Published in final edited form as:

Alcohol. 2011 February ; 45(1): 1–9. doi:10.1016/j.alcohol.2010.08.010.

ELISA assays and alcohol: increasing carbon chain length can interfere with detection of cytokines

Kristine von Maltzan and

Louisiana State University, LSU Health Sciences Center, Department of Cellular Biology and Anatomy, 1501 Kings Highway, Shreveport, LA 71104

Stephen B. Pruett, Ph.D.

Mississippi State University, College of Veterinary Medicine, Department of Basic Sciences, 240 Wise Center Drive, PO Box 6100, Mississippi State, MS 39762, Tel.: (662) 325-6653, Fax: (662) 325-8884 or (662) 325-1031, pruetts@cvm.msstate.edu

Abstract

Enzyme-linked immunosorbent assays (ELISA) are frequently used in studies on cytokine production in response to treatment of cell cultures or laboratory animals. When an ELISA assay is performed on cell culture supernatants, samples often contain the treatment agents. The purpose of the present study was to determine if some of the agents evaluated might inhibit cytokine detection by interfering with the ELISA, leaving the question of whether cytokine production was inhibited unanswered. Mouse and human cytokine ELISA kits from BD Biosciences were used according to the manufacturer's instructions. Cytokine proteins were subjected to one to five carbon alcohols at 86.8 mM (methanol, ethanol, 1-propanol, 2-propanol, n-butanol, and n-pentanol). After treating cell cultures with alcohols of different carbon chain lengths, we found that some of the alcohols interfered with measurement of some cytokines by ELISA, thus making their effects on cytokine production by cells in culture unclear. Increasing carbon chain length of straight chain alcohols positively correlated with their ability to inhibit detection of TNF- α and IL-10, but not with the detection of IL-6, IL-8, and IL-12. To avoid misinterpretation of treatment effects, ELISA assays should be tested with the reference protein and the treatment agent first, before testing biological samples. These results along with other recent results we obtained using circular dichroism indicate that alcohols with 2 or more carbons can directly alter protein conformation enough to disrupt binding in an ELISA (shown in the present study) or to inhibit ligand induced conformational changes (results not shown). Such direct effects have not been given enough consideration as a mechanism of ethanol action in the immune system.

Keywords

ELISA; alcohols; ethanol; hydrophobicity; cytokines

© 2010 Elsevier Inc. All rights reserved.

Correspondence should be sent to: Stephen B. Pruett, pruetts@cvm.msstate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Enzyme-linked immunosorbent assays (ELISA), which are based on antigen-antibody reactions, are widely applied in laboratory medicine and in biomedical research. They allow rapid detection and quantitation of proteins in biological samples. In the field of immunology, ELISA has many applications, including quantifying cytokine secretion of cells and laboratory animals in response to pathogens. Samples commonly include cell culture supernatants or animal blood.

Our group studies the effects of ethanol on the innate immune system. In particular, we focus on the pathogen induced cytokine response, mediated through Toll-like receptors (TLR). TLRs are pathogen recognition receptors that reside on the cell membrane or in intracellular compartments. On ligand binding, they trigger the innate immune response by activating production of cytokines, chemokines, and other mediators of inflammation. Using a commercial ELISA, our group and others found that ethanol decreases the host response to pathogens, measured by TNF- α secretion by macrophage-like cells or in laboratory animals. (Dai, 2006); (Stoltz et al., 2002).

Biological effects of other lower straight and branched chain alcohols had been tested on cell cultures (Bordeleau et al., 2005) and laboratory animals (Feller and Crabbe, 1991). In a study by Song et al., the effects of methanol, ethanol, 1-propanol, and n-butanol on TNF- α secretion were tested in an epithelial cell line by ELISA and other methods. The authors came to the conclusion, that inhibition of TNF- α secretion by the tested alcohols is due to the length of their carbon chain, increasingly disrupting the cell membrane (Song et al., 2005).

We used a similar approach to determine the role of hydrophobicity in the mechanism by which ethanol inhibits the pathogen induced cytokine response in cell culture assays. Therefore, we investigated the potency of other alcohols to inhibit the LPS and poly I:C induced cytokine response. If carbon chain length played a role, this could have given us an indication of the mechanism of action. Methanol, ethanol, 1-propanol, 2-propanol, n-butanol, and n-pentanol were used at 86.8 mM, which corresponds to 0.4 % ethanol. This ethanol concentration is higher than average values reported in humans, but such values do occur in humans and are not extremely rare (Urso et al., 1981).

