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Inhibitory Effects of Ethanol on the NLRP3 Inflammasome

An Honors Thesis Presented

By

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To

The College of Arts and Sciences

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Abstract

Immunosuppression is a major complication of alcoholism and contributes to increased rates of opportunistic infections and sepsis associated with the addiction. The NLRP3 inflammasome is a central intracellular pattern recognition receptor within the innate immune system, which leads to the cleavage and secretion of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18. Ethanol has been reported to inhibit IL-1β secretion, and here we verify that the alcohol can specifically inhibit activation of the NLRP3 inflammasome resulting in attenuated IL-1β and caspase-1 cleavage and secretion, as well as ASC secretion in response to several agonists. These results were found to be independent of the activation of $GABA_A$ receptors or the inhibition of NMDA receptors. Ethanol was only partially able to prevent IL-1β secretion subsequent to NLRC4 activation and was incapable of preventing NLRP1b dependent IL-1β secretion, which are both largely independent of the adapter protein ASC, and ethanol was shown to prevent the formation of ASC specks. Treatment of cells with ethanol resulted in markedly decreased global tyrosine phosphorylation, while administration of the tyrosine phosphatase inhibitor sodium orthovanadate prior to ethanol restored IL-1β secretion. Multiple alcohol containing organic compounds exerted inhibitory effects on the NLRP3 inflammasome parallel to ethanol; however, isoamyl alcohol’s non-alcohol analog, 2-methylbutane, did not. Together, these results show that ethanol antagonizes the NLRP3 inflammasome at an apical event in its activation potentially through the stimulation of protein tyrosine phosphatases. As other short chain alcohols retain this ability, this effect could be dependent on the hydroxyl group of these compounds.

Introduction

Inflammasomes are a family of large multi-protein intracellular pattern recognition receptors (PRRs) that respond to a wide variety of exogenous pathogen associated molecular patterns (PAMPs) and endogenous danger associated molecular patterns (DAMPs), facilitating the secretion of the pro-inflammatory cytokines, IL-1β and IL-18, as well as a form of inflammatory cell death known as pyroptosis (1). Unlike many innate immune pathways, stimulation of a functional inflammasome requires two steps. During priming (step 1), activation of the transcription factor NF-κB, downstream of the stimulation of many PRRs, leads to the production of several components of the inflammasome and the secretion of the pro-inflammatory cytokine TNFα (2). Activation of the inflammasome (step 2) requires the exposure of cells to a separate set of PAMPs and DAMPs, which work through unique signaling pathways leading to the oligomerization of one of several different Nucleotide Oligomerization Domain (NOD)-Like Receptor (NLR) proteins, the adaptor protein Apoptosis-associated Speck-like protein containing a CARD (ASC), and pro-caspase-1 into an organized inflammasome complex (3). This oligomerization is mediated by homotypic PYRIN-PYRIN domain binding between NLRs and ASC, and CARD-CARD interactions between ASC and pro-caspase-1, resulting in the formation of a discrete ASC speck within stimulated cells (4). These ASC specks form rapidly and irreversibly within activated cells and are a platform for efficient pro-IL-1β and pro-IL-18 cleavage. While the activity of all inflammasomes is thought to be enhanced by the incorporation of ASC into their complexes, NLRP1 and NLRC4 contain their own CARD domains and can interact directly with pro-caspase-1 independent of ASC (5). This assembly allows for the conversion of pro-caspase-1 into an active caspase-1 enzyme, which cleaves pro-IL-1β and pro-IL-18 into their mature, secreted forms. These cytokines then function to promote vasodilation, attract and stimulate neutrophils, induce fever, and activate the acute phase response within an organism (6). Some consider the secretion of IL-1β and IL-18 to be a third step in the process of inflammasome activation. Both IL-1β and IL-18 are leaderless proteins, which despite years of research and many proposed models, still do not have a well-defined mode of release (7). The final outcome of inflammasome formation, pyroptotic cell death, is believed to amplify the immune response while depleting pathogens of their host leukocyte niche (8).

The NLRP3 inflammasome is capable of responding to a particularly diverse set of PAMPs and DAMPs, including ATP, nigericin, alum, asbestos, silica, and cholesterol crystals (9-13). These agonists activate the inflammasome through disparate pathways, such as K^+ efflux and lysosomal rupture, eventually converging on ASC phosphorylation and multimerization (14, 15). As a result, this inflammasome, expressed predominantly by macrophages, but also monocytes, neutrophils, dendritic cells, some lymphocytes, and cells that are not leukocytes, plays a major function in immune homeostasis (16). Beyond its protective roles in response to pathogens, over-activation of the NLRP3 inflammasome has been implicated in the pathogenesis of an array of diseases such as atherosclerosis, diabetes, gout, and multiple sclerosis (17-19). Similarly, gain of function mutations in NLRP3 lead to the set of debilitating diseases known as Cryopyrin-Associated Periodic Syndrome (CAPS) (20). Although many inhibitors of signal 1 are known, until recently few compounds capable of directly inhibiting signal 2 were discovered.

Alcohol use disorders were estimated to be the third most common non-genetic cause of mortality in the U.S. in the year 2000 (21). Alcohol abuse predisposes individuals to opportunistic infections and organ damage, which are the two most prominent alcohol-related medical complications (21). In trauma and post-surgical patients, alcohol exposure occurring prior to or at the time of injury enhances morbidity and mortality due to increased rates of sepsis and shock, and chronic alcoholics account for 50% of all Acute Respiratory Distress Syndrome patients (22, 23). Furthermore, light to moderate alcohol consumption is associated with decreased risks of developing coronary artery disease and atherosclerosis, illnesses commonly associated with systemic inflammation (17). The pattern of drinking differentially affects the consequences of alcohol abuse. Binge alcohol consumption suppresses host innate immune defense, while chronic alcohol consumption suppresses innate and adaptive immune systems, yet activates chronic inflammation (24).

Ethanol is a known inhibitor of signal 1 (the consequences of PRR signaling) and its consumption is associated with decreased circulating levels of TNFα and IL-1β (25, 26). Recently, ethanol, but not its metabolite acetaldehyde, was found to also be capable of inhibiting signal 2 for the NLRP3 and AIM2 inflammasomes (17). The methods through which ethanol exerts its immunosuppressive effects are still unclear, yet given the central role that inflammasomes play in the immune response, it is possible that direct inhibition of signal 2 could be an important target of alcohol induced immunosuppression.

Ethanol is known to have a wide range of effects when administered to cells. At high doses, it can alter membrane fluidity and can diffuse across the plasma membrane to interact with cytosolic proteins (27). Some known intracellular effects of acute ethanol administration include tyrosine phosphatase and adenylyl cyclase activation (28, 29). At lower doses, ethanol is thought to interact with a variety of cell surface receptors, particularly neurotransmitter receptors, in an agonistic or antagonistic manner (30, 31). During chronic exposure to ethanol, gene expression can be altered, potentially contributing to the differences that chronic alcoholism and binge drinking exert on immune function (32).

The goal of this study was to further elucidate the mechanism of ethanol's inhibition of the NLRP3 inflammasome, primarily using the J774 mouse macrophage and THP-1 human monocyte cell lines and a protocol resembling binge drinking in humans. Experiments were designed to assess ethanol's ability to directly inhibit signal 2, rather than its already well-defined capacity to prevent NF-κB and signal 1 activation. By identifying pathways involved in ethanol's blockade of this key innate immune complex, we hope to better understand and determine potential sites of therapeutic intervention in ethanol mediated immunosuppression and also to identify potential targets for future NLRP3 inflammasome inhibitors.

