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Suppression of Innate Immunity by Acute Ethanol Administration: A Global Perspective and a New Mechanism Beginning with Inhibition of Signaling through TLR3[1]

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Excessive consumption of ethanol (EtOH) suppresses innate immunity, but the mechanisms have not been fully delineated. The present study was conducted to determine whether EtOH suppresses TLR signaling in vivo in mice and to characterize the downstream effects of such suppression. Degradation of IL-1R-associated kinase 1 induced by a TLR3 ligand in peritoneal cells (~90% macrophages) was suppressed by EtOH. Phosphorylation of p38 kinase in peritoneal macrophages (F4/80$^+$) was suppressed, as was nuclear translocation of p-c-Jun and p65 in peritoneal cells. EtOH decreased IL-6 and IL-12 (p40), but did not significantly affect IL-10 in peritoneal lavage fluid or in lysates of peritoneal cells. Changes in cytokine mRNAs (by RNase protection assay) in macrophages isolated by cell sorting or using Ficoll were generally consistent with changes in protein levels in cell lysates and peritoneal lavage fluid. Thus, suppression of TLR signaling and cytokine mRNA occurred in the same cells, and this suppression generally corresponded to changes in i.p. and intracellular cytokine concentrations. DNA microarray analysis revealed the suppression of an IFN-related amplification loop in peritoneal macrophages, associated with decreased expression of numerous innate immune effector genes (including cytokines and a chemokine also suppressed at the protein level). These results indicate that EtOH suppresses innate immunity at least in part by suppressing TLR3 signaling, suppressing an IFN-related amplification loop, and suppressing the induction of a wide range of innate effector molecules in addition to proinflammatory cytokines and chemokines. ***The Journal of Immunology*****, 2004, 173: 2715–2724.**

One of the characteristic effects of excessive ethanol (EtOH)[3] consumption is increased susceptibility to infections (1). Studies in animals and humans have revealed a wide range of effects of ethanol on the immune and inflammatory systems (1). Among the most consistent and profound effects of acute ethanol exposure (binge drinking) are decreased production of proinflammatory cytokines and chemokines and decreased accumulation of leukocytes at sites of infection or inflammation (2–4). Recent results have demonstrated that recognition of microbial components by TLR expressed by macrophages or other cell types (5) plays a key role in initiating inflammation.

This laboratory has demonstrated that various innate immune effector mechanisms induced through TLR3 are suppressed by EtOH (6), and other laboratories have demonstrated that this is also the case for TLR4 (7, 8). There are reports indicating that EtOH inhibits TLR4 signaling in monocytes or macrophages in vitro (8, 9). However, recent results demonstrate that signaling can be fundamentally different in vivo and in vitro (10) even when the same ligand is used (11). These observations prompted us to examine the effects of EtOH on signaling through TLR3 in a system in which initiation of signaling and the action of EtOH occur in vivo. It was hypothesized that EtOH would suppress TLR3 signaling and that this would lead to predictable suppression of cytokine expression at the protein and mRNA levels depending on which transcription factors were inhibited and on their known roles in the induction of particular cytokines or chemokines.

Studies of the mechanisms by which EtOH inhibits innate immunity and inflammation have focused on the suppression of individual mediators. In the course of studies in a number of laboratories, several mediators have been identified whose induction is suppressed by EtOH. However, the global effects of EtOH on the induction of innate effector mechanisms through a TLR have not been evaluated. Thus, DNA microarrays were used to examine the global effects of EtOH on polyinosinic-polycytidylic acid (poly(I:C))-induced changes in gene expression in peritoneal macrophages. The results indicate that signaling through TLR3; induction of IL-6, IL-12 (p40), and CXCL9 mRNA and protein; induction of an IFN-related signal amplification loop; and induction of mRNAs for a wide range of innate immune effector proteins (many of which are known to be induced by IFNs) are suppressed in an immunologically relevant cell type by EtOH.

Materials and Methods

Animals

Female C57BL/6×C3H ($B6C3F_1$) mice were used in this study, as in previous EtOH-related studies in this laboratory (6, 12, 13). The mice were obtained through the National Cancer Institute animal program, and they were allowed to acclimate for at least 2 wk before use in experiments at 8–12 wk of age. Sentinel mice periodically housed in the same room as experimental animals in this study have been negative for infectious agents during the period of this study. Mice were given free access to food (Purina lab chow; Ralston Purina, St. Louis, MO) and water and were maintained on a 12-h light, 12-h dark cycle. Animal care and use were in accord with National Institutes of Health and Louisiana State University Health Sciences Center policies.

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[3] Abbreviations used in this paper: EtOH, ethanol; EU, endotoxin unit; IRAK, IL-1R-associated kinase; IRF-7, insulin response factor 7; ISGF3-γ, IFN-stimulated gene factor 3-γ; poly(I:C), polyinosinic polycytidylic acid; RPA, RNase protection assay.

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Treatment of animals and isolation of cells, peritoneal lavage fluid, and serum

The TLR3 ligand, poly(I:C) (14), was used at 100 μg/mouse in these studies. The poly(I:C) used in most experiments was obtained from Sigma-Aldrich (St. Louis, MO; catalogue no. P0913-sterile), but some experiments were conducted with poly(I:C) purchased from Invivogen (San Diego, CA), which had undetectable endotoxin levels and is labeled LPS-free poly(I:C) hereafter. This agent was dissolved in PBS (Sigma-Aldrich.) and administered i.p. or i.v. Intraperitoneal injection was used in most experiments to insure that the cells of interest (peritoneal macrophages) were activated and because this route most closely models a condition common to alcoholics, Gram-negative peritonitis. However, i.p. administration of poly(I:C) increased the percentage of neutrophils in the peritoneal cavity, necessitating an additional step (centrifugation over Ficoll) to remove these cells before molecular analyses. To prevent possible loss of intracellular cytokines before production of cellular lysates, i.v. administration of poly(I:C) was used for those experiments (described below). The route of administration and the type of poly(I:C) (LPS-free or standard) are noted in the tables and figure legends. Poly(I:C) preparations were tested for endotoxin using a kinetic *Limulus* amebocyte lysate assay kit (Cambrex, Walkersville, MD). Endotoxin levels are reported in *Results*.

EtOH was administered by oral gavage as a 32% (v/v) solution in tissue culture grade water as reported in several previous studies from this laboratory (6, 13, 15). An EtOH dosage of 6 g/kg was selected for the present study. This dosage produces blood EtOH levels that represent the upper range of values reported in human binge drinkers (0.4%) (13, 16), but it should be noted that the effects of EtOH on poly(I:C)-induced cytokine production are dose-responsive, and significant effects occur at dosages as low as 4 g/kg (6). This dosage produces peak blood EtOH levels of ~0.2% (13), a value quite common among binge drinkers (16). In all studies described here, EtOH was administered immediately before administration of TLR ligand. Groups that received TLR ligand but no EtOH received the vehicle for EtOH (water) by gavage to control for handling and dosing-related stress. Group sizes for these experiments are indicated in the figure legends or the tables. At an appropriate time after treatment (as indicated by preliminary experiments to determine times at which the suppression of at least one cytokine was maximal or near maximal), mice were anesthetized with halothane and exsanguinated, and the contents of the peritoneal cavity were sampled by peritoneal lavage. To obtain samples for cytokine assessment, 1 ml of PBS was injected i.p., the abdominal area was massaged to distribute the fluid, the skin over the peritoneal cavity (but not the peritoneal lining) was removed to allow visualization of the fluid, and a sample (~0.7 ml) from the peritoneal cavity was removed using a needle (25 gauge) and syringe. After centrifugation (300 $\times$ *g* for 5 min), the cell pellet was saved, and the supernatant was stored at −20°C until needed for cytokine or chemokine assay. If analysis of cells was to be part of the experiment, and an additional 7 ml of PBS was injected into the peritoneal cavity, and the steps outlined above were repeated to obtain the remaining cells. The cell pellets from the 1- and 7-ml lavages were pooled and used for subsequent analysis. For experiments in which agents were administered i.p., mononuclear cells were isolated after centrifugation (20 min) over Ficoll (Histopaque 1083; Sigma-Aldrich) to remove granulocytes.