Supernatants of TLR-ligand and alcohol treated cells were examined by TNF- α ELISA. The detection of TNF- α negatively correlated with the carbon chain length of each alcohol. This effect was first thought to be due completely to inhibition of TNF- α secretion. To determine if the tested alcohols interfered with detection of TNF- α by ELISA, a solution of the TNF- α standard protein from the ELISA kit was used instead of cell culture supernatants, and treated with the alcohol series at 86.8 mM. The results demonstrate that the TNF- α ELISA was significantly inhibited by all alcohols larger than ethanol, and an IL-10 ELISA was significantly inhibited by all alcohols larger than methanol. Thus, some results with regard to the effect of ethanol on IL-10 and human TNF- α production previously reported by us and others may need to be reconsidered.

Materials and Methods

Cell culture

RAW264.7 macrophage like cells were purchased from American Type Culture Collection (ATCC). Human U937 lymphoma cells were a kind gift from Dr. Andrew Yurochko, LSUHSC Shreveport. HEK-null cells stably transfected with the pUNO-mcs vector, and HEK293-mTLR3 cells, stably transfected with mouse TLR3 (#293-null, 293-mtlr3) were

purchased from Invivogen (San Diego, CA, USA). All cell lines were grown in 75 cm² tissue culture flasks from Sarstedt INC. (Newton, NC 28658-0468, USA). For RAW264.7 and U937, cells up to the 10th passage were cultured in RPMI 1640 with L-glutamine (# 61870-036, Invitrogen) supplemented with 10 % FBS (#10437-028, Invitrogen) and penicillin-streptomycin (# P4333, Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C and 5 % CO₂. HEK293 cells were grown in DMEM with 4.5 g/l Glucose (# 11960-077, Invitrogen), 10% FBS, supplemented with 10 µg/ml of blasticidin (# ant-bl-1, Invivogen), and 1:500 Normocin (# ant-nr-1, Invivogen).

For the cell culture assays, cells were seeded into NUNC 24-well plates (# 142475, Thermo Fisher, Rochester, NY, USA) at 1×10⁶/ml, 1 ml/well, and incubated 24 h. Appropriate wells were treated with alcohol and incubated 30 min. Then, LPS or poly I:C were added to appropriate wells, and incubated further 2 h. Ultra Pure Lipopolysaccharide (LPS) from E. coli O111:B4 was purchased from List Biological Laboratories (# 421, List Biological Laboratories, INC., Campbell, CA, USA). Poly I:C was purchased from Invivogen (# tlr-pic-5). Methanol, ethanol, 1-propanol, 2-propanol, n-butanol, and n-pentanol were purchased from Sigma-Aldrich. Appropriate wells were treated first with 86.8 mM of each alcohol for 30 min, then with 100 ng/ml LPS, or with 50 µg/ml poly I:C. Supernatants were harvested after 2 h, and ELISA was performed within the next 30 min. Previous studies (results not shown) indicated that evaporation of alcohols at 2.5 hr was minimal, so the concentration of each alcohol added to ELISA was very near the concentration in the well.

ELISA

Mouse and human cytokine ELISA kits were purchased from BD Biosciences (San Jose, CA, USA).

The BD OptEIA Mouse TNF Mono/Mono kit # 555268 detects free TNF, and contained a monoclonal anti-mouse TNF capture antibody, a monoclonal biotinylated anti-mouse TNF detection antibody, streptavidin-horseradish peroxidase conjugate enzyme reagent, and recombinant mouse TNF standard.

The BD OptEIA Mouse TNF Set II # 558534 detects free and receptor bound TNF, and contained a purified anti-mouse TNF capture antibody, a biotinylated anti-mouse TNF detection antibody, streptavidin-horseradish peroxidase conjugate enzyme reagent, and recombinant mouse TNF standard.

The BD OptEIA human TNF kit # 555212 detects free TNF, and contained a monoclonal anti-human TNF capture antibody, a monoclonal biotinylated anti-human TNF detection antibody, streptavidin-horseradish peroxidase conjugate enzyme reagent, and recombinant human TNF standard.

