

Ethanol-induced inhibition of cytokine release and protein degranulation in human neutrophils

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Abstract: Ethanol impairs immune responses in humans and animal models, in vivo and in vitro. In particular, ethanol inhibits some key functions of human polymorphonuclear neutrophils (PMN). We investigated the impact of ethanol on cytokine production by highly purified PMN. In a time- and concentration-dependent manner, ethanol inhibited the production of interleukin (IL)-8 protein and mRNA and also hindered tumor necrosis factor α (TNF- α) release by modulating the expression of the TNF- α -converting enzyme involved in TNF- α shedding. This disruption of PMN cytokine release by ethanol may contribute to the increased risk of infection in alcoholic patients. Degranulation of hepatocyte growth factor (HGF) was also impaired by a clinically relevant ethanol concentration (0.8%), an action that may delay the repair of alcoholic liver damage. These findings suggest that ethanol may modulate three major cytokines involved in alcoholic liver diseases, IL-8, TNF- α , and HGF, via three different mechanisms. *J. Leukoc. Biol.* 72: 1142-1147; 2002.

Key Words: IL-8 · TNF- α · HGF · TACE

INTRODUCTION

Ethanol impairs immune responses in humans and animal models, in vivo and in vitro. In particular, ethanol has a dose-dependent, inhibitory effect on some key functions of human polymorphonuclear neutrophils (PMN), such as the oxidative burst [1, 2], adhesion molecule expression, and chemotaxis [1, 3], and elastase release [4–6]. The production of key immune mediators is also altered by ethanol. Acute ethanol exposure in vitro can inhibit proinflammatory cytokine production by various cells. In particular, tumor necrosis factor α (TNF- α) and interleukin (IL)-8 production are down-regulated by clinically relevant ethanol concentrations in mast cells [7], blood monocytes [8–10], and monocytic cell lines [11, 12].

The purpose of this study was to investigate the impact of ethanol on cytokine production by highly purified human blood PMN. These cells synthesize and/or secrete numerous cytokines [13], including IL-8, which has a key role in recruiting circulating PMN to inflammatory sites [14]; TNF- α , the first cytokine to be released during inflammatory reactions [15]; and

hepatocyte growth factor (HGF), a major mediator of tissue regeneration [16]. These three cytokines are also involved in the pathogenesis of severe alcoholic hepatitis (AH), a setting in which we recently found that IL-8, TNF- α , and HGF release by blood and liver PMN is abnormally regulated [17, 18].

Thus, we postulated that ethanol might modulate IL-8 and TNF- α production by blood PMN and thereby play a role in the immunosuppression and susceptibility to infection observed in alcoholic subjects. We also examined the influence of ethanol on the release of HGF and two markers of degranulation, myeloperoxidase (MPO) and lactoferrin (LF), to determine the mechanism of tissue repair in such patients. PMN were studied in the basal state and after optimal ex vivo stimulation, as described previously [14, 16, 17].

MATERIALS AND METHODS

Preparation of purified human blood PMN

With their informed consent, blood was obtained from volunteer staff members of our department who were healthy, had no history of alcoholism, and had not ingested ethanol for at least 7 days. PMN were purified as described previously [14]. Briefly, leukocytes were rapidly isolated in sterile conditions by sedimentation on a separating medium containing 9% Dextran T500 (Pharmacia, Uppsala, Sweden) and 38% Radioselectan (Schering Plough, Lannoy, France). The leukocyte-rich suspension was then centrifuged on a Ficoll-Paque density gradient (Pharmacia). PMN were further purified from the pellet as follows: After removal of contaminating erythrocytes by hypotonic lysis, the PMN preparation was incubated with pan-anti-human human leukocyte antigen class II antibody-coated magnetic beads (Dynal, Oslo, Norway) for 30 min at 4°C with gentle rotation to deplete monocytes, B cells, and activated T cells. Pure PMN were resuspended in RPMI-1640 medium containing 2 mmol/l glutamine, 25 IU/ml penicillin, 25 μ g/ml streptomycin, and 5% heat-inactivated fetal calf serum (BioWhittaker, Verviers, Belgium) or in Hanks' balanced salt solution (HBSS; Gibco-Life Technologies, Grand Island, NY), depending on the experiment.

