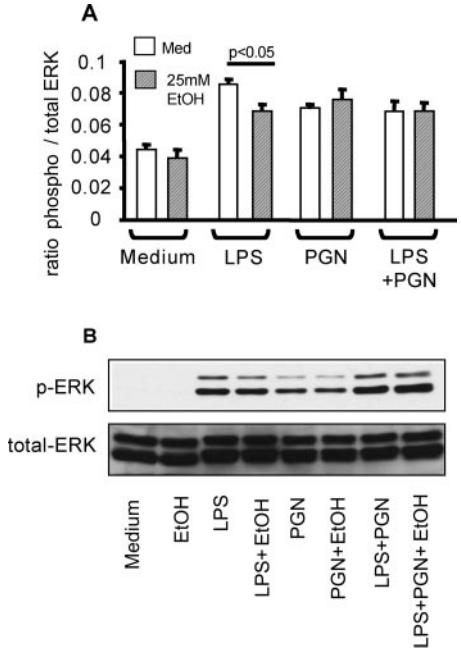

FIGURE 6. Acute alcohol inhibits TLR4-induced phosphorylation of ERK1/2 kinase. *A*, Normal human monocytes were stimulated with purified LPS (1 μg/ml) or PGN (1 μg/ml) or their combination for 15 min in the presence or absence of 25 mM alcohol. Phosphorylated and total ERK1/2 kinase levels were detected by using a specific ELISA. The graph shown depicts the ratio of phospho:total ERK in each sample. Mean ± SD of four experiments is shown. *B*, CHO/CD14/TLR2 cells were stimulated, and 30 μg of total cell lysates were subjected to Western blot analysis with phospho-ERK (*top panel*) or total ERK Ab (*bottom panel*).

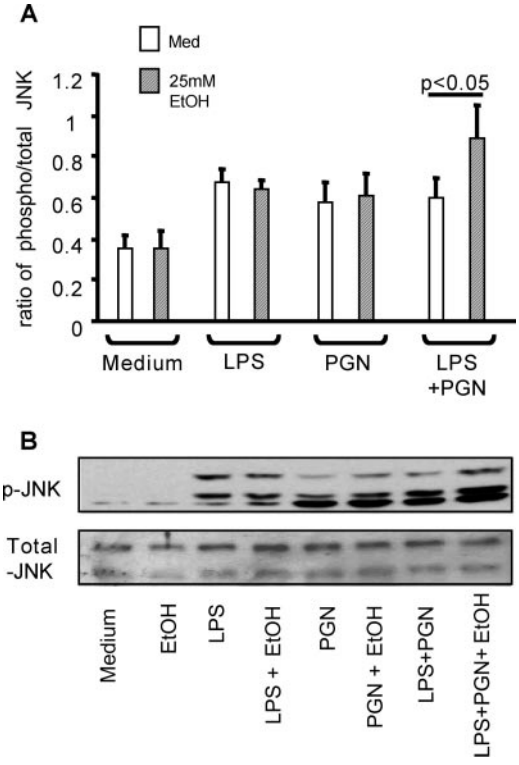

FIGURE 7. Acute alcohol augments TLR2- plus TLR4-induced phosphorylation of JNK. *A*, Normal human monocytes were stimulated with purified LPS (1 μg/ml) or PGN (1 μg/ml) or their combination for 15 min in the presence or absence of 25 mM alcohol. Phosphorylated and total JNK kinase levels were detected by using a specific ELISA. The graph shown depicts the ratio of phospho:total JNK as mean ± SD of four experiments in each sample. *B*, CHO/CD14/TLR2 cells were stimulated, and 30 μg of total cell lysates were subjected to Western blot analysis with phospho-JNK (*top panel*) or total JNK Ab (*bottom panel*).

attenuation of TNF-α production was prevented in TLR4-stimulated cells in the presence of the ERK1/2 inhibitor (Fig. 9). Monocyte pretreatment with the JNK II inhibitor attenuated TLR2- and/or TLR4-induced TNF-α production, and it prevented the alcohol-induced up-regulation of TNF-α production in monocytes stimulated with both TLR2 and TLR4 ligands (Fig. 9). Investigation of p38 MAPK phosphorylation revealed no alcohol-induced changes in TLR2- and/or TLR4-stimulated monocytes (data not shown), and addition of SB202190, a p38 MAPK inhibitor, failed to modify the effects of alcohol on TNF-α production induced by TLR4 or combined TLR2 and TLR4 ligand stimulation (Fig. 8).