Materials and Methods

Reagents

LPS, isoamyl alcohol, 2-methylbutane, picrotoxin, 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid, and monoclonal anti-mouse β-actin antibodies were all purchased from Sigma-Aldrich (St. Louis, MO). ASC and caspase-1 antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX), anti-phosphotyrosine antibodies from Cell Signaling (Danvers, MA), and anti-mouse IL-1β antibodies from R&D Systems (Minneapolis, MN). Recombinant mature mouse IL-1β was also purchased from R&D Systems. Biotin conjugated anti- mouse and rabbit secondary antibodies were from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire) and anti-goat IgG from Jackson ImmunoResearch (West Grove, PA). For inflammasome stimulation, ATP, nigericin, alum Imject, alum powder, apoSAA, and anthrax lethal factor and protective antigen were purchased from Amersham Biosciences (Piscatawy, NJ), Invivogen (San Diego, CA), Thermo Scientific (Waltham, MA), Natural Provisions Market

(Williston, VT), PeproTech Inc. (Rocky Hill, NJ) and BEI Resources (Manassas, VA) respectively. Muscimol was acquired from MP Biomedicals (Santa Ana, CA), and ethanol from Pharmco AAPER (Brookfield, CT).

Cell Culture

J774 cells purchased from American Type Culture Collection (ATCC, Manassas, VA) were maintained in DMEM media (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco), 1% L-Glutamine (Gibco), and 1x Primocin (Invivogen, San Diego, CA). Cells were not used beyond passage 20 to reduce variability between the experiments. EGFP and ASC-EGFP stably transfected THP-1 cells were kindly gifted to us by Dr. Mark D. Wewers (Ohio State University) (33) and were maintained in RPMI medium (Gibco) supplemented with 10% FBS (Gibco), 1% L-Glutamine (Gibco), 1% Pen/Strep (Gibco), and 5µM β-ME (Sigma).

For experiments in which cell supernatants were examined by ELISA, cells were plated at $2.5x10^5$ cells/well in a 48-well plate in 250µl of media and allowed to grow overnight. The following day, the media was removed, fresh media was added and cells were treated as indicated within the figure for each experiment. Cell supernatants were harvested at the end of each experiment, spun down at 6,000 rpm for 10 minutes to pellet cellular debris, transferred to new tubes, and frozen at -20˚C until analysis.

For experiments analyzed through western blotting, J774 cells were plated at $3x10^6$ cells/well in 2ml of media in a 6-well plate and allowed to grow overnight. The next day, the media was removed, cells were washed twice in warm PBS and placed in serum free media. The cells were treated as indicated within each experiment’s figure. Supernatants were spun down at 6,000 rpm, transferred to new tubes, and frozen at -20˚C. The cells were then washed twice with

PBS and lysed in RIPA buffer (50mM Tris pH 8, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) containing 1mM sodium orthovanadate (Sigma-Aldrich), 1x protease cocktail inhibitor (Sigma-Aldrich), and 1x PMSF (Sigma-Aldrich) on ice for 10 minutes with scraping. Cellular debris was removed through centrifugation at 6,000 rpm for 10 minutes, the lysates were transferred to new tubes, and frozen at -20˚C. Before running on a gel, protein concentrations in the lysates were quantified by a detergent compatible protein assay. For all experiments using J774 cells the previously determined dose of LPS for half maximal IL-1β secretion, upon stimulation, was used (37ng/ml).

For the visualization of ASC specks, ASC-EGFP and EGFP THP-1 cells were plated at $2.5x10^5$ cells/well in 12-well plates and differentiated with PMA (Sigma-Aldrich) for 24 hours. The cells were then washed with PBS and placed in fresh media for an additional 48 hours. The THP-1 cells were then treated as indicated and imaged under bright field and fluorescence microscopy using an Eclipse TS100 microscope and DS-QiMc digital camera (Nikon, Melville, NY). Five images were taken per group, along with their corresponding bright field images, and the number of ASC specks per visual field per cell were counted.

IL-1β and TNFα ELISAs

ELISAs were conducted according to manufacturer's protocols (BD Biosciences, San Jose, CA). Briefly, 96-well high-binding plates (Corning, Kennebunk, ME) were coated overnight at 4˚C with IL-1β or TNFα capture antibody diluted in coating buffer (100mM $NaHCO_3$, 33.6mM Na_2CO_3, pH 9.5) overnight. The following day, plates were washed three times and blocked in 10% FBS/PBS for 1 hour at room temperature. The plates were then washed three times and standards and samples were added for 2 hours. After an additional three washes, diluted detection antibodies were added to the plates for 1 hour. The plates were again washed three times and Streptavidin-HRP was added for 30 minutes. After washing the plates four times, substrate solutions A and B (R&D Systems) were mixed at a 1:1 ratio, added to the plate, and 2M H_2SO_4 was added to stop the development of the reaction. Plates were then read on a BioTek PowerwaveX (Winooski, VT) instrument at 450nm with a λ correction of 570nm using the program Gen5 1.1.

Cell Death Assay

Cell death was assessed via a lactate dehydrogenase (LDH) assay in cell culture supernatant using CyTox96 assays, according to manufacturer's directions (Promega, Madison, WI). Briefly, in a 96-well plate (Corning) a standard curve of 100% cell death was created with J774 cell lysates and the samples were loaded at a 1:5 dilution in assay buffer at a total volume of 50µl. To this, 25µl of substrate mix was added, the plate was incubated in the dark for 30 minutes, and 25µl of stop solution was added to each well. The plate was then read using a BioTek PowerwaveX instrument at 490nm using the program Gen5 1.1.

Protein Quantification

Protein was quantified using a detergent compatible assay (Bio-Rad, Hercules, CA), according to manufacturer's directions. Briefly, working reagent A was prepared by mixing Assay Reagent S with Assay Reagent A at a ratio of 1:25. Sample, BSA standard or blank were added to a 96-well plate, and mixed with working reagent A at a ratio of 1:5. To this, 200µl of assay reagent B was added and the plates were incubated for 15 minutes on a shaker at room temperature. The plates were subsequently read using a BioTek PowerwaveX instrument at 750nm using the program Gen5 1.1.

Chloroform-Methanol Protein Precipitation from Cell Supernatants

Supernatants from cells cultured in 6-well plates in serum free media were divided into Eppendorf tubes with 500µl of media per tube. An equal volume of methanol to supernatant (500µl) and ¼ volume of chloroform (125µl) was added, and the samples were vortexed for 20 seconds. Samples were then spun down at 20,000g for 10 minutes at room temperature and the upper phase of the mixture was removed, keeping the intermediate protein phase intact. To this, 500µl of methanol was added, the samples were vortexed for 20 seconds, and spun down at 20,000g for 5 minutes at room temperature. The liquid phase was removed and the pellet was dried at 55˚C for 1-5 minutes. The pellet was then resuspended in 15µl of PBS, duplicate samples were pooled together and protein concentrations were measured by a detergent compatible protein assay before being mixed with 4x Lamelli sample buffer containing β-ME, and vortexed an additional 20 seconds.