Peritoneal cell lysates

Peritoneal cells from individual mice were centrifuged, then resuspended in PBS with 0.5% Tween 20 (120 μl), followed by sonication for 15 s using a needle-type probe. Supernatant was removed after centrifugation at 14,000 $\times$ *g* in a microcentrifuge, and it was stored at −20°C until assayed for cytokines.

Western blot analysis

Peritoneal cells from individual mice (typically 2–3 $\times$ 10^6/mouse) were used to make extracts for analysis by Western blot. Whole cell extracts and nuclear extracts were prepared by standard procedures as described in our previous study (17). Western blot analysis was performed as described previously (17), and bands were visualized using anti-IL-1R-associated kinase 1 (anti-IRAK-1), anti-p65, and anti-phospho-c-Jun (all from Santa Cruz Biotechnology, Santa Cruz, CA), followed by an appropriate peroxidase-conjugated secondary Ab (also from Santa Cruz Biotechnology). Bands were visualized using the ECL chemiluminescence system (Amersham Biosciences, Arlington Heights, IL) to expose photographic film. Band densities were quantified using National Institutes of Health Image software and the gel analysis macro obtained from the National Institutes of Health Image web page. Actin was used to control for loading variations in the assessment of IRAK-1 in mice treated with poly(I:C) (Fig. 1), but it was not used in subsequent assays. A DNA microarray experiment revealed that EtOH alone decreased actin mRNA expression in the spleen (results not shown), and the literature is unclear regarding the existence of reliable alternatives. Thus, we elected to take greater care (using four replicates for each sample) in the bicinchoninic acid (Pierce Chemical Co.) protein assay used to quantify protein in each sample and in loading the gels (any gel in which blue dye was visible anywhere except within the appropriate well of the gel was discarded) in lieu of using a loading control of questionable validity.

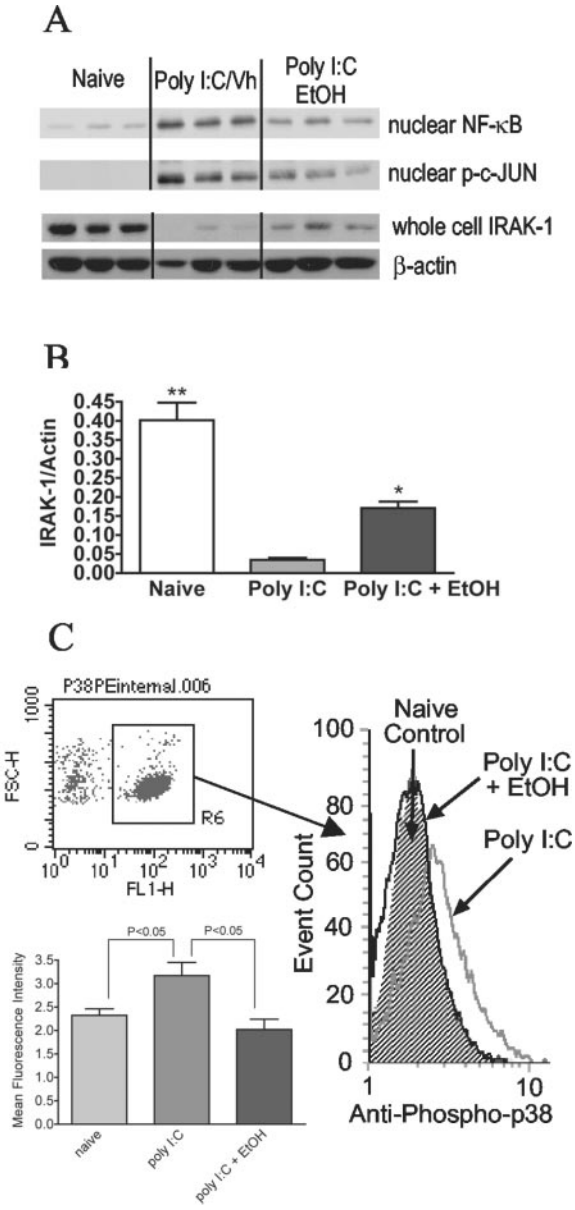

FIGURE 1. EtOH inhibits signaling through TLR3. Mice were treated with poly(I:C) (Sigma-Aldrich; i.p.) and EtOH (6 g/kg) by gavage, and peritoneal cells were isolated after centrifugation over Ficoll to remove neutrophils. Cells were obtained 30 min (for assessment of p-c-Jun, p65, and phosphorylated p38) or 1 h (for IRAK-1) after treatment (*A*). A graphic representation of the IRAK-1 to β-actin ratio is shown in *B*. Whole cells or nuclear extracts were obtained for Western blots, or cells were labeled externally with anti-F4/80-FITC and internally with anti-phospho-p38-PE for flow cytometric assessment. Gating on F4/80$^+$ cells, a histogram of the phospho-p38 results, and a graphical representation of these results are shown in *C*. Results shown in the graphs represent the mean ± SE of fluorescence intensity for three mice per group. The results in this study are representative of results from at least one additional experiment.

Quantitation of cytokine in serum and peritoneal lavage fluid by ELISA

Cytokine ELISA Ab pairs and standards for IL-6, IL-10, and IL-12 (p40) were obtained from BD Pharmingen (San Diego, CA). An Ab pair and standards for analysis of CXCL9 was obtained from R&D Systems (Minneapolis, MN). Assays were performed as described in our previous study (6).

Flow cytometry

Intracellular phosphorylated p38 was assessed using a PE-labeled anti-phosphorylated p38 Ab from BD Pharmingen. Cells were surface-labeled with anti-F4/80-FITC (Caltag Laboratories, Burlingame, CA) to identify macrophages (18). The protocol was provided by BD Pharmingen (19). Briefly, cells were fixed with 2% paraformaldehyde, labeled with anti-F4/80, washed, permeabilized with 90% cold methanol, stained intracellularly with anti-phospho-p38-PE, washed, and analyzed by flow cytometry. As recommended by BD Pharmingen (19), unstimulated cells, rather than isotype controls, were used as negative controls in this system.

RNase protection assay (RPA)

Cellular RNA extracts were prepared from peritoneal mononuclear cells (after centrifugation over Ficoll to remove granulocytes) from two mice per group. To obtain a more purified cell population, peritoneal cells pooled from groups of five mice were labeled with anti-F4/80-FITC, and cells positive for this marker were isolated by FACS. The postsort purity was 99% as indicated by flow cytometry. RNA was extracted from cells, and RPA was performed as described previously (6) using a custom probe set purchased from BD Pharmingen. The mRNAs for the p40 component of IL-12 and for a single isotype of IFN-α (IFN-α4) were detected by this kit.