PMN culture

Purified PMN (5×10^6 /ml in complete RPMI medium) were pretreated with absolute ethanol (Merck Eurolab-Nogent sur Marne, France) at various concentrations (0–1.5% weight/vol) for 1 h at 37°C in air 5% CO₂. PMN were then stimulated with lipopolysaccharide (LPS; *Escherichia coli* 055:B5; Sigma

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Chemical Co., St. Louis, MO) with or without 250 IU/ml human interferon- γ (IFN- γ ; R&D Systems, Abingdon, UK) for up to 24 h. Cell-free supernatants were harvested and stored at -70°C until assay. Cell viability was confirmed by trypan blue exclusion.

Quantification of IL-8 mRNA

Highly purified PMN (7×10^7) were incubated for 1 h in standard culture medium, with or without LPS (100 ng/ml) and/or 0.8% ethanol. In some experiments, PMN were preincubated for 15 min with $5 \mu\text{g/ml}$ actinomycin D (Sigma Chemical Co.) to block transcription. Total cellular RNA was isolated from PMN with the RNA-B[®] kit (Bioprobe System, Montreuil sous Bois, France) according to the manufacturer's instructions. Briefly, cells were lysed in guanidium thiocyanate, and RNA was extracted with chloroform, then precipitated with isopropanol, and washed with 75% ethanol. The final preparation was re-dissolved in water, and the RNA concentration was determined spectrophotometrically at 260 nm. Total RNA (20 μg) was analyzed by electrophoresis on a 1% agarose-formaldehyde gel to check RNA purity and integrity. IL-8, TNF- α , and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNAs were then quantified in each sample by using Quantikine[®] mRNA kits (R&D Systems, Minneapolis, MN), as described previously [14]. Briefly, 0.1 μg each RNA sample was hybridized with gene-specific, biotin-labeled oligonucleotide capture probes (IL-8 or GAPDH) and digoxigenin-labeled detection probes in microplates placed in a 65°C water bath for 60 min. The hybridization solutions were then transferred to streptavidin-coated microplates, and the RNA/probe hybrids were captured at room temperature for 60 min. After a wash to remove unbound material, an antidigoxigenin alkaline phosphatase conjugate was added for 60 min. After washing steps, a substrate solution and then an amplifier solution were added, and color was developed. Color development was stopped, and the intensity (proportional to the amount of IL-8 or GAPDH mRNA) was measured at 490 nm with wavelength correction at 650 nm. Results were expressed in pg/mL IL-8 or GAPDH mRNA/ μg total RNA. The detection limits were 2.6 pg/mL IL-8 and 1.9 pg/mL GAPDH mRNA.

Flow cytometric analysis of TNF- α -converting enzyme (TACE) expression in PMN

Highly purified PMN ($5 \times 10^6/\text{ml}$ in complete RPMI medium) were pretreated with 0.8% absolute ethanol for 1 h at 37°C and then stimulated with LPS (100 ng/ml), with or without IFN- γ (250 IU/ml) for up to 24 h. At time zero (before culture initiation) and after 2 h, 6 h, 12 h, and 24 h of culture, PMN were washed in phosphate-buffered saline (PBS) supplemented with 0.5% human serum albumin (HSA; LFB, Courtaboeuf, France) and were then resuspended in PBS/HSA; Fc receptors were blocked with purified human immunoglobulin G (IgG; $1 \mu\text{g}/10^5$ PMN; Tegeline[®], LFB) for 15 min at room temperature. Samples were then incubated on ice with a fluorescein-conjugated anti-human TACE monoclonal antibody (clone #111633, R&D Systems, Minneapolis, MN) for 45 min. After one wash with ice-cold PBS/HSA, PMN were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry. Nonspecific binding was determined on cells incubated with the same concentration of a fluorescein-labeled, irrelevant IgG1 antibody (R&D Systems). Flow cytometry was done using a Becton Dickinson FACScan (Immunocytometry Systems, San Jose, CA) equipped with a 15-mV, 488-nm argon laser. Ten thousand events were counted per sample, and the fluorescence pulses were amplified by four-decade logarithmic amplifiers. All the results were obtained with a constant photomultiplier gain. The data were analyzed with LYSIS II software, and the mean fluorescence intensity was used to quantify the responses.