Western Blots

Cell culture supernatants were based on equivalent volumes, whereas cell lysate samples were normalized to total protein, and prepared by diluting 1:4 in 4x β-ME sample buffer, heating at 100˚C for 5 minutes, vortexing for 20 seconds, and brief centrifugation to remove the condensation from the tubes. Samples were then loaded onto gels, run in 1x TGS running buffer, and transferred to nitrocellulose (for probing with ASC, caspase-1 and phosphotyrosine antibodies) or PVDF (for probing with IL-1β antibody) membranes. Blots were blocked in 5% milk/TBST (IL-1β, ASC and β-actin), 0.5% milk/TBST (caspase-1) or 3% BSA/TBST (phosphotyrosine) for 2 hours at room temperature, and placed in primary antibody overnight at 4˚C. Blots were then washed three times in TBST for 10 minutes and placed in biotin-conjugated secondary antibody for 2 hours at room temperature. The blots were again washed and incubated in ECL reagent (Thermo Scientific) for 5 minutes before exposing to X-ray film and developing.

Ponceau Staining

J774 cells were treated as indicated and lysates and supernantants were prepared as previously described and separated by SDS-PAGE. Proteins within the gel were transferred onto nitrocellulose and washed with TBS for 5 minutes. The membrane was then placed in ponceau stain (0.1% ponceau S (Sigma-Aldrich) in 5% acetic acid) for 5 minutes, and washed with water tree times for 5 minutes each.

Statistical Calculations

All experiments were repeated at least twice and representative results are presented. Results were analyzed by two-tailed unpaired t test, one-way ANOVA or two-way ANOVA and Bonferroni post hoc test using GraphPad Prism 5 for Windows (GraphPad). A p value <0.05 or <0.0001was considered statistically significant.

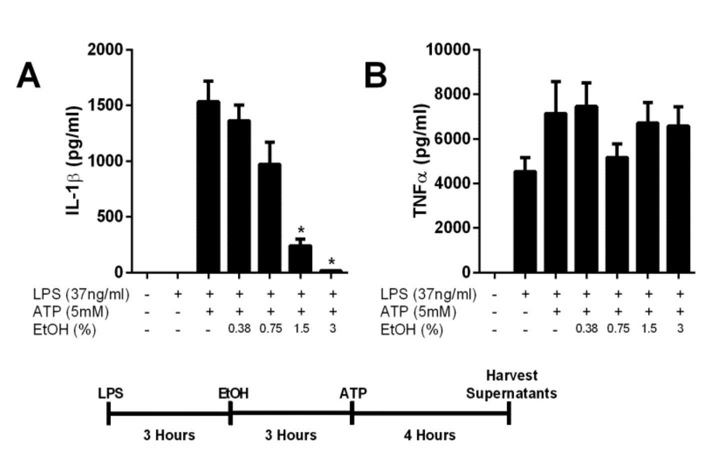

Figure 1. IL-1β (**A**) and TNFα (**B**) ELISAs on supernatants from J774 cells treated as indicated with LPS, ethanol, and ATP. *****<0.0001 by one-way ANOVA relative to the LPS+ATP treated group. EtOH = ethanol.

Results

Ethanol Can Inhibit ATP-Induced IL-1β Secretion

The combination of priming macrophages with the TLR4 agonist lipopolysaccharide (LPS) and stimulating with the DAMP ATP is a well characterized method of inducing NLRP3 inflammasome activation (9). In order to test whether ethanol is capable of inhibiting IL-1β secretion subsequent to NLRP3 inflammasome stimulation, J774 cells were primed with LPS for 3 hours, pre-treated with ethanol for an additional 3 hours, and stimulated with ATP for 4 hours. Ethanol significantly and dose dependently inhibited the secretion of IL-1β relative to cells treated with LPS and ATP alone (**Figure 1A**). In addition, TNFα production was unaffected by ethanol, suggesting that NF-κB activation and priming of the inflammasome were not influenced by the alcohol at this time point (**Figure 1B**). Therefore, it is likely that ethanol acts on either signal 2 (activation) or signal 3 (secretion) to prevent IL-1β secretion from the NLRP3 inflammasome.

Ethanol Exposure does not Promote Cell Death

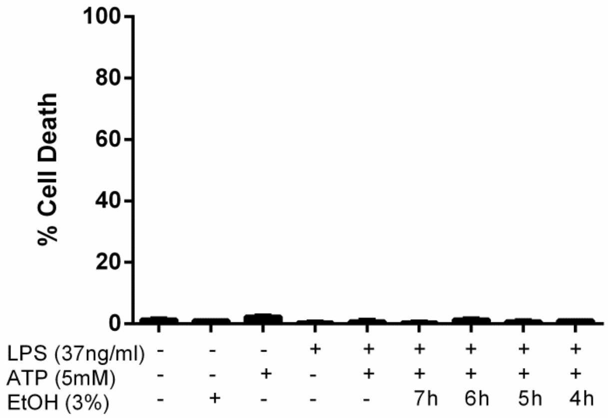

Figure 2. An LDH assay on the supernatants of J774 cells treated with LPS, ATP, and ethanol as indicated to measure the effect on ethanol on cell death. EtOH = ethanol.

To ensure that the doses of ethanol used in these experiments would not be toxic to our cell line, an LDH assay was run on the supernatants of LPS primed J774 cells pre-treated with ethanol for up to three hours and stimulated with ATP. Ethanol administration to J774 cells alone for 7 hours and for 4-7 hours during inflammasome activation did not induce measureable cell death by LDH assay (**Figure 2**). These results validated that the maximum dose of ethanol used in our studies (3% v/v) did not induce a loss of cellular viability. Therefore, it was determined that doses of ethanol at and below 3% were acceptable for use in these experiments.

Ethanol Does Not Interfere with the IL-1β or TNFα ELISAs

It was feasible that the presence of ethanol in the supernatants could interrupt protein structure and inhibit an ELISA's ability to detect IL-1β. To rule this out, three standard curves of recombinant mature IL-1β were run on an ELISA in the presence of 0, 1.5, and 3% ethanol. Treatment with 3% ethanol was capable of interfering with the detection of IL-1β, generating calculated IL-1β levels that were up to 28% below the expected value (**Figure 3A**). However, this is significantly less than the 92% decrease in IL-1β secretion observed from J774 cells primed with LPS and pre-treated with 3% ethanol before stimulation with ATP when compared to cells treated with LPS and ATP alone (**Figure 3B**). These results indicate that the decrease in IL-1β that we measure through ELISAs is primarily the result of a reduction in the amount of secreted IL-1β protein.