The BD OptEIA mouse IL-6 kit # 555240 contained a monoclonal anti-mouse IL-6 capture antibody, a monoclonal biotinylated anti-mouse IL-6 detection antibody, streptavidin-horseradish peroxidase conjugate enzyme reagent, and recombinant mouse IL-6 standard.

The BD OptEIA human IL-8 kit # 555244 contained a purified anti-human IL-8 capture antibody, a biotinylated anti-human IL-8 detection antibody, streptavidin-horseradish peroxidase conjugate enzyme reagent, and recombinant human IL-8 standard.

The BD OptEIA mouse IL-10 # 555252 contained a monoclonal anti-mouse IL-10 capture antibody, a monoclonal biotinylated anti-mouse IL-10 detection antibody, streptavidin-horseradish peroxidase conjugate enzyme reagent, and recombinant mouse IL-10 standard.

The BD OptEIA mouse IL-12 (p40) kit # 555165 contained a monoclonal anti-mouse IL-12 capture antibody, a monoclonal biotinylated anti-mouse IL-12 detection antibody, streptavidin-horseradish peroxidase conjugate enzyme reagent, and recombinant mouse IL-12 (p40) standard.

In all ELISAs, the substrate solution was tetramethylbenzidine (TMB), the reaction was stopped with $2\text{NH}_2\text{SO}_4$, and absorbance was read at 450 nm. The assays were carried out according to the manufacturer's specifications. For all assays, NUNC maxisorp 96 well ELISA plates were used (# 439454, Thermo Fisher, Rochester, NY, USA).

Statistical Analysis

Differences between groups were analyzed by One-Way ANOVA and Newman-Keuls multiple comparison test. $P < 0.05$ was considered significant. Analysis was done with Prism 4.0 software (GraphPad Software, INC, La Jolla, CA, USA).

Results

To determine the lowest concentration of ethanol that inhibits TNF- α secretion, mouse macrophage-like RAW264.7 cells were seeded into 24-well plates at $1 \times 10^6/\text{ml}$, 1 ml per well, and incubated 24 h. Medium was mixed with a serial dilution of ethanol from 86.8 mM to 2.71 mM of ethanol. Old medium was removed from the cells by suction. Medium with ethanol was pipetted on the monolayer of cells and incubated 30 min. Then, LPS 50 ng/ml was pipetted into each appropriate well and incubated 2 h. Cell culture supernatants were collected and ELISA was performed. All concentrations of ethanol significantly inhibited the LPS-induced production of TNF- α (Figure 1A).

The effect of ethanol on human macrophage like cells is shown in Figure 1B. A human monocyte-like cell line, U937, was differentiated to a macrophage-like phenotype with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), seeded into 24 well plates and incubated 48 h. The monolayers were washed twice with medium, incubated with 86.8 mM ethanol for 30 min, treated with LPS and incubated further 2 h. Supernatants were collected and human TNF- α ELISA performed. Ethanol significantly inhibited TNF ($P < 0.001$).

Next, RAW264.7 cells were incubated with 86.8 mM methanol, ethanol, 1-propanol, 2-propanol, and n-butanol (Figure 2 A and B). The cell viability test by trypan blue exclusion showed, that n-pentanol at this concentration is cytotoxic, so results for n-pentanol were omitted. After 30 min incubation, cells were challenged with either LPS (Figures 2A, 2C, 2D), or poly I:C (Figure 2B) for two hours. Cell culture supernatants were collected and ELISA was performed immediately. For TNF- α detection, an ELISA kit was used, that detects free (non-receptor bound) TNF- α . All tested alcohols significantly inhibited TNF- α concentration, except methanol in the poly I:C-treated group, and inhibition increased with increasing chain length. To test, if longer chain alcohols inhibit TNF- α in a dose-dependent fashion like ethanol (Figure 1A), we performed titrations with 1-propanol (Figure 2C) and butanol (Figure 2D). The best curve fit of the dose-response profile was different for these two alcohols. Propanol inhibited TNF- α in a sigmoidal fashion, shown after linear regression analysis (Figure 2C). Inhibition was significant up to 10.42 mM. Butanol inhibited TNF- α at all concentrations tested. Even at 2.71 mM, there was significant inhibition of TNF- α compared to cells that were treated with LPS only ($P < 0.001$).