PMN degranulation

Purified PMN ($5 \times 10^6/\text{ml}$ in HBSS) were preincubated for 10 min at 37°C under gentle agitation with varying concentrations of ethanol (0–0.8%), with or without recombinant human IL-10 (0–100 ng/ml). A portion of the cells was kept for 15 min in medium alone, on ice or at 37°C , and the remainder was incubated at 37°C for 5 min with $5 \mu\text{g/ml}$ cytochalasin B (Sigma Chemical Co.) and then with 10^{-6} N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma Chemical Co.) for 10 min with gentle agitation. The tubes were then centrifuged at $+4^{\circ}\text{C}$, and the cell-free supernatants were stored at -70°C until HGF, LF, and MPO assay.

Enzyme-linked immunosorbent assay (ELISA)

IL-8, TNF- α , HGF, MPO, and LF were measured by using commercial ELISA kits (Quantikine[®] human IL-8 and HGF kits, R&D Systems, Abingdon, UK; human TNF- α ELISA, Immunotech, Marseille, France; human LF and MPO ELISAs, Oxys International Inc., Portland, OR), according to the manufacturer's instructions. The detection limits were as follows: 10 pg/mL IL-8 and TNF- α ; 40 pg/mL HGF; and 1 ng/ml LF and MPO.

Statistical analysis

Results are expressed as means \pm SEM. Ethanol-treated and -untreated groups were compared by using ANOVA followed by multiple comparison of means with Fischer's least significance procedure. Paired comparisons were made using Wilcoxon's paired test. *P* values of <0.05 were considered significant.

RESULTS

Ethanol-induced inhibition of IL-8 production by PMN after 24 h of culture

In the absence of ethanol, IL-8 production by PMN was low in the basal state, increasing upon stimulation with LPS (100 ng/ml), alone or combined with IFN- γ (250 IU/ml; **Fig. 1**). Regardless of the concentration, ethanol had no significant effect on IL-8 release by PMN cultured in the absence of exogenous stimuli. Ethanol significantly inhibited LPS- and LPS + IFN- γ -induced IL-8 production in a concentration-dependent manner up to 0.8%. As no further significant inhibition was observed with higher concentrations, 0.8% ethanol was used in subsequent experiments.

To optimize PMN stimulation, cells were stimulated with increasing concentrations of LPS (from 0.1 ng/ml to 1 $\mu\text{g/ml}$) with or without 0.8% ethanol pretreatment. In the absence of ethanol, IL-8 production reached a plateau at 100 ng/ml LPS (**Fig. 2**). At this concentration, 0.8% ethanol pretreatment inhibited IL-8 production by 72% on average; the concentration of 100 ng/ml was thus used in subsequent experiments.

IL-8 down-regulation by ethanol was observed at the protein and the mRNA levels. The following results represent one

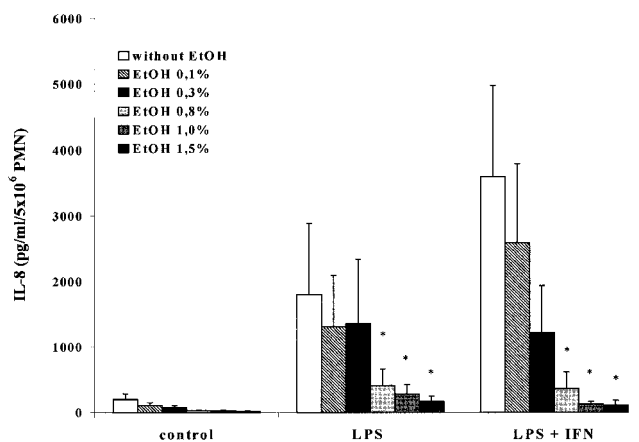


Fig. 1. Concentration effect of LPS on IL-8 production by PMN in the presence or absence of ethanol. PMN were pretreated with 0.8% ethanol for 1 h and then stimulated with various concentrations of LPS. The supernatants were collected after 24 h and assayed for IL-8. Results are expressed as the means \pm SEM of six independent experiments. *, *P* < 0.05 compared with cells incubated with LPS alone.