Discussion

Inflammatory cell activation is an important initial step in host defense against invading pathogens (8, 9). TLRs, expressed on monocytes, macrophages and other innate immune cells sense pathogen-derived molecular patterns and upon ligand-induced stimulation, initiate a cascade of intracellular signaling events leading to proinflammatory responses (8–10, 26). Alcohol use alters host immunity and predisposes to infections with various pathogens including *Streptococcus pneumoniae*, *Klebsiella*, and *Listeria monocytogenes* (reviewed in Refs. 1–4). Recognition of these organisms depends on TLR4 and/or TLR2 signaling (29–31). In this study, we found that acute alcohol treatment exerted different effects on proinflammatory cell activation depending on the stimulatory signals triggered by TLR2 and/or TLR4 activation. Acute alcohol treatment selectively inhibited proinflammatory pathways induced by LPS, a TLR4 ligand, but not by PGN, a TLR2 ligand. To our surprise, alcohol had an opposite effect on monocytes stimulated with the combination of TLR2 and TLR4 ligands because it augmented NF-κB activation, TNF-α mRNA, and protein levels. These results imply that depending on the complexity of pathogen-derived signals, alcohol can attenuate or augment inflammatory responses. We also identified that the molecular targets for attenuation of TLR4-triggered inflammatory pathways included reduced IRAK-1 and ERK1/2 kinase activity and increased levels of the inhibitory IRAK-M in monocytes. In contrast, augmentation of combined TLR2- and TLR4-induced inflammation by alcohol was mediated through increased phosphorylation of IRAK-1 and JNK kinases, reduced IRAK-M levels, and increased NF-κB and AP-1 activation.

Due to the complexity of pathogens and the restricted recognition profile of TLRs, multiple TLRs can be triggered during the interaction of microbes with the innate immune system. For example, *Mycobacterium tuberculosis*, a microorganism that is poorly eliminated in people with alcoholic use, has ligands for both TLR2 and TLR4 (32, 33). In alcoholic liver disease, increased gut permeability contributes to LPS-induced activation of Kupffer cells (7). Gut-derived pathogens include both Gram-negative organisms, a source of LPS, and Gram-positive bacteria, which are primarily recognized by TLR2 (8, 9). Although the molecular pathways linked to activation of individual TLRs are well characterized, little is known about the effect of stimulation by multiple TLR ligands on monocytes or their modulation by alcohol. In the present study, we show novel findings on the direct effects of alcohol on signal transduction pathways induced by a single TLR4 or TLR2 activation compared with combined TLR2 and TLR4 stimulation. Although TLR4 activation with phenol-purified LPS

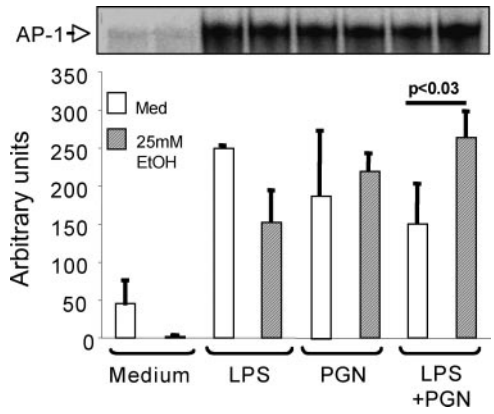

FIGURE 8. AP-1 binding is increased by acute alcohol in the presence of TLR2 plus TLR4 stimulation. CHO/CD14/TLR2 cells were stimulated with purified LPS (1 μg/ml) or PGN (1 μg/ml) or their combination for 1 h in the presence or absence of 25 mM alcohol. Nuclear extracts were subjected to an EMSA using a labeled AP-1 oligonucleotide. A 20-fold excess of the unlabeled AP-1 fragment was included as a cold competitor. A representative EMSA of a total of three experiments is shown here. Densitometric analysis of the AP-1 bands presented as mean ± SD of three experiments.