DNA microarray

Peritoneal cells isolated from groups of four mice were pooled, and mononuclear cells were isolated by centrifugation over Ficoll as noted above. The following groups were used: one naive (untreated) control group, two groups treated with poly(I:C) (i.p.) and the vehicle for EtOH (water by gavage), and two groups treated with poly(I:C) plus EtOH (6 g/kg by gavage). EtOH was administered immediately before poly(I:C), and peritoneal mononuclear cells were harvested 2 h later. Total RNA was checked for degradation with an Agilent 2100 Bioanalzyer (Agilent Technologies, Palo Alto, CA).

Details on the preparation of cDNA, cRNA, fragmentation of cRNA, and analysis using a murine genome U74Av2 array (Affymetrix, Santa Clara, CA) as well as the data obtained can be found on the Gene Expression Omnibus database (accession no. naive, GSM21605; poly(I:C) group 1, GSM21606; poly(I:C) group 2, GSM21607; poly(I:C) plus EtOH group 1, 21608; poly(I:C) plus EtOH group 2, GSM21609; overall description, GSE1308). It should be noted that the raw fluorescence values were deposited, whereas the log ratios were used to find the fold differences reported in this study.

Statistical analysis

Data with continuous variables were analyzed by ANOVA, followed by Newman-Keuls post-hoc test as implemented using PRISM software (GraphPad, San Diego, CA).

Results

Analysis of endotoxin in poly(I:C) preparations

Endotoxin was undetectable (using an assay with a sensitivity of 0.02 endotoxin unit (EU)/ml) in LPS-free poly(I:C) (from Invivogen). Poly(I:C) from Sigma-Aldrich, at the concentration used for administration to mice, had an endotoxin concentration of 38.8 EU/ml. To confirm that the amount of LPS in this poly(I:C) preparation was not sufficient to activate macrophages, serum and peritoneal fluid levels of IL-6, IL-12, and IL-10 were measured in mice treated with an equivalent dose of LPS (0.2 ml at 38.8 EU/ml = 7.8 EU/mouse). No significant change in IL-6, IL-12, or IL-10 was observed compared with naive controls (data not shown). It should be noted that the quality assurance standard for items such as Characterized FBS from Sigma-Aldrich is 25 EU/ml, suggesting that the level of contamination in the Sigma-Aldrich poly(I:C) preparation was probably inconsequential. Even so, selected experiments were performed using endotoxin-free poly(I:C) from Invivogen. The results from experiments with both poly(I:C) preparations are shown in Table I, and they are generally comparable (i.e., both poly(I:C) preparations induced IL-6>IL-12>IL-10, and EtOH significantly suppressed the IL-6 and IL-12 responses and did not significantly affect IL-10). However, the suppression of IL-6 by EtOH was greater for LPS-free poly(I:C) than for standard (Sigma-Aldrich) poly(I:C).

EtOH suppresses signaling through TLR3

The results shown in Fig. 1 demonstrate that i.p. administration of poly(I:C) (Sigma-Aldrich) caused degradation of IRAK-1, which was suppressed by concomitant administration of EtOH. Degradation of IRAK-1 is secondary to hyperphosphorylation, and phosphorylation of IRAK-1 occurs early in TLR signaling, just after receptor-ligand binding and attraction of adapter molecules (e.g., MyD88) (20). As explained in *Materials and Methods*, actin was not used as a loading control for all experiments in this study, but it was used in IRAK-1 evaluation in samples from the poly(I:C) experiment (Fig. 1). Cytospin preparations were counted for most experiments in this study, and the percentage of macrophages exceeded 77% in all cases (in most groups of mice treated with TLR ligands, the value was ~90%).

To determine whether a later step in TLR signaling was inhibited and to confirm that signaling changes were occurring in macrophages, phosphorylation of the MAPK p38 was examined using flow cytometry. In vivo administration of poly(I:C) caused a significant increase phosphorylated p38 in cells gated for surface expression of F4/80 (macrophages). This activation was suppressed by EtOH to naive (untreated) control levels (Fig. 1).

Table I. *Effects of EtOH (6 g/kg) on cytokine induction by ligands for different TLRs*

Treatment Groups (group size)	Serum Cytokine/Chemokine Concentrations (pg/ml)				Peritoneal Cytokine/Chemokine Concentrations (pg/ml)			
	IL-6	IL-10	IL-12 (p40)	CXCL9	IL-6	IL-10	IL-12 (p40)	CXCL9
Naive (2)	0 ± 0[a]	0 ± 0	546 ± 43[a]	ND	20 ± 20[a]	0 ± 0[b]	7 ± 3[a]	10 ± 4[a]
Poly(I:C) i.p. (5)	8,916 ± 1,985	0 ± 0	14,625 ± 3,359	ND	2,844 ± 341	63 ± 37	238 ± 65	141 ± 29
Poly(I:C) + EtOH (5)	6,449 ± 1,706	583 ± 233[a]	2,713 ± 740[a]	ND	1,925 ± 181[a]	142 ± 29	69 ± 13[a]	27 ± 5[a]
Naive (2)	ND	ND	ND	ND	0 ± 0[a]	27 ± 1	50 ± 11[a]	ND
LPS-free poly(I:C) i.v. (5)	ND	ND	ND	ND	1,769 ± 278	65 ± 20	202 ± 47	ND
LPS-free poly(I:C) + EtOH (5)	ND	ND	ND	ND	0 ± 0[a]	36 ± 8	141 ± 28[a]	ND
Naive (3)	0 ± 0[a]	44 ± 19	144 ± 35[a]	ND	0 ± 0[a]	27 ± 2	27 ± 7[a]	ND
LPS-free poly(I:C) i.p. (3)	13,025 ± 1,935	28 ± 2	8,151 ± 1,756	ND	2,303 ± 641	27 ± 1	135 ± 23	ND
LPS-free poly(I:C) + EtOH (3)	1,801 ± 173[a]	159 ± 20[a]	2,089 ± 237[a]	ND	85 ± 33[a]	44 ± 3[a]	60 ± 8[a]	ND

[a] Significantly different from group treated with LPS or poly(I:C) ($p < 0.01$, by ANOVA followed by the Newman-Keul's post-hoc test).
[b] Significantly different from group treated with LPS or poly(I:C) ($p < 0.05$, by ANOVA followed by the Newman-Keul's post-hoc test).

Later steps in TLR3 signaling were also inhibited by EtOH. The amount of phosphorylated c-Jun (a component of AP-1) and the amount of p65 (a component of NF-κB) in the nucleus of peritoneal cells was increased 30 min after administration of poly(I:C) in vivo, and EtOH suppressed this increase (Fig. 1).