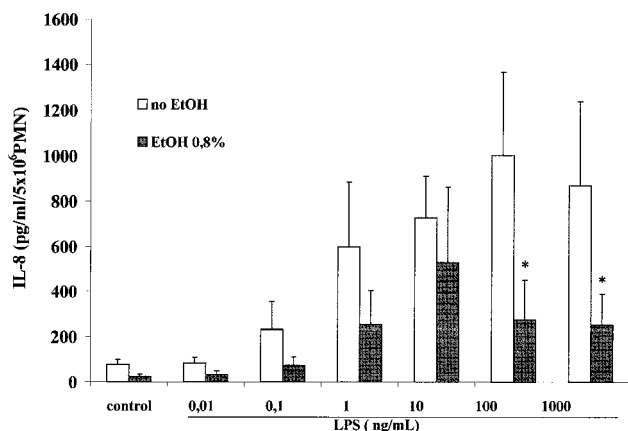


Fig. 2. Ethanol (EtOH) inhibits LPS and LPS + IFN- γ -induced IL-8 production by PMN in a concentration-dependent manner. PMN were pretreated with various concentrations of ethanol for 1 h and were then stimulated with LPS (100 ng/ml) or LPS + IFN- γ (250 IU/ml). The supernatants were collected after 24 h and assayed for IL-8. Results are expressed as the means \pm SEM of six independent experiments. *, $P < 0.05$ compared with stimulated cells not pretreated with ethanol.

typical experiment out of three. IL-8 mRNA was detectable after 1 h in control PMN and PMN incubated with ethanol alone (43 and 47 pg/ml/ μ g total RNA, respectively). IL-8 mRNA accumulation induced by LPS was inhibited by ethanol (141 and 75 pg/ml/ μ g total RNA, respectively). Addition of the transcription inhibitor actinomycin D at the beginning of the culture period strongly reduced IL-8 mRNA accumulation induced by LPS in the presence and absence of ethanol (32 and 49 pg/ml/ μ g total RNA, respectively). GAPDH mRNA levels were not modified in any of the culture conditions (data not shown).

Ethanol-induced inhibition of TNF- α production by PMN after 24 h of culture

Maximal TNF- α production by purified human PMN was lower than maximal IL-8 production. Combination of LPS (100 ng/ml) with IFN- γ (250 IU/ml) was needed to significantly increase TNF- α production relative to unstimulated PMN and PMN stimulated with LPS alone (**Fig. 3**). Only in these optimal conditions of stimulation did ethanol significantly decrease TNF- α secretion, in a concentration-dependent manner up to 0.8% (**Fig. 3**).

We did not investigate the mechanism underlying this ethanol-induced down-regulation by means of transcription analysis, as TNF- α mRNA levels are very low in human PMN [13]. As TNF- α secretion involves enzymatic processing of the membrane-bound form, we investigated this shedding mechanism by quantifying TACE expression at the PMN surface. As shown in **Table 1**, TACE was expressed in basal conditions and was significantly up-regulated after 24 h of LPS + IFN- γ stimulation; LPS alone did not significantly increase TACE expression (data not shown). PMN preincubation with 0.8% ethanol did not modify basal expression but significantly inhibited stimulation-induced up-regulation after 24 h of culture