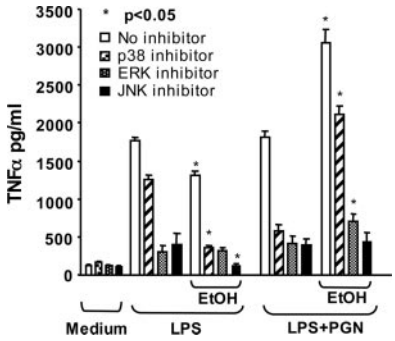

FIGURE 9. JNK inhibitor II inhibits acute alcohol-induced up-regulation of TNF-α in TLR2- plus TLR4-stimulated monocytes. Normal human monocytes were incubated with p38 inhibitor (SB202190, 10 μM), ERK inhibitor (PD98059, 50 μM), or JNK inhibitor (JNK inhibitor II, 40 μM) for 4 h followed by stimulation with LPS or LPS (1 μg/ml) plus PGN (1 μg/ml) in the presence or absence of 25 mM alcohol. After 16-h stimulation, monocyte supernatants were collected and assayed for TNF-α in an ELISA. The data are represented as mean ± SE ($n = 5$). The * represent a p value <0.05 between the same groups with or without alcohol.

and TLR2 stimulation with PGN both induced substantial TNF-α induction in monocytes, their combination resulted in minimal additive effects. This is not unexpected considering that TLR2 and TLR4 share the common, MyD88-dependent pathway for NF-κB activation (26). We concluded that alcohol affected the MyD88-dependent pathway of the TLR signaling based on changes observed in IRAK-1 kinase activity. However, a possible role for TIR domain-containing adaptors, other than MyD88, cannot be ruled out in alcohol-induced modulation of TLR signaling (34). The MyD88-independent pathway, used by TLR3 and TLR4, involves the adaptor molecule TIR domain-containing adaptor-inducing IFN-β and leads to activation of IFN regulatory factor-3 and a set of genes including IFN-β and IFN-γ-inducible protein 10 (8, 9, 35). Inhibition of TLR3 ligand-induced inflammatory responses has recently been shown in peritoneal macrophages after in vivo exposure to acute alcohol (36). Our observation showed that alcohol-induced inhibition of TLR4-induced pathways was associated with inhibition of IRAK-1 kinase activity, a component of the MyD88-dependent pathway. Although TLR4 activation also activates a MyD88-independent pathway through IL regulatory factor-3 that leads to IFN-β production and NF-κB activation, these pathways are induced in a delayed fashion and would not be reflected in TNF-α production measured after 16-h stimulation in our experiments (26).

IRAK-1 Ser/Thr kinase phosphorylation is involved in the induction of NF-κB by LPS via the MyD88-dependent pathway (26). In the case of simultaneous stimulation with TLR2 and TLR4, we found that IRAK-1 kinase activity was enhanced by acute alcohol. Our results also showed that LPS-induced IRAK activation was inhibited, whereas PGN-induced IRAK-1 activation was not affected by alcohol. This observation was similar to down-regulation of IRAK-1 kinase activity by acute alcohol previously reported by Yamashina et al. (37) in Kupffer cells. In that study, alcohol given 1 h before an in vivo LPS challenge inhibited IRAK kinase activity; and this was concomitant to reduced NF-κB activation and TNF-α secretion in Kupffer cells (37).

Our experiments revealed a novel alcohol-mediated regulation of IRAK-M, a negative TLR signaling molecule (13). We found that acute alcohol altered the mRNA and protein levels of IRAK-M in a reciprocal fashion compared with its effects on IRAK-1 kinase activity. The opposite regulation of IRAK-1 kinase activity and IRAK-M levels by alcohol suggests an evidence for the involvement of these signaling elements in alcohol-induced regulation of TLR-mediated inflammatory pathways. Furthermore, our immunoprecipitation experiments using an anti-IRAK-1 Ab showed decreased IRAK-1:TRAF-6 association in alcohol plus TLR4-treated cells as compared with TLR4 stimulation alone, indicating inhibition of down-stream signaling. Conversely, the sustained presence of IRAK-1:TRAF6 complexes at 15 min and 20 h after alcohol plus TLR2 and TLR4 treatment in monocytes indicates ongoing activation of the down-stream pathway, resulting in increased activation of NF-κB. Hence, our data suggest that IRAK-M plays an important monocyte/macrophage-specific role in determining how alcohol modulates the TLR2- and TLR4-mediated responses.