For linear alcohols (all except 2-propanol), there was a clear relationship between chain length and decrease in TNF- α in RAW 264.7 cell cultures stimulated by LPS or poly I:C (Figure 2A and 2B). Longer chain alcohols inhibited TNF- α production (or detection) to a greater extent than short chain alcohols. There was significant inhibition of TNF- α down to

a concentration of 10.85 mM of 1-propanol in a sigmoidal fashion (Figure 2C), as determined by ELISA from cell culture supernatants (standard curve: $R^2=0.995$). Butanol inhibited TNF- α at all concentrations as determined by ELISA from cell culture supernatants (standard curve: $R^2=0.997$) (Figure 2D). However, it remained possible that these effects represented interference with the ELISA by the alcohols remaining in culture supernatants.

To evaluate the possibility that the alcohols in the cell culture supernatants interfered with ELISA detection of TNF- α , recombinant mouse TNF- α reference protein from an ELISA kit was prepared in cell culture medium at 1000 pg/ml. Cell culture medium was also used to prepare a serial dilution of standards for the standard curve, starting with 1000 pg/ml. Cell culture medium alone did not contain TNF- α (Figure 3A). Butanol added to the blocking step of the 96-well plate did not remove the capture antibody (Figure 3B). The other alcohols were tested in the same way and were found not to affect the TNF- α capture antibody (data not shown). Ethanol and the branched chain alcohol 2-propanol had no effect on mouse TNF- α detection. However, ethanol and 2-propanol significantly inhibited human TNF- α detection. Propanol, butanol, and pentanol inhibited detection significantly ($P<0.05$ for 1-propanol, $P<0.001$ for butanol and pentanol). The inhibition was stronger with increasing carbon chain length (Figure 3A). On the basis of these results, we repeated the experiment with a different mouse TNF- α ELISA kit that detects free and receptor bound TNF- α , as well as with a human TNF- α kit (as used above for U937 cells). For kit II, ethanol had no effect. One-propanol, 2-propanol, and butanol inhibited detection significantly ($P<0.001$). Pentanol was not tested with kit II. Detection of the human TNF standard protein was significantly inhibited by all tested alcohols apart from methanol. 1-propanol, butanol and pentanol inhibited beyond detection. The results with TNF- α prompted us to evaluate ELISA kits for other cytokines. A mouse IL-10 ELISA kit was subjected to the alcohol series. A solution of IL-10 standard protein was made in assay diluent (PBS + 10 % FBS). Here, all tested alcohols inhibited IL-10 detection. The inhibition by the straight chain alcohols became stronger with increasing carbon chain length, although there was no significant difference between butanol and pentanol (Figure 4, $P<0.001$ for all alcohols). Again, 2-propanol inhibited less than 1-propanol.

Next, mouse IL-6, human IL-8, and mouse IL-12 ELISA kits were tested with the alcohol series. None of these cytokine ELISA kits were affected by any of the alcohols (Figure 5A, 5C, 5E). However, cell culture experiments revealed, that IL-6 and IL-8 secretion were inhibited by some of the tested alcohols. RAW264.7 cells were treated with the alcohol series (86.8 mM) and LPS like above. IL-6 ELISA was performed on cell culture supernatants. For ethanol and LPS only, the IL-6 concentration was above 886.6 pg/ml, which resulted into a color intensity that exceeded the absorbance maximum of the plate reader. Therefore, inhibition of ethanol could not be evaluated. 1-propanol and butanol significantly inhibited IL-6 detection (Figure 5B). HEK293-mTLR3 cells were treated with different concentrations of ethanol from 43.4 mM to 173.6 mM, and poly I:C as described above. All concentrations significantly inhibited IL-8 secretion. Remarkably, in this system there was no significant relationship between the ethanol dose and the extent of IL-8 inhibition. The TLR4 ligand LPS had no effect, as this cell line does not express TLR4 (Figure 5D). The same experiment was performed with the HEK293-null cell line, and IL-8 was not detected (data not shown). None of the alcohols at 86.8 mM inhibited detection of IL-12 (5E), suggesting that our previous studies using the same ELISA kit and showing decreased IL-12 production following acute exposure to ethanol at this concentration detected changes in cellular production of IL-12, not altered quantitation in the ELISA.