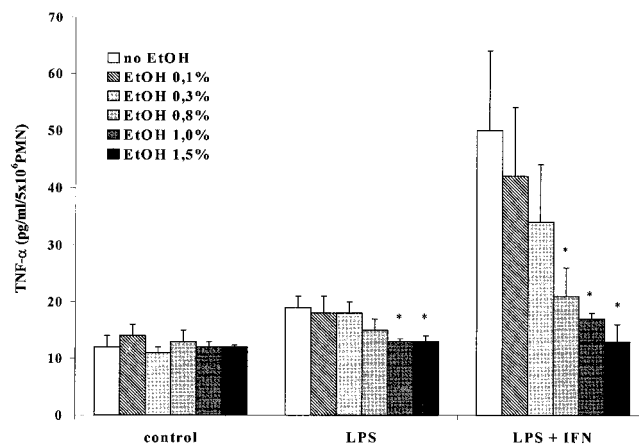


Fig. 3. Ethanol inhibits LPS and LPS + IFN- γ -induced TNF- α production by PMN in a concentration-dependent manner. PMN were pretreated with various ethanol concentrations for 1 h and were then stimulated with LPS (100 ng/ml) or LPS + IFN- γ (250 IU/ml). The supernatants were collected after 24 h and assayed for TNF- α . Results are expressed as the means \pm SEM of six independent experiments. *, $P < 0.05$ compared with cells not pretreated with ethanol.

Time course of ethanol inhibition of LPS + IFN- γ -induced IL-8 and TNF- α production

Upon LPS + IFN- γ stimulation, small amounts of IL-8 and TNF- α were detected in cell-free supernatants after 2 h and gradually increased for up to 24 h (**Table 2**). Ethanol significantly prevented this effect as early as 6 h of culture. In the presence of LPS alone, similar conclusions can be obtained concerning IL-8 release; however, the levels of TNF- α were too low to observe any significant effect of 0.8% ethanol, even after 6 h of culture (data not shown). Moreover, as shown in **Table 1**, the LPS + IFN- γ -induced TACE up-regulation was observed as early as 6 h of culture and was totally prevented by 0.8% ethanol. TACE expression was not significantly modified at any time of culture in the presence of ethanol.

Ethanol-induced inhibition of HGF, LF, and MPO release by PMN

As shown in **Figure 4**, PMN treated with cytochalasin B and fMLP (to ensure total degranulation) released large amounts

TABLE 1. Time Course of TACE Expression at the PMN Surface before Cell Culture (0) and after Stimulation with LPS + IFN- γ in the Presence or Absence of 0.8% Ethanol

Duration of stimulation (h)	LPS + IFN- γ	LPS + IFN- γ + EtOH
0: Basal state	19 \pm 5	15 \pm 4
2	25 \pm 8	17 \pm 6
6	47 \pm 10*	19 \pm 7 [#]
12	56 \pm 13*	20 \pm 5 [#]
24	57 \pm 7*	22 \pm 4 [#]

Results are means \pm SEM of median of fluorescence intensity in four experiments. * Significantly increased compared with TACE expression before stimulation. [#] Significantly decreased compared with LPS + IFN- γ -stimulated cells in the absence of ethanol.

TABLE 2. Time Course of Ethanol Inhibition of LPS + IFN- γ -Induced IL-8 and TNF- α Production (pg/ml/5x10⁶ PMN)

Duration of stimulation (h)	IL-8 release		TNF- α release	
	LPS + IFN- γ	LPS + IFN- γ + EtOH	LPS + IFN- γ	LPS + IFN- γ + EtOH
2	120 \pm 18	132 \pm 27	12 \pm 5	13 \pm 4
6	783 \pm 52	217 \pm 22*	39 \pm 7	19 \pm 6
12	2415 \pm 63	315 \pm 35*	55 \pm 7	20 \pm 5*
24	4036 \pm 91	391 \pm 29*	61 \pm 13	22 \pm 7*

Results are expressed as the means \pm SEM of four independent experiments. * Significantly decreased compared with LPS + IFN- γ -stimulated cells in the absence of ethanol.

of mediators from gelatinase/specific granules (LF and HGF) and azurophil granules (MPO) relative to unstimulated PMN maintained at 37°C. Ethanol significantly inhibited LF, HGF, and MPO degranulation in a concentration-dependent manner.

DISCUSSION

Ethanol inhibits several critical PMN functions in vitro, such as β 2 integrin CD11b/CD18 adhesion molecule expression and chemotaxis [1, 5], the oxidative burst [1, 2], and elastase degranulation [4]. Here, we demonstrate that ethanol can also modulate the production and release of three cytokines (IL-8, TNF- α , and HGF) by human PMN in vitro via different mechanisms.