EtOH alters induction of cytokines through TLR3

EtOH substantially modified the expression IL-6 and IL-12 (p40) in serum and peritoneal lavage fluid and the expression of IL-10 in serum. Several features of the results shown in Table I are of particular interest. In mice treated with poly(I:C), EtOH caused a significant, large increase in IL-10 concentration in serum, but a smaller increase (or decrease in the case of i.v. administration of poly(I:C)) in peritoneal fluid. This suggests that IL-10 in serum is derived from different cell types or subpopulations than IL-10 in the peritoneal cavity. In contrast, EtOH decreased IL-6 (except in serum of mice treated with Sigma-Aldrich poly(I:C), i.p.) and IL-12 responses, as indicated by serum cytokine concentrations. The pattern was generally similar in peritoneal lavage fluid, except that IL-6 induced by Sigma-Aldrich poly(I:C) (i.p.) was suppressed in the peritoneal cavity, but was not significantly suppressed in the serum. After results from DNA microarrays indicated that CXCL9 mRNA induction was suppressed by EtOH, this chemokine was also evaluated at the protein level. CXCL9 was induced in peritoneal fluid by poly(I:C), and EtOH significantly decreased this induction (Table I). Although cytokine concentrations were uniformly lower in peritoneal lavage fluid than in serum, this does not represent the actual, effective concentration. For example, if the 1-ml volume of the peritoneal lavage fluid diluted the endogenous peritoneal fluid by 1/10 (which seems a reasonable estimate), the actual concentrations i.p. would approach those in serum in most cases. EtOH plus the vehicle for poly(I:C) (i.p.) did not affect serum or peritoneal concentrations of IL-12 (p40) or IL-10 2 h after treatment with 6 g/kg (shown). EtOH (6 g/kg) plus the vehicle for poly(I:C) also did not affect serum IL-6 concentrations 0.5, 1, or 6 h after treatment (data not shown). Values in these cases were not significantly different from those in naive controls.

EtOH suppresses cytokine induction through TLR3 similarly in peritoneal cell lysates and peritoneal lavage fluid

Even though the results presented in Table I and those from another laboratory indicate that cytokines measured in peritoneal lavage fluid are not primarily derived from blood (21), it remained possible that EtOH-induced changes in i.p. cytokines were not directly connected to the observed changes in signaling parameters in peritoneal macrophages. For example, some of the i.p. cytokines could be derived from cell types in the peritoneum per se (e.g., fibroblasts), not from cells isolated in peritoneal lavage fluid (mostly macrophages). To confirm that changes in cytokines in peritoneal fluid reflect changes in cytokines produced by the same cells used to measure signaling, cells were isolated by lavage from the peritoneal cavity 2 h after i.v. administration of poly(I:C) (LPS-free). The cytokine concentrations in peritoneal lavage fluid and in lysates from peritoneal cells obtained from the lavage fluid were determined. Differential counts of the peritoneal cells were also obtained, and all these data are shown in Fig. 2. The pattern of effects was generally similar for poly(I:C)-induced cytokines in peritoneal lavage fluid and cellular lysates. The effect of EtOH on IL-12 (p40) production in cell lysates was not quite significant in the experiment shown, but it was significant in a second experiment in which the IL-12 concentrations in cell lysates were 321 $\pm$ 98 pg/ml for the poly(I:C) group and 46 $\pm$ 9 for poly(I:C) + EtOH group. Cell lysates from the naive group had a higher concentration of IL-10 than cells from the other two groups, a situation not

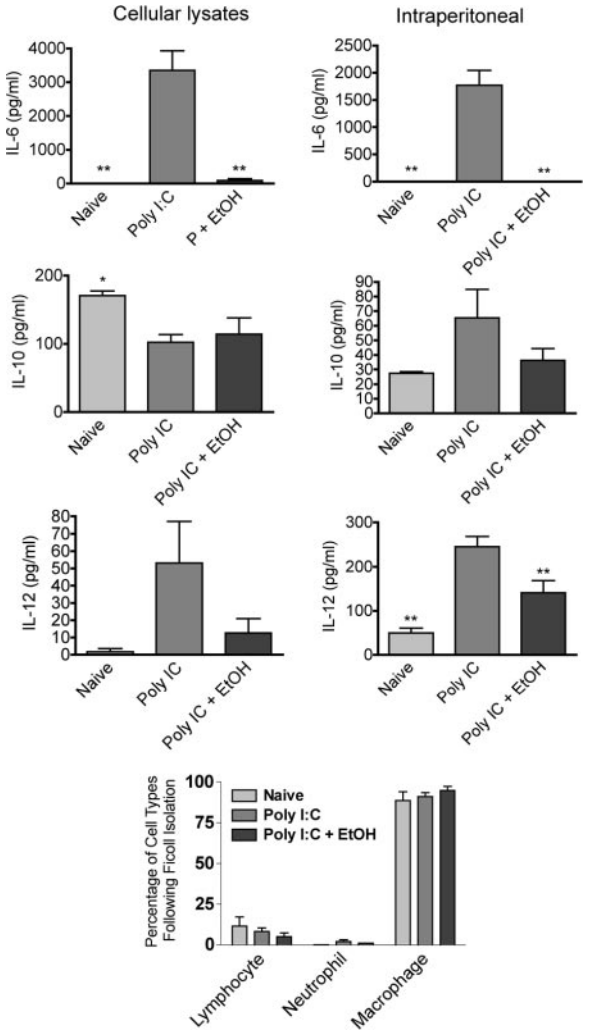

FIGURE 2. Effects of EtOH on cytokine concentrations in peritoneal cell lysates and on cytokine concentrations in peritoneal lavage fluid are comparable. Mice were treated with LPS-free poly(I:C) (i.v.) and EtOH or vehicle for EtOH (water). Peritoneal lavage was performed 2 h later, and cells and lavage fluid were obtained. Cells were lysed, and cytokine concentrations in cellular lysates and in peritoneal lavage fluid from the same mice were measured by ELISA. Results shown are the mean $\pm$ SE ($n = 5$ mice/group for poly(I:C) and poly(I:C) plus EtOH groups and $n = 2$ for the naive group). Values significantly different from the poly(I:C)-treated group are indicated: *, $p < 0.05$; **, $p < 0.01$. Results for peritoneal lavage fluid for this experiment were also presented in Table I (LPS-free poly(I:C), i.v.); they are shown to facilitate comparison with intracellular cytokine levels.

mirrored by IL-10 concentrations in the peritoneal fluid. However, this is consistent with reports that IL-10 may be present in unstimulated cells, and its release can be regulated post-transcriptionally (22). The cells in all groups were >85% macrophages (Fig. 2). These results demonstrate that intracellular cytokine concentrations as well as signaling parameters (Fig. 1) were affected in peritoneal cells isolated by lavage.

EtOH suppresses poly(I:C)-induced IL-12 throughout the normal period of IL-12 production

Evaluating the time course of all cytokines was beyond the scope of the present study, but one was evaluated to estimate the duration of the effect of EtOH. Suppression of poly(I:C)-induced IL-12 (p40) production by EtOH was noted at 3 h in serum and at 3 and

6 h in peritoneal fluid (Fig. 3) in addition to the 2 h point noted in Table I. In addition, it seems that IL-12 production remains elevated for a longer period of time in peritoneal cavity than in serum, again suggesting that IL-12 detected in the peritoneal cavity was not primarily derived from the blood. Similarly, a previous study demonstrated that changes in poly(I:C)-induced IL-10 and IFN-α in serum persist essentially as along as the cytokine is detectable (6). The more substantial suppression of i.p. IL-12 at 3 h than at 2 h (Table I) is not surprising, as one would expect a decrease in mRNA expression at 2 h (Fig. 4) to require additional time to be fully reflected by a change in the protein concentration.