TLR2 and TLR4 activate a common signaling pathway that results in the activation of NF-κB and MAPKs such as ERKs, p38, and JNK (26, 27). We found selective down-regulation of TLR4-induced ERK1/2 by alcohol. Inhibition of TLR4-induced ERK1/2 phosphorylation by acute alcohol in human monocytes was similar to reduced ERK1/2 activation reported in macrophages after i.p. administration of alcohol (19). The same study reported attenuation of TLR2-induced (zymosan) ERK1/2 phosphorylation in peritoneal macrophages of alcohol-treated mice, which was different from our observation of no significant alcohol-induced changes in TLR2-stimulated (PGN) human monocytes. These differences could be related to different TLR2 coreceptor use (8, 9, 12) or to the timing between alcohol and TLR ligand administration. In our experiments, alcohol was given at the same time with the TLR ligands to avoid induction of tolerance or alcohol-induced modulation of expression of TLR2 and -4 or their coreceptors, CD14 and MD2 (38, 39). Earlier studies showed up-regulation of CD14 in rat Kupffer cells after acute ethanol administration (40), and induction of gut-derived LPS was also shown after in vivo alcohol administration that can provide a mechanism for cross-tolerization to TLR2 stimulation (7, 38, 39).

Transcriptional activation of TNF-α by LPS requires activation of a distinct set of transcription factors including NF-κB, and AP-1 (21, 28). LPS stimulation of JNK leads to phosphorylation of c-Jun and subsequent binding of c-Jun to the CRE/AP-1 site on the TNF-α promoter (41, 42). We found that alcohol augmented JNK

and AP-1 activation in cells stimulated with both TLR2 and TLR4 ligands, which likely contributed to increased TNF-α mRNA levels and TNF-α production (43). MAPK inhibitor studies further confirmed the role of JNK activation in the effects of alcohol on the enhanced production of TNF-α in combined TLR2- and TLR4-stimulated monocytes. Recent evidence suggests that chronic alcohol feeding increased ERK1/2 in phosphorylation Kupffer cells in animal models (44). Based on our observation that acute alcohol inhibited ERK only in LPS-stimulated cells, we speculate that ERK may be the primary target of alcohol-induced regulation of inflammation when selective TLR4 activation is present. These results demonstrate that alcohol may inhibit or increase inflammation depending on the complexity of TLR-mediated inflammatory signals.

Disclosures

The authors have no financial conflict of interest.