IL-8 production by isolated PMN was inhibited by ethanol in a concentration-dependent manner in suboptimal (LPS alone) and optimal (LPS+IFN- γ) conditions of stimulation. Time-course studies suggested that the inhibitory effect of ethanol occurred after as little as 6 h of culture, during the early phase of IL-8 production and before the appearance of autocrine regulatory mechanisms partially mediated by TNF- α [15]. This inhibitory effect of ethanol on IL-8 production in vitro has already been observed with human mast cells [7], endothelial cells [19], and monocytes [8]; this dysregulation in monocytes appeared to be mediated in part by inhibition of LPS-induced p38 mitogen-activated protein (MAP) kinase [8]. We found that ethanol inhibits IL-8 production by stimulated PMN at the protein and the mRNA levels. Ethanol decreased IL-8 mRNA levels after 1 h of culture relative to LPS alone. A transcription-dependent mechanism could be involved, at least in part, as the inhibitory effect of actinomycin D was similar to that of ethanol.

Our results also show that ethanol inhibits TNF- α release by PMN in a concentration- and time-dependent manner after optimal stimulation with LPS + IFN- γ . In suboptimal conditions of stimulation, with LPS alone, a different kinetics of TNF- α release was observed, as described previously [15]: Maximal release was low and occurred at 6 h, i.e., too early to observe a significant inhibitory effect of ethanol. This inhibition has already been observed in whole blood [20], mast cells [7], and monocytes [8–10] and was attributed to inhibition of p38 MAP kinase and nuclear factor- κ B activation [9, 21]. In a recent study, ethanol did not significantly reduce TNF- α mRNA levels in two monocytic cell lines [11], and therefore, we postulated that ethanol modulated TNF- α release by PMN by acting at a post-transcriptional step. In various cell types, the 17-kDa-secreted TNF- α protein is produced by proteolytic cleavage of the 26-kDa membrane-bound precursor by TACE, a disintegrin and metalloproteinase family enzyme [22]. We demonstrate here that TACE is expressed at the human PMN surface and thus, may be involved in the shedding of mem-