EtOH suppresses poly(I:C)-induced cytokine expression at the mRNA level

If EtOH-induced suppression of signaling was involved in altering cytokine production, it would be expected that changes in cytokine mRNA levels would correspond at least approximately to the changes in cytokine protein levels already noted. To determine whether this was the case, mRNA from peritoneal cells was quantified using RPA. Mice were treated with poly(I:C) (i.p.) and EtOH. Two hours after treatment, cells were harvested by peritoneal lavage. Whole cell lysates were prepared from peritoneal cells that had been isolated using a Ficoll gradient. Lysates were also prepared from peritoneal cells that were pooled from five mice per group and purified to 99% F4/80$^+$ by cell sorting. Samples of RNA prepared from Ficoll-isolated cells from two mice per group and a single sample from each group of pooled, sorter-purified cells were then analyzed by RPA. The values shown in the graph are the mean ± SE for each of the three samples in each treatment group (two samples from Ficoll-isolated cells and one from sorter-purified cells in each group; Fig. 4). Changes in cytokine mRNA values generally corresponded to changes in the protein values reported in Table I for IL-6, IL-12 (p40), and IL-10 concentrations in peritoneal lavage fluid. The results shown in this study for peritoneal macrophages are quite similar to results reported previously for spleen cells (6). Induction of mRNA for IFN-α, IFN-β, IFN-γ, and IL-15 was significantly suppressed by EtOH. Results for sorter-purified and Ficoll-isolated macrophages were substantially different only in the case of IL-10. The basis for this difference is not known, but IL-10 mRNA has an unusually short half-life (22). The additional time required to purify cells by cell sorting (~5 h) may have been sufficient to allow degradation of IL-10 mRNA. Even so, the relative difference between the poly(I:C) and poly(I:C) plus EtOH groups in IL-10 mRNA was similar for sorter-purified and Ficoll-isolated cells. These results indicate that the changes in mRNA for the cytokines evaluated in this study were similar in cell sorter-purified and Ficoll-isolated macrophages, suggesting that results obtained with Ficoll-isolated cells were not substantially affected by contaminating cell types. It should also be noted that the results from this experiment, the results from the first listing for poly(I:C) in Table I, the results for the signaling experiments in Fig. 1, and results from the time-course study of IL-12 (p40) expression in Fig. 3 were obtained using standard poly(I:C)

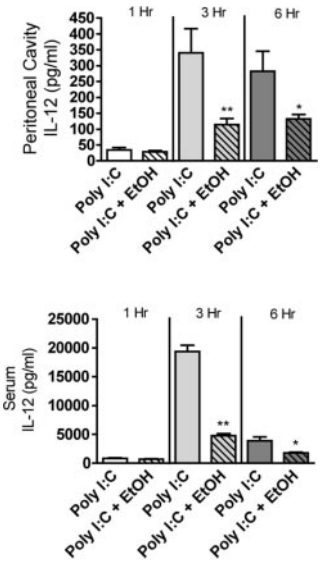

FIGURE 3. Time course of suppression of the IL-12 (p40) concentration in mice treated with poly(I:C) (Sigma-Aldrich; i.p.) and EtOH (6 g/kg) or vehicle for EtOH (water). Mice were treated with EtOH immediately before poly(I:C). Serum and peritoneal lavage fluid were obtained from different groups (n = 5/group) at the indicated times. Values shown are the mean ± SE. Values significantly different from the poly(I:C)-treated group are indicated: ∗, $p < 0.05$; ∗∗, $p < 0.01$).

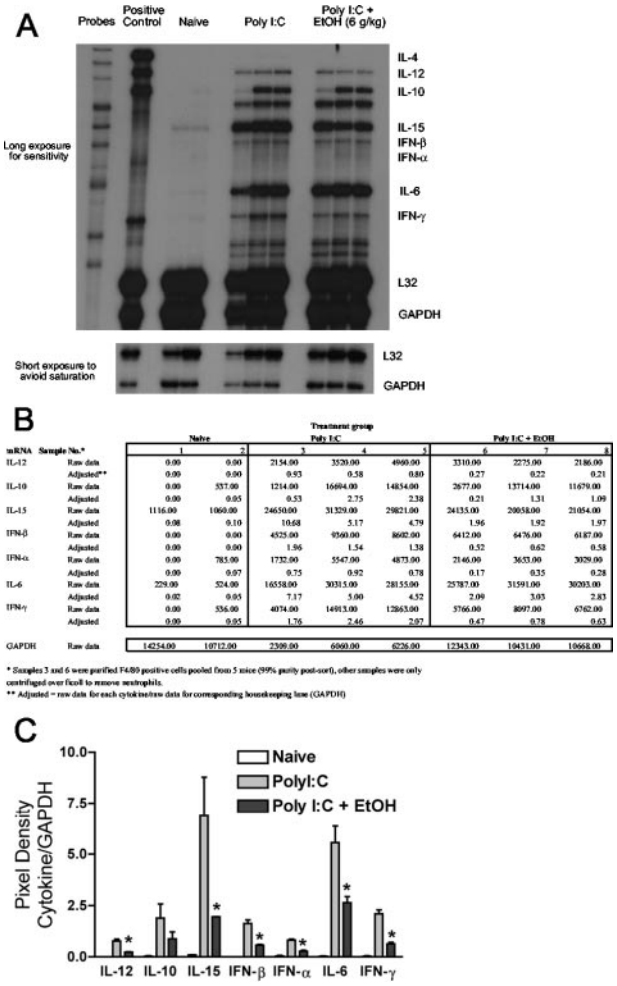

FIGURE 4. EtOH (6 g/kg) alters cytokine mRNA in peritoneal cells after treatment with poly(I:C) (Sigma-Aldrich; i.p.) in vivo. An RPA was performed, and the resultant film (*A*) was scanned and analyzed by National Institutes of Health Image (*B*). The three lanes for each treatment group (poly(I:C) and poly(I:C) plus EtOH) include a sample from cell sorter-purified peritoneal macrophages (the *left lane* in each group) and two samples from Ficoll-isolated peritoneal cells (obtained from different mice). The results in *B* were averaged, and the mean ± SE are shown in *C*. Values significantly different from those of the poly(I:C) group are indicated: ∗, $p < 0.05$.

administered i.p. Cytokine protein and mRNA results were generally comparable. An exception is that EtOH caused a nonsignificant decrease in IL-10 mRNA (Fig. 4), whereas EtOH caused a nonsignificant increase in IL-10 protein (Table I).