Discussion

An apparent positive correlation between the carbon chain length and the inhibitory effect on pathogen activated cytokine response could have been the result of direct inhibition of the TNF- α and IL-10 ELISAs by the alcohols. One to five carbon straight chain alcohols, and one branched chain alcohol were tested on their effect on ELISA detection of a small number of cytokine proteins. When a fixed concentration of mouse TNF- α standard was incubated with different carbon chain lengths alcohols, the results were largely similar to the results found with cell culture supernatants. Repeated assays with different mouse TNF ELISA kits and one human TNF ELISA kit showed, that methanol, ethanol, and the branched chain alcohol 2-propanol did not inhibit ELISA detection of mouse TNF α , but ethanol and 2-propanol significantly inhibited the detection of human TNF. In all three tested kits, 1-propanol, n-butanol, and n-pentanol had an increasingly inhibitory effect with increased carbon chain length.

Titration of ethanol, 1-propanol and butanol to cell cultures before treatment with LPS showed, that the inhibitory effect on TNF- α ELISA detection was linear. For these studies, ELISA kit II was used, that detects free and receptor bound TNF- α . Whilst the results for ethanol are believed to be genuine inhibition of TNF- α secretion, the results with 1-propanol and butanol largely reflect inhibition of protein binding to the capture antibody, or inhibition of detection antibody binding to the protein. For 1-propanol, significant inhibition could be seen up to a concentration of 10.85 mM. For butanol, no minimum concentration was determined. Even at 2.71 mM, inhibition was significant.

Production of IL-6 and IL-8 was significantly decreased when alcohols were added to cell cultures and supernatants were tested by ELISA. In studies on the effects of ethanol on IL-10, IL-6, and IL-12 secretion, ethanol significantly inhibited the LPS and poly I:C induced secretion of IL-12, IL-10 and IL-6 by cultured mouse peritoneal macrophages (Pruett et al., 2005). IL-10 was inhibited beyond detection, and there was about 80% inhibition of IL-6 and IL-12. Here, we saw an about 40 % inhibition of the IL-10 ELISA standard by ethanol, and no inhibition of the IL-8, IL-6 and IL-12 standards, so that the inhibition observed in cultured macrophages is still valid. In studies with RAW264.7 cells, Dai et al. (Dai et al., 2005) found significant inhibition of the LPS induced TNF- α secretion by ethanol. We found no inhibition of the mouse ELISA standards by ethanol, but inhibition of the human TNF standard. Thus, the results from this and other laboratories indicating significant effects of ethanol and other alcohols on cytokine production in vitro are very likely to be mostly valid. However, the results also indicate that the effects on some cytokines cannot be reliably evaluated because of direct inhibition of the ELISA.

A recent report indicating significant immunosuppressive effects of 2-propanol in vitro (Desy et al., 2008) may need to be assessed with the effects reported here in mind. However, in that study, suppressive effects of isopropanol were confirmed in vivo at times after dosing at which most of the alcohol should have already been cleared. Thus, it is likely that the immunosuppression reported is relevant, but evaluation of some of the cytokines following in vitro exposures may not be quantitatively accurate. Some of our own results that relied on quantitation of TNF- α in vitro may also have been affected by this phenomenon (Dai et al., 2005); (Dai and Pruett, 2006) (Dai, 2006), but inhibition of other key cellular responses, not just decreased TNF- α production, were noted, suggesting that the overall results were valid even if the amount of TNF- α inhibition may not have been accurate. It seemed possible that alcohols inhibited the ELISA immediately after adding the test samples by causing the capture antibody to detach from the plate. However, results here which ethanol and the other alcohols were added during the blocking step indicated that they did not interfere with binding of the capture antibody to the ELISA plate.

A likely explanation for the observed phenomenon could be an alteration of proteins at the binding site of the capture and/or detection antibody. Effects of different alcohols on proteins have been discussed in the literature. Xu et al. found that ethanol in the range of 0.2% to 5.0% caused a change in the interaction between human serum albumin and a tested drug. The addition of the more hydrophobic alcohol 1-propanol caused a change in protein-drug interaction at a much lower concentration, which pointed to hydrophobic interaction as a possible mechanism of action (Xu et al., 2004).

Bordeleau et al. (Bordeleau et al., 2005) studied the ability of different alcohols to prevent lysophosphatidylcholine (LPC)-induced Ca^{2+} overload in rat cardiomyocytes, loaded with a calcium sensitive fluorophore. They tested the straight chain alcohols ethanol, n-propanol, n-butanol, and n-pentanol as well as some branched chain alcohols at 88 mM. These experiments showed, that the increase in lag time after LPC before Ca^{2+} accumulation was inversely proportional to the length of the carbon chain, but only for straight chain alcohols up to four carbons. The authors attributed this effect to a small lipophilic pocket on a protein, or to lateral pressure perturbation in the membrane.