References

1. Nelson, S., and J. K. Kolls. 2002. Alcohol, host defence and society. *Nat. Rev. Immunol.* 2: 205–209.
2. Thun, M. J., R. Peto, A. D. Lopez, J. H. Monaco, S. J. Henley, C. W. Heath, and R. Doll. 1997. Alcohol consumption and mortality among middle-aged and elderly U.S. adults. *N. Engl. J. Med.* 337: 1705–1714.
3. Szabo, G. 1999. Consequences of alcohol consumption on host defence. *Alcohol Alcohol* 34: 830–841.
4. Cook, R. J. 1998. Alcohol abuse, alcoholism and damage to the immune system: a review. *Alcohol Clin. Exp. Res.* 22: 1927–1943.
5. McClain, C. J., Z. Song, S. S. Barve, D. B. Hill, and I. Deaciuc. 2004. Recent advances in alcoholic liver disease. IV. Dysregulated cytokine metabolism in alcoholic liver disease. *Am. J. Physiol.* 287: G497–G502.
6. McClain, C. I., D. B. Hill, Z. Song, I. Deaciuc, and S. Barve. 2002. Monocyte activation in alcoholic liver disease. *Alcohol* 27: 53–61.
7. Rao, R. K., A. Seth, and P. Sheth. 2004. Recent advances in alcoholic liver disease. I. Role of intestinal permeability and endotoxemia in alcoholic liver disease. *Am. J. Physiol.* 286: G881–G884.
8. Akira, S., and S. Sato. 2003. Toll-like receptors and their signaling mechanisms. *Scand. J. Infect. Dis.* 35: 555–562.
9. Kopp, E., and R. Medzhitov. 2003. Recognition of microbial infection by Toll-like receptors. *Curr. Opin. Immunol.* 15: 396–401.
10. Takeda, K., and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol.* 17: 1–14.
11. Yamamoto, M., K. Takeda, and S. Akira. 2004. TIR domain-containing adaptors define the specificity of TLR signaling. *Mol. Immunol.* 40: 861–868.
12. Janssens, S., and R. Beyaert. 2003. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Mol. Cell* 11: 293–302.
13. Kobayashi, K., L. D. Hernandez, J. E. Galán, C. A. Janeway, R. Medzhitov, and R. A. Flavell. 2002. IRAK-M is a negative regulator of Toll-like receptor signaling. *Cell* 110: 191–202.
14. Mandrekar, P., D. Catalano, and G. Szabo. 1999. Inhibition of LPS-mediated NF-κB activation by ethanol in human monocytes. *Int. Immunol.* 11: 1781–1790.
15. Szabo, G., D. Catalano, and P. Mandrekar. 1997. Ethanol-induced regulation of NF-κB in monocytes. *Alcohol Clin. Exp. Res.* 21: 988–994.
16. Blanco-Colio, L. M., M. Valderrama, L. A. Alvarez-Sala, C. Ortego, M. Hernández-Presa, P. Cancelas, J. Gómez-Gerique, J. Millán, and J. Egido. 2000. Red wine intake prevents NF-κB activation on peripheral blood mononuclear cells of healthy volunteers during postprandial lipemia. *Circulation* 102: 1020–1026.
17. Mandrekar, P., D. Catalano, A. Dolganiuc, K. Kodys, and G. Szabo. 2004. Inhibition of myeloid dendritic cell accessory cell function and induction of T cell anergy by alcohol correlates with decreased IL-12 production. *J. Immunol.* 173: 3398–3407.
18. Dolganiuc, A., S. Oak, K. Kodys, D. T. Golenbock, R. W. Finberg, E. Kurt-Jones, and G. Szabo. 2004. Hepatitis C core and nonstructural 3 proteins trigger Toll-like receptor 2-mediated pathways and inflammatory activation. *Gastroenterology* 127: 1513–1524.
19. Goral, J., and E. J. Kovacs. 2005. In vivo ethanol exposure down-regulates TLR2-, TLR4-, and TLR9-mediated macrophage inflammatory response by limiting p38 and ERK1/2 activation. *J. Immunol.* 174: 456–463.
20. Latz, E., A. Visitin, E. Lien, K. A. Fitzgerald, B. G. Monks, E. A. Kurt-Jones, D. T. Golenbock, and T. Espevik. 2002. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the Toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J. Biol. Chem.* 277: 47834–47843.
21. Hayden, M. S., and S. Ghosh. 2004. Signaling to NF-κB. *Genes Dev.* 18: 2195–2224.
22. Dobrovolskaia, M. A., A. E. Medvedev, K. E. Thomas, N. Cuesta, V. Toshchakov, T. Ren, M. J. Cody, S. M. Michalek, N. R. Rice, and S. N. Vogel. 2003. Induction of in vitro reprogramming by Toll-like receptor (TLR) 2 and TLR 4 agonists in murine macrophages: effects of TLR "homotolerance" versus "heterotolerance" on NF-κB signaling pathway components. *J. Immunol.* 170: 508–519.
23. Ziegler-Heitbrock, L. 2001. The p50-homodimer mechanism in tolerance to LPS. *J. Endotoxin Res.* 7: 219–222.
24. Fan, H., and J. A. Cook. 2004. Molecular mechanisms of endotoxin tolerance. *J. Endotoxin Res.* 10: 71–84.