EtOH suppresses expression of genes associated with a wide range of innate immune defense mechanisms, and inhibition of an IFN-related amplification loop may play a role

EtOH is known to affect the induction of genes for a few cytokines, chemokines, and other molecules, but innate responses to TLR ligands are known to alter the expression of hundreds of genes (23), and the effects of EtOH on most of these have not been evaluated. To determine whether EtOH affects the expression of other genes activated by administration of a TLR ligand, DNA microarrays were used to analyze EtOH-induced changes in gene expression in peritoneal macrophages from mice treated with poly(I:C) (Sigma-Aldrich; i.p.). Immune/inflammation- or stress-related genes for which EtOH increased or decreased expression 2-fold or more are shown in Tables II and III. Macrophages were obtained by peritoneal lavage from two groups of four mice treated with poly(I:C) (i.p.), from two groups of four mice treated with poly(I:C) plus EtOH, and from one group of four mice that remained untreated (naive). Mononuclear cells from each group were isolated by centrifugation over Ficoll. Cells from each group of four mice were pooled to yield one sample for each group of mice (one naive, two poly(I:C), and two poly(I:C) plus EtOH). The results are shown for immune- or inflammation-related mRNAs that were altered by 2-fold or more in poly(I:C)- plus EtOH-treated mice compared with poly(I:C)-treated mice. The change in the poly(I:C) group 1 (P1) relative to the naive group is shown to indicate whether the genes affected by EtOH had been up- or down-regulated by poly(I:C) alone.

The percentage of false positive genes whose expression differed by 2-fold or more was 23 of 12,488 genes (0.18%) comparing samples P1 and P2, and 25 of 12,488 genes (0.20%) comparing samples E1 and E2. None of these discordant genes is among those listed in Table II or III. The consistency of the results is also indicated by the similarity of values in Tables II and III comparing all possible combinations of poly(I:C) and poly(I:C) plus EtOH groups. The reliability of the results is further indicated by the generally good agreement between these data and those obtained by RPA (Fig. 4). Changes in IL-10, IL-15, IFN-γ, and IFN-β mRNA were similar by DNA microarray and RPA. The mRNAs for IFN-α and IL-12 were not detectable by DNA microarray, and these mRNAs were the least abundant, as detected by RPA. Only in the case of IL-6 were divergent results obtained. The DNA microarray experiment detected IL-6 mRNA, which was substantially up-regulated by poly(I:C), but EtOH did not significantly decrease mRNA levels. This contrasts with results obtained by RPA for IL-6 mRNA and by ELISA for IL-6 protein, which indicate decreased expression in EtOH-treated groups (Table I and Fig. 4). Possible reasons for this divergent result are noted in *Discussion*.

Table II. *Peritoneal cell genes down-regulated 2-fold or more (for at least three of four comparisons) by EtOH*

	Fold Decrease in EtOH-Treated Mice				
	P1 vs E1[a]	P1 vs E2	P2 vs E1	P2 vs E2	P1 Relative to Naive
Cytokines and chemokines					
IL-1β	2.17	3.48	1.68	2.81	58.89
IL-1R type II (decoy receptor)	2.43	2.36	2.01	2.30	13.09
IL-1R antagonist	2.71	3.10	2.89	3.39	135.30
IFN-β	2.19	2.14	2.48	2.38	4.63
IFN-γ	3.01	26.17	1.51[b]	7.36[b]	5.39
IL-15[c]	1.95	2.19	1.93	2.23	11.63
CCL7[c]	2.00	2.68	2.33	2.83	276.28
CCL12[c]	2.97	2.33	4.47	2.71	14.52
CXCL9[c]	3.10	3.36	3.94	4.59	98.36
Signaling-related and Transcription Factors					
STAT 11[c]	2.58	2.95	2.85	3.36	3.32
N-Myc and Stat interactor (Nmi)	2.14	2.45	2.39	2.83	2.53
IRF7[c]	2.03	2.22	2.57	2.60	16.68
IFN-γ-inducible factor[c]	4.17	6.59	4.89	6.92	0.24
RhoB	2.60	2.27	2.91	2.50	0.97
Inhibitor of DNA binding 1	3.41	5.31	3.66	5.82	1.52
Immune function related					
Inducible NO[c]	2.53	1.62	3.51	2.51	5.66
PGE E synthase[c]	2.43	3.27	2.68	3.66	24.42
IFN-inducible GTPase (MX-1)[c]	3.27	3.58	4.17	4.53	32.45
dsRNA-specific adenosine deaminase[c]	2.31	2.25	2.55	2.45	5.78
Calcium-binding protein myeloid-related protein 8[c]	4.72	5.66	3.58	3.86	83.29
Calcium-binding protein MRP14	5.54	6.23	3.71	4.56	280.14
Granzyme B	3.78	2.30	2.97	2.27	9.71
IgG F_c RI (high affinity)[c]	2.53	2.75	3.97	4.17	6.32
VCAM-1	2.91	3.76	2.55	3.27	73.52
Others					
Tissue inhibitor of metalloproteinase	2.48	2.19	2.38	2.14	4.63
Heat shock protein 1B	4.86	4.72	4.14	3.84	2.75
Matrix metalloproteinase 1	2.14	2.64	5.58	7.52	2.77
IFN-stimulated protein (Igs20)[c]	2.38	2.07	2.79	2.48	4.43

[a] P1, poly(I:C)-treated group 1; E1, poly(I:C)- plus EtOH-treated group 1; P2, poly(I:C)-treated group 2; E2, poly(I:C)- plus EtOH-treated group 2; Naive, untreated mice.
[b] These values are not precisely quantitative, because one of the mRNA for one of the E groups was interpreted as absent by the Affymetrix software.
[c] Genes indicated by Medline searches to be induced by IFN (in macrophages or monocytes).

Table III. *Gene expression in peritoneal macrophages increased 2-fold or more by EtOH*

	Fold Increase in Gene Expression				
	P1 vs E1[a]	P1 vs E2	P2 vs E1	P2 vs E2	P1 Relative to Naive
MAPK-interacting serine/threonine kinase 2	3.16	3.03	3.51	3.86	0.16
Ets-1 (transcription factor)	2.11	2.01	2.91	2.87	0.19
Ceramide kinase	2.79	2.69	3.29	2.95	0.19
GTPase-activating protein GAPIII	2.07	2.19	2.83	3.03	0.26
MAPK 14 (p38b)	2.79	2.45	3.46	2.79	0.30
Coronin-actin binding-phagocytosis related	2.51	2.39	2.33	2.11	0.30
Kruppel-like factor 9	3.05	4.03	3.43	5.98	0.37
Spred-1 (inhibitor of Ras)	2.38	2.93	2.01	2.57	0.65
FK506-binding protein, 51 kDa	2.99	3.14	3.58	3.16	NC
Cathepsin E	2.01	2.48	2.01	2.48	1.56
T cell death-associated gene 51	2.83	3.18	4.03	3.36	2.91
Adrenomedullin	3.73	3.12	3.86	3.46	5.28
GADD45	2.64	2.04	3.61	2.91	15.78
4–188 ligand for TNF receptor superfamily member 9	3.16	3.58	3.34	3.76	24.59

[a] P1, poly(I:C)-treated group 1; E1, poly(I:C)- plus EtOH-treated group 1; P2, poly(I:C)-treated group 2; E2, poly(I:C)- plus EtOH-treated group 2; Naive, untreated mice.