Bucci et al. (Bucci et al., 2006) found a hydrophobic binding pocket for alcohol in the *Drosophila* odorant binding protein LUSH. In H_2O , this protein was partially unfolded, and addition of n-alcohols increased folding and stability. In this study, a linear correlation between alcohol carbon chain length and protein stability was observed, particularly among the residues around the alcohol-binding pocket. Although this protein was derived through natural selection to respond to odorants, including ethanol, this illustrates that ethanol at relevant concentrations can affect protein structure.

Heat shock proteins play an important role in protein folding and act as molecular chaperones, helping proteins to gain their proper conformation. Chaudhuri et al. suggested in studies with *Escherichia coli*, that unfolded proteins in the cytoplasm elicited a heat shock response (Chaudhuri et al., 2006). Some of the alcohols used in our experiments were reported in the literature to cause misfolding of proteins, which initiated a heat shock response. Curran et al. investigated the correlation between alcohol carbon chain length and the heat shock response in *Saccharomyces cerevisiae* treated with methanol, ethanol, n-propanol, n-butanol, and n-pentanol. They found that with increasing carbon chain length, less alcohol is required to elicit the maximum heat shock reaction. The authors suggested that this effect was attributed to increasing interference with the lipid bilayer of the cell membrane with increasing hydrophobicity (Curran and Khalawan, 1994). However, this explanation may not be conclusive, and conformational alterations to cellular proteins could have been responsible for the heat shock response. Neuhaus-Steinmetz et al. investigated methanol, ethanol, 1-propanol, butanol and other alcohols for their capacity to induce synthesis of certain heat shock proteins, and found a strong correlation with increased carbon chain length. The authors speculated that this could be due to a direct effect of alcohols on protein folding (Neuhaus-Steinmetz et al., 1994). In our experiments, three to five carbon straight chain alcohols at a dose of 86.8 mM inhibited ELISA detection of TNF- α and IL-10. The human TNF- α ELISA was inhibited even by ethanol. An explanation for this may be structural alteration of the TNF- α , the capture antibody or the detection antibody caused by the alcohols, and consequent inhibition of antibody binding. The increased sensitivity of the ELISA for human TNF- α as compared to both assays for mouse TNF- α may indicate greater sensitivity of human than mouse TNF- α to conformational change caused by ethanol. Evidence has been reported that ethanol at the concentration used here causes significant alteration in protein conformation due to slight alterations in the solvent properties of water (Martin et al., 2008) or binding of alcohols to specific binding sites (Jung and Harris, 2006), but it is obvious that the degree of change is specific for different proteins.

Taken together, hydrophobicity increases with carbon chain length of alcohols, correlating with the potency of alcohols to cause conformational changes in some proteins. This may distort epitopes for antibody binding and interfere with detection of proteins by ELISA. The results reported here demonstrate the importance of evaluating agents that are tested for their effects on cytokine production in vitro to determine if they are affecting the production of cytokines by cells in culture or the quantitation of cytokines by acting directly in the ELISA. Of course, the alcohol to which humans are exposed most often and in greatest abundance is ethanol. Ethanol apparently is only marginal in altering protein conformation sufficiently to directly disrupt ELISA (significant inhibition was noted only for IL-10 and human TNF- α). However, disruption of other proteins not tested here may be greater. In fact, we have evidence from circular dichroism studies that the conformational change in TLR3 caused by binding its ligand is largely reversed by a relevant concentration of ethanol (unpublished results). This suggests that direct action of ethanol on proteins should be seriously considered as a possible mechanism of ethanol-induced modulation of the immune system.

Acknowledgments

This work was supported by NIAAA grant AA009505.