25. Wesche, H., X. Gao, X. Li, C. J. Kirschning, G. R. Stark, and Z. Cao. 1999. IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family. *J. Biol. Chem.* 274: 19403–19410.
26. Akira, S., and K. Takeda. 2004. Toll-like receptor signaling. *Nat. Rev. Immunol.* 4: 499–511.
27. Johnson, G. L., and R. Lapadat. 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298: 1911–1912.
28. Shaulian, E., and M. Karin. 2002. AP-1 as a regulator of cell life and death. *Nat. Cell Biol.* 4: E131–E136.
29. Knapp, S., C. W. Wieland, C. vantVeer, O. Takeuchi, S. Akira, S. Florquin, and T. vanderPoll. 2004. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. *J. Immunol.* 172: 3132–3138.
30. Chalifour, A., P. Jeannin, J. F. Gauchat, A. Blaecke, M. Malissard, T. Nguyen, N. Thieblemont, and Y. Delneste. 2004. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers α-defensin production. *Blood* 104: 1778–1783.
31. Torres, D., M. Barrier, F. Bihl, V. J. Quesniaux, I. Mailleet, S. Akira, B. Ryffel, and F. Erard. 2004. Toll-like receptor 2 is required for optimal control of *Listeria monocytogenes* infection. *Infect. Immun.* 72: 2131–2139.
32. Heldwein, K. A., M. D. Liang, T. K. Andresen, K. E. Thomas, A. M. Marty, N. Cuesta, S. N. Vogel, and M. J. Fenton. 2003. TLR2 and TLR4 serve distinct roles in the host immune response against *Mycobacterium bovis* BCG. *J. Leukocyte Biol.* 74: 277–286.
33. Takeda, K., O. Takeuchi, and S. Akira. 2002. Recognition of lipopeptides by Toll-like receptors. *J. Endotoxin Res.* 8: 459–463.
34. Yamamoto, M., S. Sato, H. Hemmi, H. Sanjo, S. Uematsu, T. Kaisho, K. Hoshino, O. Takeuchi, M. Kobayashi, T. Fujita, et al. 2002. Essential role for TIRAP in activation of the signaling cascade shared by TLR2 and TLR4. *Nature* 420: 324–329.
35. O'Neill, L. A. J., K. A. Fitzgerald, and A. G. Bowie. 2003. The Toll-IL-1 receptor adaptor family grows to five numbers. *Trends Immunol.* 24: 286–289.
36. Pruett, S. B., C. Schwab, Q. Zheng, and R. Fan. 2004. Suppression of innate immunity by acute ethanol administration: a global perspective and a new mechanism beginning with inhibition of signaling through TLR3. *J. Immunol.* 173: 2715–2724.
37. Yamashina, S., M. D. Wheeler, I. Rusyn, K. Ikejima, N. Sato, and R. G. Thurman. 2000. Tolerance and sensitization to endotoxin in Kupffer cells caused by acute ethanol involve Interleukin-1 receptor-associated kinase. *Biochem. Biophys. Res. Comm.* 277: 686–690.
38. Medvedev, A. E., A. Lentschat, L. M. Wahl, D. T. Golegbock, and S. N. Vogel. 2002. Dysregulation of LPS-induced Toll-like receptor 4-MyD88 complex formation and IL-1 receptor-associated kinase 1 activation in endotoxin-tolerant cells. *J. Immunol.* 169: 52095216.
39. Sato, S., O. Takeuchi, T. Fujita, H. Tomizawa, K. Takeda, and S. Akira. 2002. A variety of microbial components induce tolerance to lipopolysaccharide by differentially affecting MyD88-dependent and -independent pathways. *Int. Immunol.* 14: 783–791.
40. Enomoto, N., K. Ikejima, B. U. Bradford, C. A. Rivera, H. Kono, D. A. Brenner, and R. G. Thurman. 1998. Alcohol causes both tolerance and sensitization of rat Kupffer cells via mechanisms dependent on endotoxin. *Gastroenterology* 115: 443–541.
41. Tsai, E. Y., J. V. Falvo, A. V. Tsytsykova, A. K. Barczak, A. M. Reimold, L. H. Glimcher, M. J. Fenton, D. C. Gordon, I. F. Dunn, and A. E. Goldfeld. 2000. A lipopolysaccharide-specific enhancer complex involving Ets, Elk-1, Sp1, and CREB binding protein and p300 is recruited to the tumor necrosis factor α promoter in vivo. *Mol. Cell Biol.* 20: 60846094.
42. Yao, J., N. Mackman, T. S. Edgington, and S. T. Fan. 1997. Lipopolysaccharide induction of the tumor necrosis factor-α promoter in human monocytic cells: regulation by Egr1, c-Jun, and NF-B transcription factors. *J. Biol. Chem.* 272: 17795–17801.
43. Swantek, J. L., M. H. Cobb, and T. D. Geppert. 1997. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor α (TNF-α) translation: glucocorticoids inhibit TNF-α translation by blocking JNK/SAPK. *Mol. Cell Biol.* 17: 6274–6282.
44. Shi, L., R. Kishore, M. R. McMullen, and L. E. Nagy. 2002. Lipopolysaccharide stimulation of ERK1/2 increases TNF-α production via Egr-1. *Am. J. Physiol.* 282: C1205–C1211.