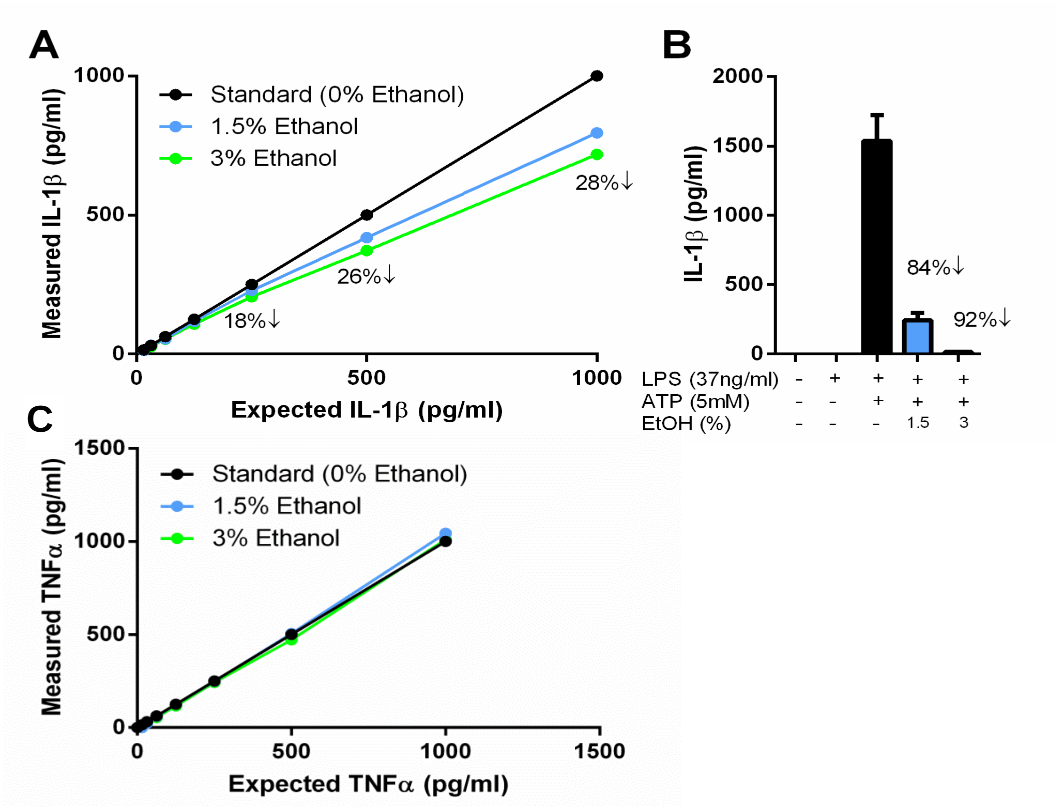

Figure 3. An IL-1β ELISA of cytokine standards treated with 0, 1.5, and 3% ethanol to measure the alcohol's ability to interfere with the assay (**A**). An IL-1β ELISA on supernatants from J774 cells unstimulated, treated with LPS (10h), with LPS (6h) and ATP (4h), or pretreated with ethanol (3h) before ATP addition (**B**). A TNFα ELISA of cytokine standards treated with 0, 1.5, and 3% ethanol (**C**). EtOH = ethanol.

Since ethanol did interfere slightly with the IL-1β ELISA's ability to detect its target protein we diluted TNFα standards in 0, 1.5, and 3% ethanol to determine its capacity to exert a similar effect on the TNFα ELISA. Ethanol showed no ability to alter the performance of the TNFα ELISAs (**Figure 3C**), which matches our results showing no change in TNFα secretion from groups treated with the alcohol.

Ethanol Can Inhibit NLRP3 Inflammasome Activation by a Variety of Agonists

To evaluate upon which pathways leading to NLRP3 inflammasome activation ethanol can act, LPS primed J774 cells were treated with ethanol prior to stimulation with several

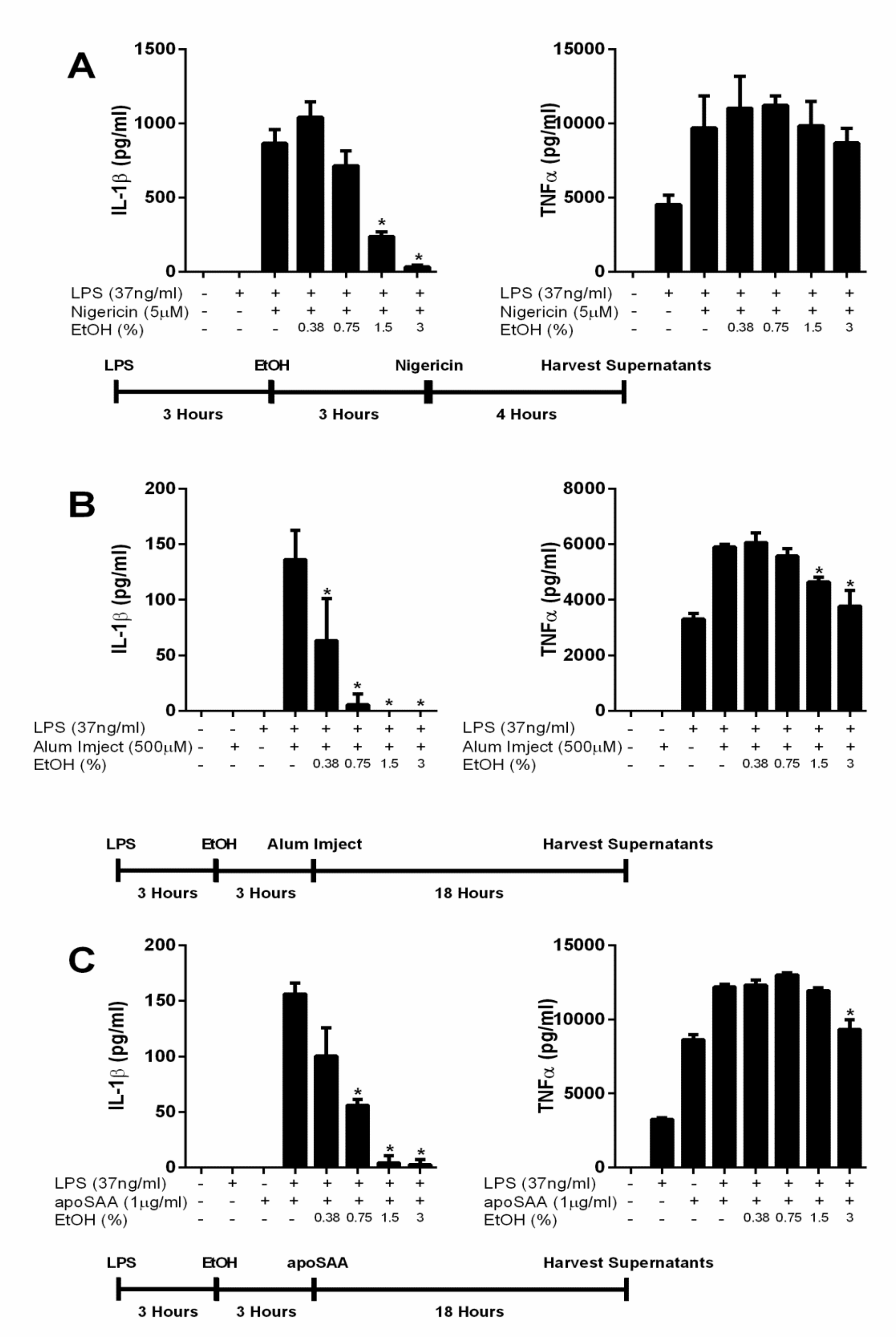

Figure 4. IL-1β and TNFα ELISAs on supernatants from J774 cells treated as indicated with LPS, ethanol, and nigericin (**A**), alum (**B**), or apoSAA (**C**) to determine ethanol's capacity to inhibit infalmmasome formation in response to different types of agonists. *****<0.0001 by one-way ANOVA relative to the LPS+Nigericin (**A**) +Alum (**B**) or + apoSAA (**C**) treated groups. EtOH = ethanol.

additional NLRP3 agonists (nigericin, alum, and apoSAA), which act though different upstream mechanisms distinct from those utilized by ATP (P2X7 receptor activation) to induce inflammasome stimulation. Ethanol significantly and dose dependently inhibited the secretion of IL-1β from cells stimulated with each of these three agonists, while having no substantial impact on TNFα production (**Figure 4**). Since ethanol is capable of preventing IL-1β secretion in response to each agonist without altering TNFα production, this implies that ethanol's actions are likely on downstream events in NLRP3 inflammasome formation, where the three pathways converge.