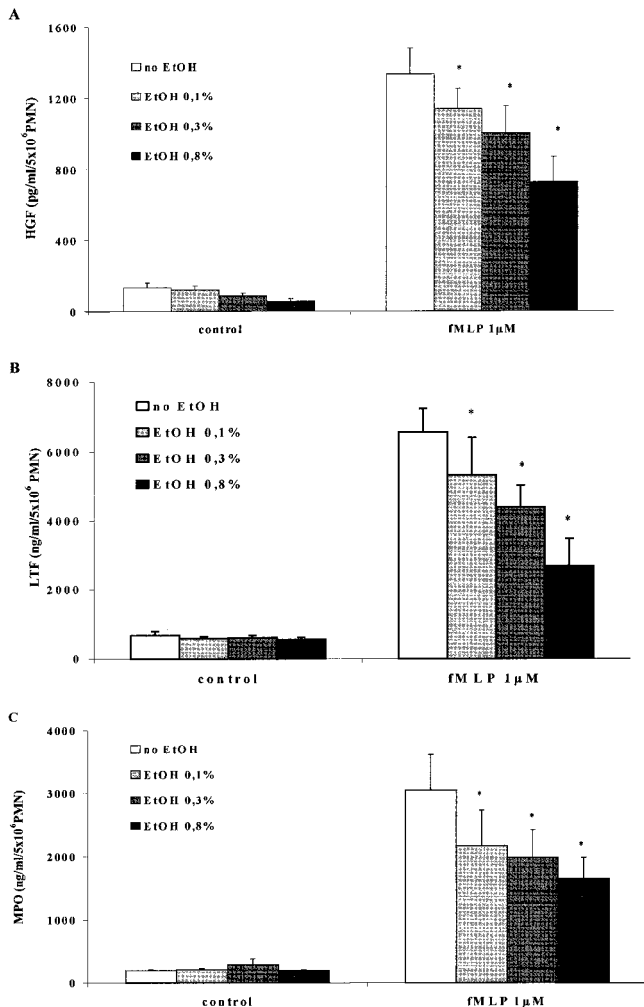


Fig. 4. Ethanol inhibits fMLP + cytochalasin B-induced PMN degranulation in a concentration-dependent manner. PMN were pretreated with various concentrations of ethanol for 10 min and were then stimulated with 1 μ M fMLP plus 5 μ g/ml cytochalasin B. The supernatants were collected after 15 min and assayed for HGF (A), LTF (B), and MPO (C). Results are expressed as the means \pm SEM of six independent experiments. *, $P < 0.05$ compared with cells not pretreated with ethanol.

brane-bound TNF- α [23]. Moreover, we found that LPS + IFN- γ up-regulated TACE expression (as already observed with monocytes stimulated with LPS alone; ref. [24]) and that ethanol inhibited this effect. Therefore, ethanol impairment of TNF- α release by stimulated PMN could be a result, at least in part, of a decrease in TACE expression, leading to decreased shedding of membrane-bound TNF- α ; indeed, time-course studies showed parallel, preventive effects of ethanol on LPS + IFN- γ -induced TACE up-regulation and soluble TNF- α release.

We recently reported that HGF is stored in secretory vesicles and gelatinase/specific granules of PMN in the pro-HGF form, which is rapidly mobilized as the mature form by fMLP plus cytochalasin b-induced degranulation [16]. Here, in similar conditions of degranulation, we found that HGF release was inhibited by ethanol in a concentration-dependent manner. Similar results were obtained with LF (another component of gelatinase/specific granules) and with MPO (a component of azurophil granules). Thus, our results suggest that ethanol impairs degranulation of both granule types induced by fMLP plus cytochalasin b. This inhibitory effect of ethanol is reminiscent of its action on elastase release [4]. Ethanol-induced down-regulation of fMLP receptors on PMN [5] could be involved in this inhibition of degranulation.

These in vitro results may have implications for the understanding of alcoholic liver diseases. Indeed, PMN IL-8, TNF- α , and HGF release was significantly inhibited by 0.8% ethanol, a clinically relevant concentration during severe intoxication [4, 8, 19]. Thus, acute ethanol intoxication may impair IL-8, TNF- α , and enzyme secretion by PMN, resulting in decreased PMN migration, oxidative burst activity, and bacterial killing, and this might explain, at least in part, the frequency and severity of bacterial infections in alcoholic patients. Furthermore, alcohol-impaired HGF release may delay the repair of alcoholic liver damage, as PMN-derived HGF participates in liver regeneration in patients with severe AH [18]. In several recent animal models of inflammation, acute ethanol intake suppressed PMN migration to inflammatory sites [25] and reduced TNF- α , IL-1 β , and chemokine production [26–28]. Impaired bacterial clearance and increased mortality have also been described in acutely alcohol-intoxicated rats with *Streptococcus pneumoniae* or *Klebsiella pneumoniae* pneumonia [29–31]; these effects were attributed to decreased production of IFN- γ , IL-12, and chemokines. However, in patients with cirrhosis and AH, we previously observed IL-8, TNF- α , and HGF hyperproduction by PMN [17, 18]. This paradoxical effect of ethanol on PMN production of cytokines, and particularly TNF- α , has been studied by Zhang et al. [12] in vitro and Yamagishi et al. [32] in vivo. These authors reported that acute ethanol dosing initially inhibited TNF- α production, as during chronic high-dose ethanol exposure, subsequently, TNF- α production was significantly up-regulated, leading to liver injury in the rat model. Thus, ethanol may affect PMN cytokine production and degranulation differently according to the type of exposure (acute or chronic, high or low dose).

In conclusion, ethanol, at clinically relevant concentrations, inhibits the release of IL-8, TNF- α , and HGF, three major cytokines involved in chronic alcoholic liver disease, by modulating IL-8 mRNA transcription, TNF- α shedding, and HGF

degranulation. This disruption of cytokine release by PMN may contribute to the increased risk of infection and the delayed liver repair observed in alcoholic patients.

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