References

- Bordeleau LJ, Gailis L, Fournier D, Morissette M, Di Paolo T, Daleau P. Cut-off phenomenon in the protective effect of alcohols against lysophosphatidylcholine-induced calcium overload. *Pflügers Arch* 2005;450(5):292–297. [PubMed: 15909177]
- Bucci BK, Kruse SW, Thode AB, Alvarado SM, Jones DN. Effect of n-alcohols on the structure and stability of the *Drosophila* odorant binding protein LUSH. *Biochemistry* 2006;45(6):1693–1701. [PubMed: 16460016]
- Chaudhuri S, Jana B, Basu T. Why does ethanol induce cellular heat-shock response? *Cell Biol Toxicol* 2006;22(1):29–37. [PubMed: 16463017]
- Curran BP, Khalawan SA. Alcohols lower the threshold temperature for the maximal activation of a heat shock expression vector in the yeast *Saccharomyces cerevisiae*. *Microbiology* 1994;140(Pt 9): 2225–2228. [PubMed: 7952173]
- Dai Q. Different Effects of Acute and Chronic Ethanol on LPS-Induced Cytokine Production and TLR4 Receptor Behavior in Mouse Peritoneal Macrophages. *J Immunotoxicol* 2006;3:217–225. [PubMed: 18958703]
- Dai Q, Pruett SB. Ethanol suppresses LPS-induced Toll-like receptor 4 clustering, reorganization of the actin cytoskeleton, and associated TNF- α production. *Alcohol. Clin. Exp. Res* 2006;30(8): 1436–1444. [PubMed: 16899048]
- Dai Q, Zhang J, Pruett SB. Ethanol alters cellular activation and CD14 partitioning in lipid rafts. *Biochem. Biophys. Res. Commun* 2005;332(1):37–42. [PubMed: 15896296]
- Desy O, Carignan D, Caruso M, de Campos-Lima PO. Immunosuppressive effect of isopropanol: down-regulation of cytokine production results from the alteration of discrete transcriptional pathways in activated lymphocytes. *J. Immunol* 2008;181(4):2348–2355. [PubMed: 18684924]
- Feller DJ, Crabbe JC. Effect of alcohols and other hypnotics in mice selected for differential sensitivity to hypothermic actions of ethanol. *J. Pharmacol. Exp. Ther* 1991;256(3):947–953. [PubMed: 2005589]
- Jung S, Harris RA. Sites in TM2 and 3 are critical for alcohol-induced conformational changes in GABA receptors. *J. Neurochem* 2006;96(3):885–892. [PubMed: 16405501]
- Martin SR, Esposito V, De Los Rios P, Pastore A, Temussi PA. Cold denaturation of yeast frataxin offers the clue to understand the effect of alcohols on protein stability. *J. Am. Chem. Soc* 2008;130(30):9963–9970. [PubMed: 18593164]
- Neuhaus-Steinmetz U, Xu C, Fracella F, Oberheitmann B, Richter-Landsberg C, Rensing L. Heat shock response and cytotoxicity in C6 rat glioma cells: structure-activity relationship of different alcohols. *Mol. Pharmacol* 1994;45(1):36–41. [PubMed: 8302278]

- Pruett SB, Fan R, Zheng Q, Schwab C. Differences in IL-10 and IL-12 production patterns and differences in the effects of acute ethanol treatment on macrophages in vivo and in vitro. *Alcohol* 2005;37(1):1–8. [PubMed: 16472714]
- Song K, Zhao XJ, Marrero L, Oliver P, Nelson S, Kolls JK. Alcohol reversibly disrupts TNF-alpha/TACE interactions in the cell membrane. *Respir. Res* 2005;6:123. [PubMed: 16246259]
- Stoltz DA, Nelson S, Kolls JK, Zhang P, Bohm RP, Murphey-Corb M, Bagby GJ. Effects of in vitro ethanol on tumor necrosis factor-alpha production by blood obtained from simian immunodeficiency virus-infected rhesus macaques. *Alcohol. Clin. Exp. Res* 2002;26(4):527–534. [PubMed: 11981130]
- Urso T, Gavalier JS, Van Thiel DH. Blood ethanol levels in sober alcohol users seen in an emergency room. *Life Sci* 1981;28(9):1053–1056. [PubMed: 7219065]
- Xu H, Yu XD, Chen HY. Analysis of conformational change of human serum albumin using chiral capillary electrophoresis. *J. Chromatogr. A* 2004;1055(1–2):209–214. [PubMed: 15560498]