Kinetics of Ethanol Inhibition

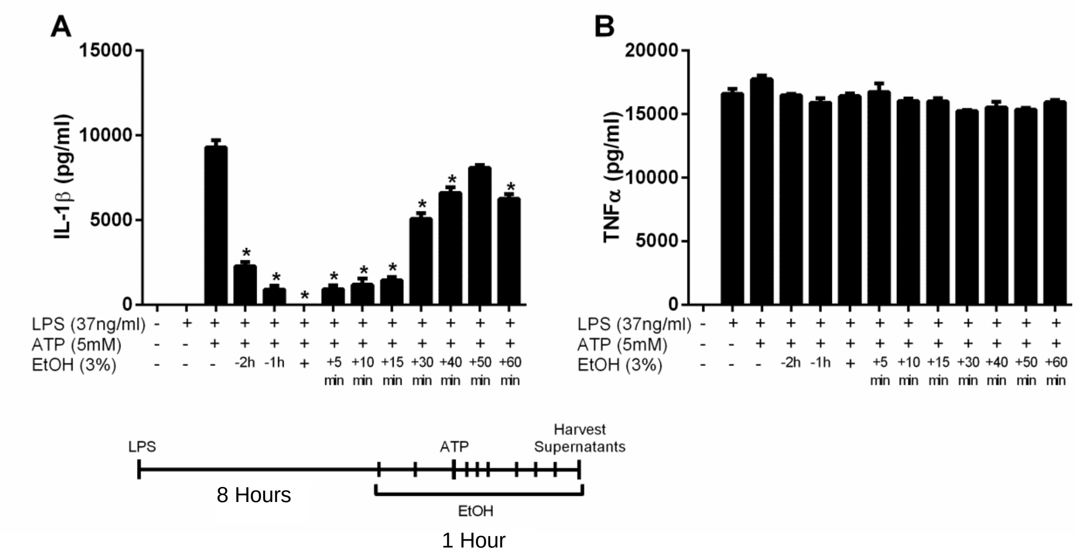

Figure 5. IL-1β (**A**) and TNFα (**B**) ELISAs on supernatants from J774 cells unstimulated, primed with LPS, treated with LPS and ATP, or treated with LPS, ATP, and ethanol at indicated time points pre and post ATP addition to determine the kinetics of ethanol's inhibition of the NLRP3 inflammasome. *****<0.0001 by one-way ANOVA relative to the LPS+ATP treated group. EtOH = ethanol.

Elucidating at which time points ethanol is capable of blocking inflammasome stimulation is important for understanding the mechanisms through which this chemical might be acting. To observe the kinetics of ethanol inhibition, ethanol (3%) was administered to LPS primed cells 2 and 1 hours before and simultaneously with ATP stimulation, as well as at several times points following ATP addition. Ethanol treatment at each time point before ATP addition and up to 15 minutes after stimulation was found to inhibit NLRP3 inflammasome activation, and consistent with previous experiments, ethanol treatment had no impact on macrophage priming and TNFα production (**Figure 5**). These results indicate that the effects of ethanol are immediate and unlikely to be due to slower cell signaling processes such as alterations in gene expression.

Ethanol Prevents the Cleavage of Pro-IL-1β into its Mature Form

Since the decrease in IL-1β production observed by ELISA could be due to retention of cleaved IL-1β within the cell rather than inhibition of caspase-1 and inflammasome action, western blots were performed to determine whether mature IL-1β could be found in the lysates or the supernatants of primed, ethanol treated macrophages stimulated with ATP. Consistent with an absence of caspase-1 enzymatic activity, there was no cleaved IL-1β visible in either the cell lysates or supernatants of ethanol treated cells (**Figure 6**). There was, however, abundant 17kDa mature and alternatively cleaved 28kDa IL-1β in cells treated with LPS and ATP alone. From LPS and ATP treated cells both with and without ethanol administration, pro-IL-1β was detected in the supernatants. This could be due to ATP induced cell death occurring at too low a level to be identified by LDH assay and subsequent leakage of pro-IL-1β from damaged cell membranes. Despite some

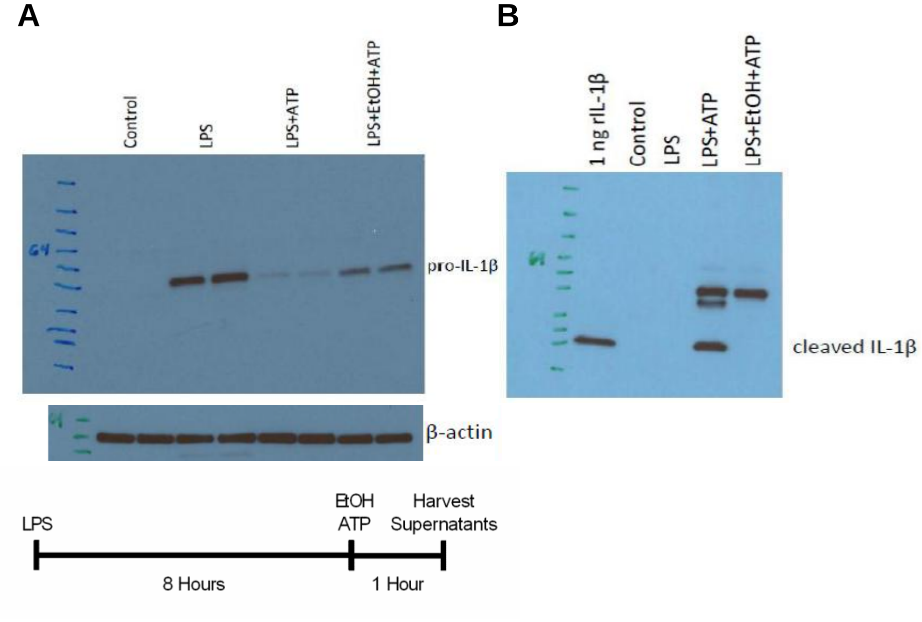

Figure 6. Western blots on J774 cell lysates treated as indicated and probed for IL-1β and β-actin (**A**), and supernatants from the same experiment probed for IL-1β (**B**). EtOH = ethanol.

secretion of pro-IL-1β, there was still a greater concentration of pro-IL-1β retained in the lysates of cells treated with ethanol verses those treated only with LPS and ATP, likely representing the pool of pro-IL-1β that was not converted to mature IL-1β and secreted.

Treatment with Ethanol Prevents ASC and Caspase-1 Secretion

The activity of functional inflammasomes results in the secretion of not only IL-1β and IL-18, but also other inflammasome components, including NLRP3, ASC, and caspase-1. To further validate that ethanol inhibits inflammasome activation, western blots of the lysates and supernatants of J774 cells were probed with anti-ASC or anti-caspase-1 antibodies. While constitutively expressed ASC was detected in the lysates of every group (**Figure 7A**), there was significantly less ASC (**Figure 7B**) and no mature caspase-1 (**Figure 7C**) identified in the supernatants of cells pre-treated with ethanol. However, ASC and caspase-1 were detected in the supernatants of LPS and ATP treated cells, indicating successful inflammasome stimulation with this protocol. Similar to pro-IL-1β, pro-caspase-1 was detected in the supernatants of both groups treated with ATP.