The up-regulation of several inflammation-related and anti-viral mRNAs was diminished by EtOH. One of the more interesting patterns was a decrease in expression of type I and type II IFNs, four important components in IFN signaling (STAT-1, N-Myc and STAT interactor protein, GIF-1, and IFN response factor 7 (IRF-7)), and several IFN-induced genes. N-Myc and STAT interactor protein (Nmi) potentiates the action of STAT-1 as a transcription factor component in the activation of gene expression by IFN-γ (24). It is particularly interesting that STAT-1 and a protein induced by GIF-1 (IFN-stimulated gene factor 3-γ (ISGF3-γ)) (25) are components of ISGF3, a transcription factor that is involved in expression of IFN-stimulated genes (26). In addition, ISGF3 is involved in the up-regulation of IRF-7, leading to a gene amplification loop in which IRF-7 enhances type I IFN production, and type I IFN production increases ISGF3 activation (26). These findings are summarized in Fig. 5. The results strongly suggest that decreased IFN production and decreased IFN signaling caused by EtOH contribute to diminished up-regulation of a substantial number of IFN-induced proteins (Table II). Although it is sometimes assumed that IFN-γ is derived exclusively from lymphocytes, macrophages can produce it as well (27, 28).

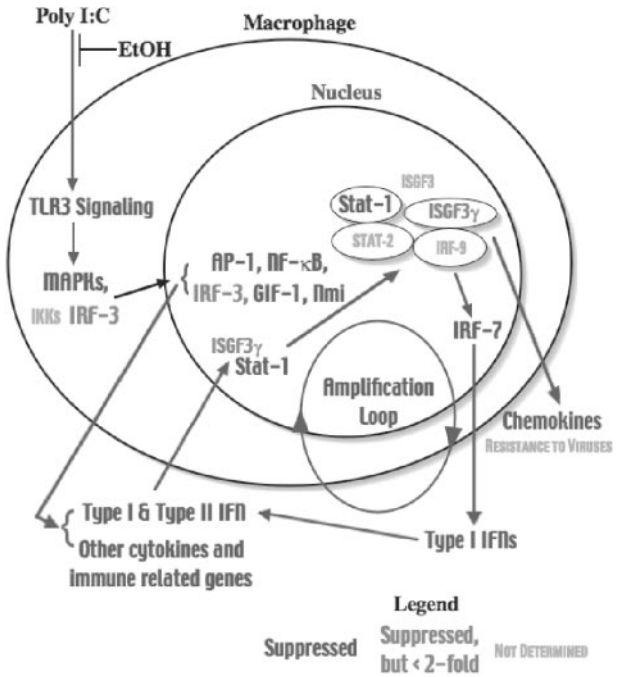

FIGURE 5. Overview of EtOH-induced suppression of IFN-mediated aspects of innate immunity. Suppression of several cytokines and one chemokine has been documented at both the mRNA (by RPA (Fig. 4) and/or by DNA microarray (Table II)) and protein levels (Table I). Suppression of TLR3 signaling was documented by Western blot and flow cytometry (Fig. 1). Suppression in this diagram may represent suppression of a signaling function (e.g., translocation of NF-κB into the nucleus) or decreased expression of a gene that codes for a signaling protein. This diagram is not intended to be complete; it is recognized that transcription of a particular gene usually depends on several transcription factors. However, loss or substantial decreases in any one of them may significantly decrease gene expression.

The expression of the chemokines CCL7, CCL12, and CXCL9 was suppressed by 2-fold or more. These chemokines are primarily induced by IFN-γ or IFN-αβ (29–31). The expression of mRNA coding for CCL2, CCL3, CCL4, CCL5, and CXCL10 was induced >2-fold by poly(I:C) and was decreased (<2-fold) by EtOH for all pairs of comparisons shown in Table II (data not shown).

Several other important mediators of inflammation, immunity, or resistance to viruses were suppressed 2-fold or more by EtOH. The expression of all these mediators was up-regulated by poly(I:C) (substantially in some cases; immune function-related genes; Table II), and EtOH suppressed this up-regulation by varying degrees. The functions of most of these mediators are summarized in *Discussion*. The net effect of these changes as well as the changes in cytokines and chemokines would seem to be anti-inflammatory or immunosuppressive.

Several genes were up-regulated by EtOH, and most of these had been down-regulated by poly(I:C) (Table III). Thus, up-regulation of these genes by EtOH only tended to restore them toward normal (unstimulated) values. One of these is of particular interest because of its involvement in TLR signaling (p38). The reversal of the poly(I:C)-induced decrease in the expression of this protein might serve to diminish the suppression of IL-12 expression by EtOH, because activation of p38 is critical in the induction of IL-12 through TLRs (32). A few genes that were up-regulated by poly(I:C) were further up-regulated by EtOH, and some are of particular interest. The increase in GADD45 may reflect a typical response of macrophages to stressful conditions (33) and would tend to prevent these cells from entering the cell cycle. The increase in adrenomedullin could contribute to decreased expression of inflammatory cytokines (34) and chemokines (35). Interestingly, T cell death-associated gene 51 (which is involved in

T cell apoptosis) shares a function with another of the genes up-regulated by EtOH, MAPK-interacting serine/threonine kinase 2: inhibition of protein synthesis (36, 37). It is possible that this contributes to the decreased levels of some of the proteins evaluated in this study.

Discussion

The results from this study indicate that EtOH suppressed TLR3 signaling and decreased the up-regulation of proinflammatory cytokines and CXCL9 at the mRNA and protein levels in peritoneal macrophages. Evaluation of signaling initiated in vivo requires rapid isolation of cells, and this precluded even relatively rapid cell purification methods (other than brief centrifugation over Ficoll). Thus, these experiments were designed to use a source of cells in which one cell type would predominate and to confirm that key end points were indeed affected in the cell type of interest, the peritoneal macrophage. The results demonstrate the expected correspondence of suppression of signaling, changes in mRNA expression, and changes in protein expression in peritoneal macrophages.

The suppression of IRAK-1 degradation (Fig. 1) suggests that EtOH suppresses an early event in TLR signaling, thereby decreasing or delaying hyperphosphorylation and degradation of IRAK-1 (20). Macrophages from IRAK-1 knockout mice do not respond to LPS (38), and we have found that EtOH suppresses IRAK-1 degradation induced by LPS (manuscript in preparation). Thus, it is clear that IRAK-1 is critical in signaling through TLR4 (and probably for TLR3 as well) in normal macrophages, and it is likely that suppression of IRAK-1 phosphorylation would adversely affect signaling.

It is not clear whether the inhibition of later signaling events (Fig. 1) is mediated solely by inhibition of early events or whether EtOH exerts multiple independent effects on signaling. In any case, MAPKs (including p38) have been demonstrated to be essential in the activation of transcription factors responsible for the expression of several cytokine genes, and IL-12 production decreases in proportion to the amount of inhibition of p38 by a specific chemical inhibitor (32). Thus, it is likely that the effects of EtOH on cytokine gene expression in the present study are mediated at least in part by suppression of signaling.

The specific action of EtOH that leads to disruption of signaling is not known. It is intriguing that recent results suggest that lipid rafts are involved in TLR signaling (39). It has been known for some time that EtOH alters membrane fluidity (40). Thus, it would not be unreasonable to speculate that EtOH may inhibit TLR signaling by disrupting lipid rafts. Experiments to evaluate this possibility are in progress.

Considering that IL-12, IL-6, and CXCL9 generally promote or enhance inflammation or immunity and that IL-10 is suppressive in most systems, the net result of the EtOH-induced changes in these parameters would probably be anti-inflammatory or immunosuppressive. These results are generally consistent with reports from other laboratories indicating suppression of proinflammatory cytokines (9) and increased IL-10 (41) associated with alcohol consumption (42). However, this is the first report in which signaling, cytokine mRNA, and cytokine protein concentration have all been evaluated in a relevant cell type in vivo. The interpretation of the results for IL-12 must include consideration of the fact that p40, not p70, was measured in these studies. It is generally thought that p40 is inactive or antagonistic, but recent results indicate that p40 is an IL-12R agonist and shares at least some of the biological activities of the p70 heterodimer (43, 44).