Figure 1. Ethanol inhibits TNF secretion of mouse and human macrophage like cells. Cell culture assays with RAW264.7 cells, and U937 cells were performed at 1×10^6 /ml. There were 6 samples per group. Bars with no shared letters are significantly different ($p < 0.05$). nd = not

detected. **A** RAW264.7 cells. Different concentrations of ethanol in a serial dilution were added to the cell culture medium, and there was a 30 min incubation before treatment with 50 ng/ml LPS for 2 h. ELISA was used to detect free TNF- α . All ethanol concentrations significantly inhibit TNF- α ($P < 0.001$). In the naïve group, TNF was not detectable. Standard curve: $R^2 = 1$. **B** U937 cells were treated with 86.8 mM ethanol and incubated 30 min, then treated with 100 ng/ml LPS for 2 h. ELISA was used to detect free TNF. Standard curve: $r^2 = 1$. Ethanol significantly inhibited TNF ($P < 0.001$).

Figure 2.

Inhibition of LPS or poly (I:C) induced TNF- α increases with increasing carbon chain length of the alcohol. Cell culture assays with RAW264.7 cells. There were 6 samples per group. In Figure A and B, all alcohols were at 86.8 mM. There was a 30 min incubation before treatment with 100 ng/ml LPS or 50 μ g/ml poly (I:C) for 2 h. ELISA was used to detect free TNF- α . All alcohols, except methanol, significantly inhibit TNF- α detection ($P < 0.001$). Bars with no shared letters are significantly different ($p < 0.05$). nd = not detected. **A)** ELISA from supernatants of cells treated with LPS (standard curve: $R^2=0.996$) **B)** ELISA from supernatants of cells treated with Poly (I:C) (standard curve: $R^2=0.992$). **C)** Effects of propanol added to RAW 264.7 cells before treatment with LPS. There is inhibition of TNF- α up to a concentration of 5.42 M in a linear fashion. ELISA from cell culture supernatants (standard curve: $R^2=0.995$). **D)** Titration of butanol before treatment with LPS. Butanol inhibits TNF- α at all concentrations tested in a linear fashion. ELISA from cell culture supernatants (standard curve: $r^2=0.997$).

Figure 3. TNF- α ELISA from 1000 pg/ml solutions of standard protein, treated with different alcohols. There were 5 samples per group. TNF- α protein samples and standard curve were prepared in cell culture medium. Each alcohol was 86.8 mM. Bars with no shared letters are significantly different ($p < 0.05$). nd = not detected. **A)** Mouse TNF- α kit I, detects free TNF- α (standard curve: $R^2=0.989$). **B)** Mouse TNF- α ELISA Kit II, detects free and receptor bound TNF (standard curve: $R^2=0.99$). Butanol bl. = butanol added to the blocking step. **C)** Human TNF ELISA kit (standard curve: $r^2=0.999$).

Figure 4. Mouse IL-10 ELISA standards. There were 5 samples per group. Samples and standards were prepared in assay diluent (PBS with 10 % FBS). Ethanol bl. = ethanol in blocking step. Each alcohol was 86.8 mM. Standard curve: $R^2=0.999$. Bars with no shared letters are significantly different ($p < 0.05$).

Figure 5.
One to five carbon chain alcohols of 86.8 mM did not affect mouse IL-6, human IL-8, or mouse IL-12 ELISA. There were five samples per group. Samples and standards were

prepared in assay diluent (PBS with 10 % FBS). In the assays with standard protein, each alcohol was 86.8 mM. Bars with no shared letters are significantly different ($p < 0.05$). nd = not detected; a.m. = absorbance maximum. **A)** Mouse IL-6 ELISA standards. Standard curve: $R^2=0.999$. Ethanol bl. step = ethanol was added to the blocking step. **B)** A cell culture experiment with RAW264.7 cells was performed. Cells were treated with one to four carbon chain alcohols and LPS as described above. IL-6 ELISA was performed from cell culture supernatants. Abs. max. = absorbance maximum reached. Standard curve: $R^2=0.912$. **C)** Human IL-8 ELISA standards. The concentration of the standard protein was 200 pg/ml. Standard curve: $R^2=0.979$. Ethanol bl. step = ethanol was added to the blocking step. **D)** A cell culture experiment was performed with HEK293-mTLR3 cells. Cells were treated with different concentrations of ethanol, and with poly (I:C) as described above. IL-8 ELISA was performed on cell culture supernatants. The concentration of IL-8 standard was 200 pg/ml. Standard curve: $r^2=0.999$. **E)** Mouse IL-12 ELISA standards. Standard curve: $r^2=1$.