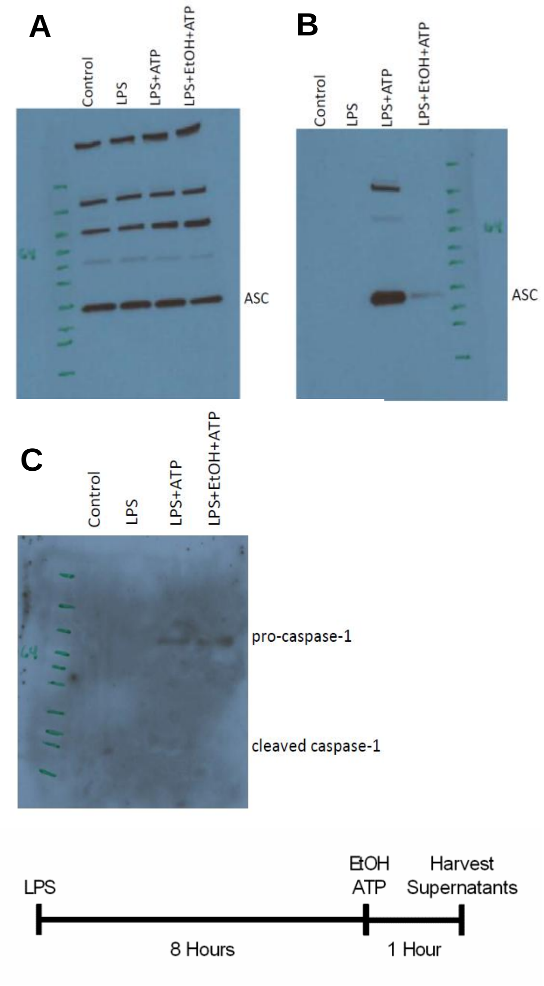

Figure 7. Western blots of lysates (**A**) and supernatants (**B** and **C**) from J774 cells treated with LPS (37ng/ml), ATP (5mM) and ethanol (3%) as indicated and probed for ASC (**A** and **B**) or caspase-1 (**C**). EtOH = ethanol.

Ethanol Cannot Reverse ATP-Induced Protein Secretion

J774 cells were treated as indicated and a Ponceau stain was performed on both cell lysates and precipitated supernatants (**Figure 8**). Even amounts of protein were detected in the lysates of each group; however, secreted proteins could only be detected in those treated with ATP. There was no discernible inhibition of general protein secretion with the addition of ethanol, indicating that the absent mature IL-1β, caspase-1, and ASC secretion observed after treatment with ethanol is due to direct inhibition of the NLRP3 inflammasome.

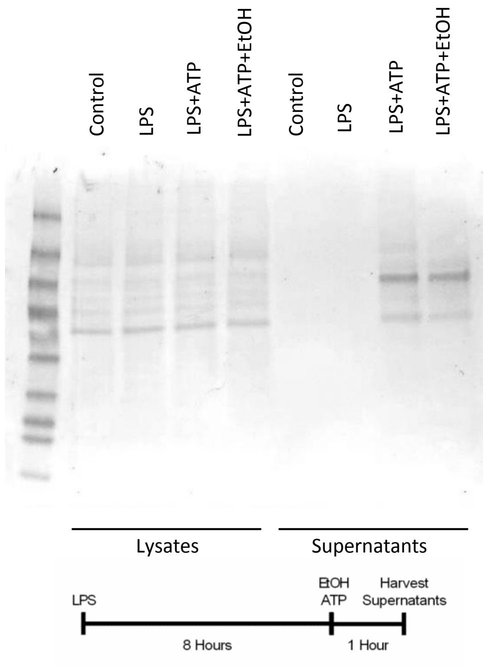

Figure 8. A Ponceau stain lysates and supernatants from J774 cells treated with LPS (37ng/ml), ATP (5mM) and ethanol (3%) as indicated. EtOH = ethanol.

Ethanol Displays Incomplete Inhibition of IL-1β Secretion from ASC Independent Inflammasomes

To determine whether ethanol's inhibition might be ASC dependent, J774 cells were treated with flagella, which contains the NLRC4

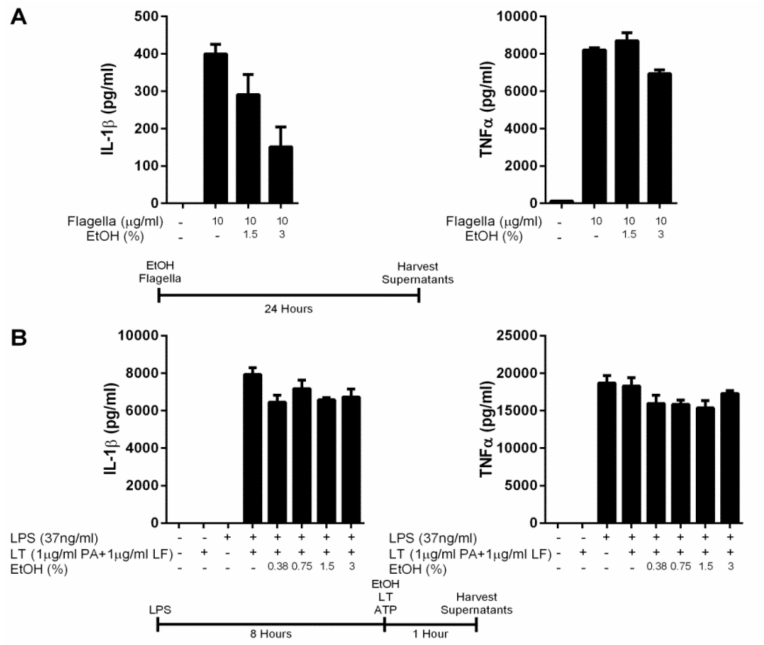

Figure 9. IL-1β and TNFα ELISAs from supernatants of J774 cells treated with the NLRC4 agonist, flagella (**A**) or the NLRP1b agonist anthrax lethal toxin (**B**) with or without ethanol. *****<0.0001 by one-way ANOVA relative to the flagella or LPS+LT treated groups. EtOH = ethanol

agonist flagellin. As flagella acts as both a signal 1 and 2 agonist, the cells were treated with ethanol (1.5-3%) at the same time as flagella (10μg/ml) to ensure that it would have sufficient time to block signal 2 at the expense of potentially also inhibiting inflammasome priming. In contrast to NLRP3 agonists, ethanol was only able to partially inhibit the NLRC4 inflammasome and IL-1β secretion (**Figure 9A**). In addition, LPS primed J774 cells were treated with the NLRP1b agonist anthrax lethal toxin (LT), a combination of anthrax lethal factor (LF 1μg/ml) and protective antigen (PA 1μg/ml), both with and without ethanol. Similar to NRLC4 inflammasomes, NLRP1b inflammasome stimulation could not be inhibited by the administration of ethanol (**Figure 9B**). As NLRC4 and NLRP1b inflammasome activation is enhanced by, but not dependent on, the adaptor protein ASC, these results could support ASC speck formation as a target of ethanol's inhibition.

Ethanol Treatment Inhibits ASC Speck Formation

Since ASC speck formation is a point of convergence for all NLRP3 inflammasome activators and we have shown that ethanol cannot completely inhibit IL-1β secretion from the partially ASC-independent NLRC4 inflammasome, we tested the ability of ethanol to prevent the formation of ASC specks. THP-1 cells stably transfected to express a fusion protein of ASC and GFP were treated with nothing, LPS, LPS and ATP, or LPS, ethanol and ATP and were observed by fluorescence microscopy to visualize the generation of ASC specks.