Decreased activation of AP-1 and NF-κB in peritoneal macrophages (Fig. 1) was predictably associated with suppressed induction of cytokines and chemokines for which these transcription factors are required: IL-6 (45), IL-12 (46), IL-15 (47), IFN-β (48), and CXCL9 (49). However, the expression of IL-10, which requires Sp1, but not AP-1 or NF-κB (50), is not suppressed by EtOH (Table I).

The results obtained from DNA microarray analysis indicate that EtOH adversely affected the induction of several genes relevant to innate immunity. The generalized decrease in chemokines noted in the present study is consistent with a previous report of suppression of MIP-2 and cytokine-induced neutrophil chemoattractant in vivo by EtOH and consequent decreases in host resistance and inflammation in rats (3). EtOH-induced suppression of MCP-3 (CCL7) production by glial cells has also been reported (51). The present study is the first indication of suppression of CCL12 or CXCL9 expression by EtOH. CXCL9 (formerly known as monokine induced by IFN-γ) is a T cell-attracting chemokine (52) that is important in resistance to certain virus infections and also has direct, defensin-like antibacterial effects (53). CCL12 (also called MCP-5) is a macrophage-derived, monocyte-attracting chemokine (54). The induction of both chemokines is dependent on IFN-γ and STAT-1 (30, 52), suggesting that decreased expression of both these molecules (Table II) may contribute to decreased expression of CCL12 and CXCL9 by EtOH.

The basis for the difference between the results for IL-6 obtained with RPA (decreased by EtOH) and those obtained by DNA microarray (no change caused by EtOH) is not clear. Cells for RPA, DNA microarray analysis, and cellular cytokine levels were obtained under the same conditions and at the same time point (2 h after dosing). The RPA results should probably be considered more reliable than DNA microarray results for IL-6, because the RPA probe for IL-6 was optimized for quantitation of this one cytokine. Although quantitation of mRNAs using DNA microarrays is generally quite reliable (55), it is well documented that the results are not always accurate for every gene and that genes that are expressed at high levels, such as IL-6 (which was the 357th highest expressed gene of 12,488), are particularly prone to inaccurate quantitative estimations (55). The reliability of the RPA results is also indicated by the observation that IL-6 protein in peritoneal fluid (Fig. 2) was decreased by EtOH to an even greater extent than IL-6 mRNA (by RPA; Fig. 4). This additional suppression of protein expression might be due in part to post-translational regulatory mechanisms, but it is also consistent with suppression of transcription, as indicated by RPA.

Although it is not possible to conclusively demonstrate functional effects on the basis of changes in mRNA levels alone, one particular set of changes identified in the DNA microarray study is striking (Fig. 5). The decreased expression of several key transcription factor components in a predictable way (i.e., suppression of upstream components was consistently associated with suppression of downstream components) strongly suggests that these changes in gene expression are functionally relevant. Further evidence for the functional impact of the IFN amplification loop illustrated in Fig. 5 is the decreased expression of numerous IFN-inducible genes (Table II). The functional importance of suppression of the IFN amplification loop is also suggested by our finding that IFN-α is suppressed to a greater degree by lower dosages of EtOH than any other poly(I:C)-induced mediator evaluated to date (6).

These results are of particular interest considering that poly(I:C) induces a very similar set of responses as some viruses (23) and that alcoholics are less able than other individuals to control replication of hepatitis C virus as well as other viruses (56). Similarly, the scheme in Fig. 5 would predict diminished responses to therapeutic IFN-α given for hepatitis C infection, which have been observed in human alcoholics (56).

Previous studies in which poly(I:C) has been used to study TLR3 signaling or global effects on gene expression (14, 23) did not include consideration of the possible role of LPS contamination, and the present study used the same poly(I:C) supplier as that noted in the paper originally identifying TLR3 as the receptor that recognizes poly(I:C) (14). Results presented in this study (comparing standard and LPS-free poly(I:C) i.p. in Table I) suggest that there may be some effect of minor LPS contamination, and that this should be considered. However, differences in the effects of EtOH on IL-6 responses in mice treated with LPS-free poly(I:C) and standard poly(I:C) may be related to other differences in these preparations (which are from different suppliers), such as average m.w. (which can vary substantially among various suppliers). Thus, resolving this issue will require a series of experiments, perhaps using LPS-free poly(I:C) compared with the same preparation plus known amounts of LPS. Until this is achieved, the results presented in this study and in previous studies evaluating global effects of TLR3 ligands cannot definitively exclude some role for LPS contamination in the effects of poly(I:C). However, that does not invalidate the fundamental conclusions regarding the effects of EtOH on TLR signaling and gene expression in the present study. In fact, simultaneous exposure to more than one TLR ligand would presumably be more relevant to innate immune responses to microbes than exposure to a single purified TLR ligand.

The mRNAs for several other proteins important in host resistance to infection were also suppressed by EtOH. It has previously been reported that inducible NO synthase mRNA is suppressed by EtOH and that this suppression can decrease resistance to infection (57). The results shown in Table II indicate that this may also be the case in the present model. In addition, the results indicate that the expression of several host resistance-related genes not previously known to be affected by EtOH in macrophages were diminished in EtOH-treated mice. These include the high affinity IgG FcR, PGE synthase, granzyme B, VCAM-1, MPR8, and MPR14 (calcium binding proteins of activated macrophages that have chemotactic and direct microbicidal activities) (58), and dsRNA-specific adenosine deaminase (an IFN-induced enzyme that is involved in inhibition of virus replication) (59). The suppression of RhoB may exacerbate deficiencies in IgG FcR-mediated phagocytosis, because Rho is required to initiate the signaling pathway that activates this process (60). Recent results demonstrate an unexpectedly predominant role for PGE synthase in some types of inflammation (61). Thus, suppressed up-regulation of this gene by EtOH may substantially limit inflammatory processes. The role of VCAM-1 as an adhesion molecule on vascular endothelial cells and of granzyme B as a component of the lytic machinery of NK cells and T cells are well recognized, but each of these proteins can also be expressed by macrophages (62, 63). The importance of their expression by these cells in host resistance is not known, but the collective suppression of all mediators listed in Table II in a cell type known to be important in innate immunity would suggest a role for this suppression in alcohol-induced immunosuppression.

Finally, the DNA microarray results presented in this study serve to support the emerging idea that is at the core of systems biology: that complex biological systems cannot best be understood by sequential use of reductionist experimental designs to identify single mediators that are involved in suppression of innate immunity or other complex functions (including some of our own previous studies). The microarray results clearly suggest that a wide range of innate immune effector proteins, particularly those induced by IFNs, are likely to be involved in deficits in innate immune functions that have been documented in poly(I:C)-treated mice exposed to EtOH (6, 12). It is likely that suppression of the IFN-related amplification loop is involved in EtOH-induced suppression of resistance to a wide range of pathogens for which TLR3 or TLR4 are involved in resistance. Studies to directly test this hypothesis are in progress.

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