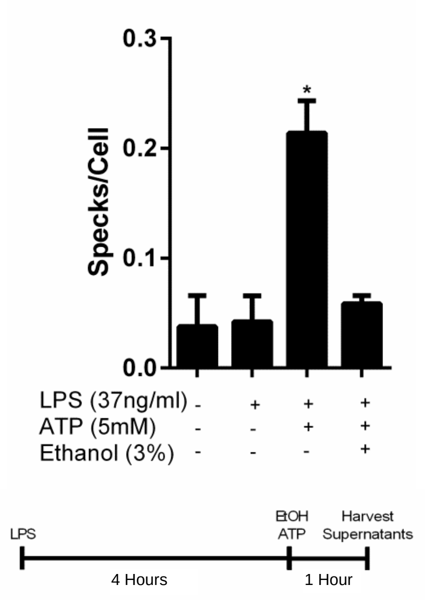

Figure 10. ASC-EGFP transfected THP-1 cells treated with LPS (100ng/ml), ethanol (3%), and ATP (5mM) as indicated and imaged using using bright field and fluorescence microscopy for total cell counts and speck formation. *****<0.05 by one-way ANOVA relative to the control group.

Five images were taken per group along with corresponding bright field images to calculate the number of specks formed per total number of cells per image. EGFP stably transfected THP-1 cells were used as a negative control and consistently demonstrated no visible speck formation under fluorescence microscopy in response to any of the four treatments. Both untreated and LPS primed ASC-EGFP THP-1 cells exhibited low levels of speck formation (**Figure 10**). Stimulation with ATP significantly increased the number of ASC specks present per cell per visual field, and this increase was nearly completely ameliorated by the administration of ethanol alongside ATP.

Ethanol Decreases Global Tyrosine Phosphorylation

Ethanol has been reported to activate tyrosine phosphatases, and the phosphorylation of ASC at Y144 (mouse) and Y146 (human) has been proven to be vital for ASC speck and inflammasome formation to occur (14). To test whether ethanol has global effects on tyrosine phosphorylation in macrophages, we treated J774 cells as indicated and performed western blots on the lysates, probing for phosphotyrosine. As anticipated, ethanol treatment greatly reduced tyrosine phosphorylation relative to controls, while having no effect on β-actin levels (**Figure 11**).

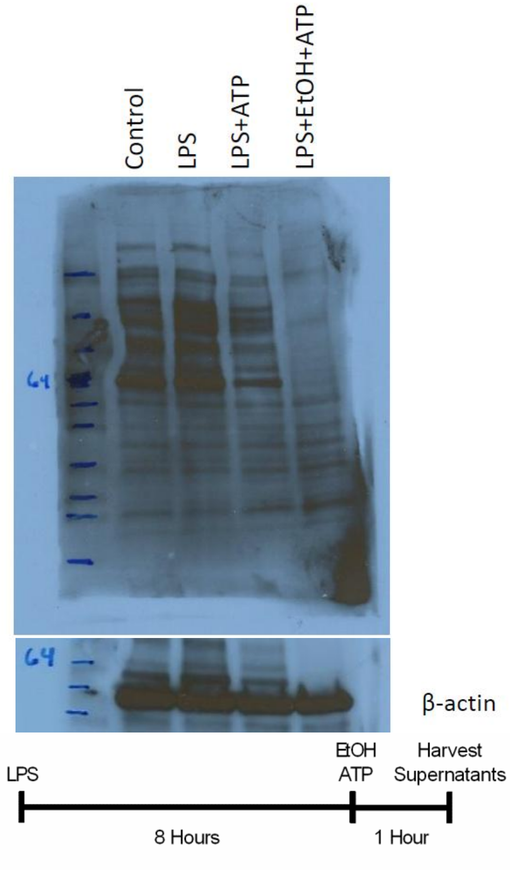

Figure 11. Western blots of lysates from J774 cells treated with LPS (37ng/ml), ATP (5mM), and ethanol (3%) as indicated and probed for phosphotyrosine and β-actin. EtOH = ethanol.

There was an unexpected decrease in tyrosine phosphorylation in LPS and ATP treated cells, possibly due to loss of select proteins secreted during inflammasome activation.

Phosphatase Blockade Ameliorates Ethanol's Inhibition of IL-1β Secretion

To evaluate whether ethanol's NLRP3 inhibitory activity might be a result of increased tyrosine phosphatase activity, J774 cells were pre-treated with sodium orthovanadate (100-1000μM) 30 minutes prior to ethanol and ATP stimulation. All doses of sodium orthovanadate used reversed the effects of ethanol's inhibition (**Figure 12**). To ensure that any increases in IL-1β detected by ELISA would not be due to cell death and leakage of pro-IL-1β into the supernatants, cells were primed with LPS and treated with sodium orthovanadate with the same doses and for the same period of time as those receiving ethanol and ATP. In these groups, no

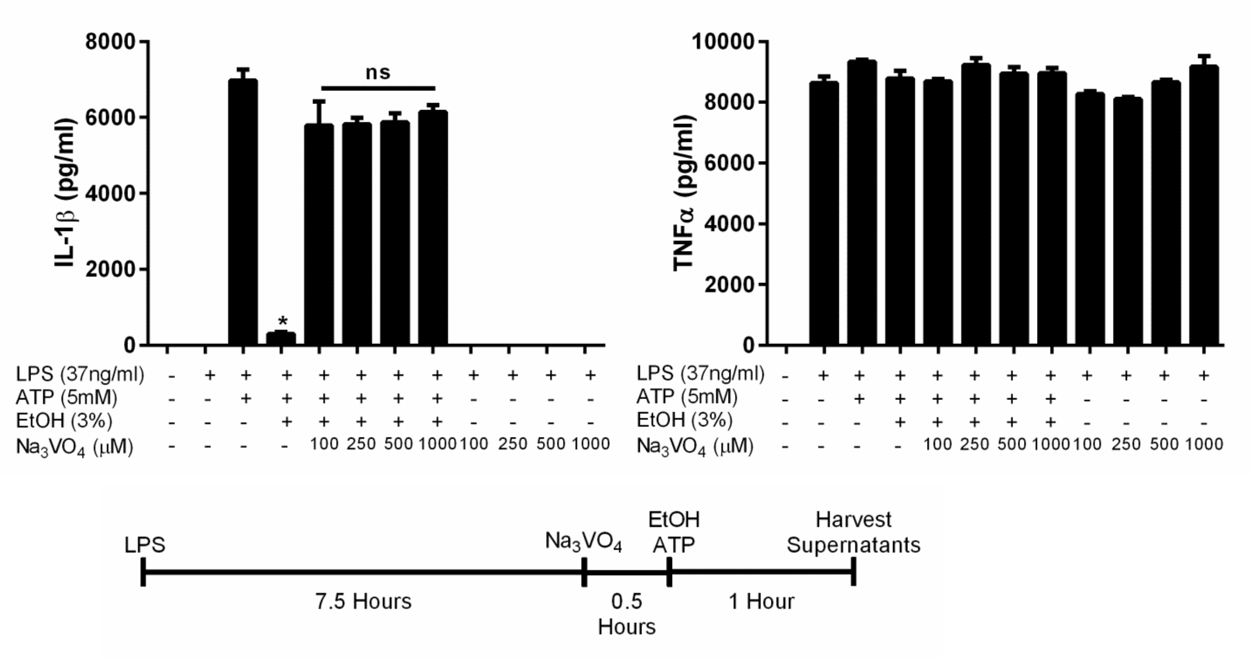

Figure 12. IL-1β (**A**) and TNFα (**B**) ELISAs on the supernatants from J774 cells untreated, primed with LPS, stimulated with LPS and ATP, LPS, ATP, and ethanol, and LPS, ATP, and ethanol pre-treated with Na_3VO_4, to block the activity of phosphatases globally. *****<0.0001 by one-way ANOVA relative to the LPS+ATP treated group. EtOH = ethanol, Na_3VO_4 = sodium orthovanadate.

IL-1β was detected in the supernatants, indicating that sodium orthovanadate's reversal of ethanol's inhibition was due to true restoration of IL-1β secretion.

GABA$_A$ Receptors Are Not Necessary or Sufficient for the Inhibitory Effects of Ethanol

Ethanol is a known agonist for GABA$_A$ receptors, which when activated function as a chloride specific ion channel (34). GABA$_A$ receptors have been found on macrophages and their activation has been shown to have anti-inflammatory effects (35). To test whether activation of

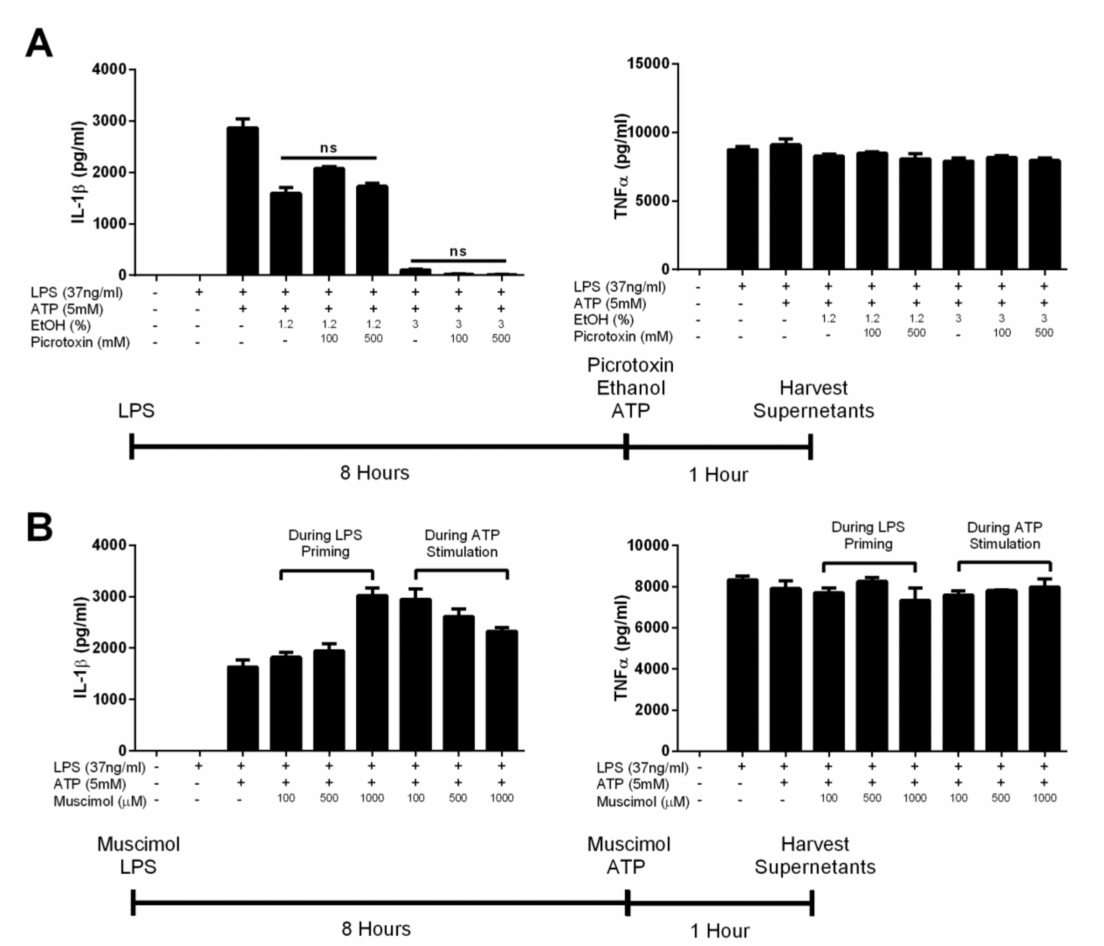

Figure 13. IL-1β and TNFα ELISAs on supernatants from J774 cells treated as indicated with LPS, ATP, ethanol, and the GABA$_A$ channel blocker picrotoxin (**A**), or from cells treated with the GABA$_A$ channel agonist muscimol prior to priming with LPS or stimulation with ATP (**B**). *****<0.0001 by one-way ANOVA relative to the LPS+ATP+EtOH 1.2% or 3% treated groups. EtOH = ethanol.

GABA_A receptors on J774 cells is responsible for ethanol's rapid immunosuppressive effects, groups were treated with the GABA_A receptor antagonist picrotoxin (100 or 500mM) immediately before the addition of ethanol (1.2% or 3%) and ATP to prevent channel opening. Blockade of GABA_A receptors had no impact on the inhibitory effects of ethanol either at its half maximal inhibitory dose of 1.2% or is fully inhibitory concentration of 3% (**Figure 13A**). This indicates that GABA_A receptors are not necessary for ethanol to inhibit NLRP3 inflammasome activation. To test whether GABA_A receptor activation was sufficient for NLRP3 inflammasome blockade, J774 cells were given the GABA_A receptor agonist muscimol (100-1000μM), directly before LPS priming or ATP stimulation. Muscimol was unable to prevent IL-1β and TNFα secretion when given before the signal 1 agonist LPS or the signal 2 agonist ATP (**Figure 13B**). From these data, we determined that GABA_A receptor activation is unable to inhibit either of the two steps needed for NLRP3 inflammasome activation to occur.

NMDA Receptor Inhibition Is Not Sufficient to Block NLRP3 Activation

NMDA receptors are ionotropic neurotransmitter receptors that open to form a non-specific cation channel and are antagonized by ethanol (31). Like GABA_A receptors, they have also been found on leukocytes, and in microglial cells their activation results in TNFα and IL-1β production (36). To determine

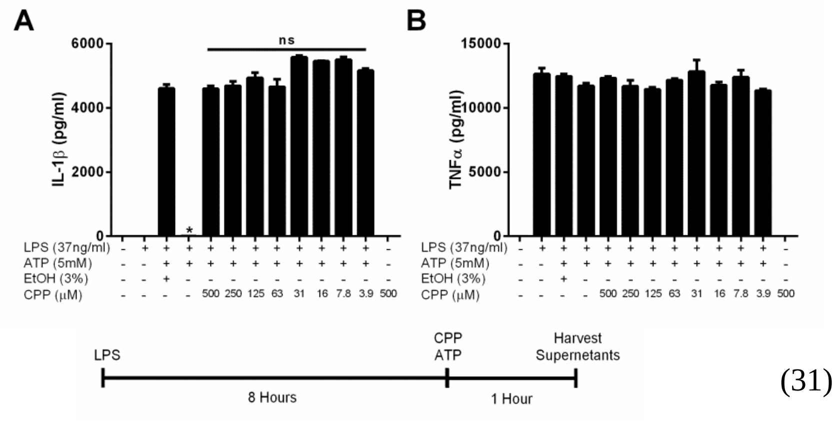

Figure 14. IL-1β (**A**) and TNFα (**B**) ELISAs from supernatants of J774 cells which were treated with LPS, ATP, ethanol, and the NMDA receptor antagonist as indicated. *****<0.0001 by one-way ANOVA relative to the LPS+ATP treated group. EtOH = ethanol, CPP = 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid.

whether NMDA receptor antagonism could block inflammasome activation, the antagonist 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP: 500-3.9μM) was administered to LPS primed J774 cells before stimulation with ATP. Antagonism of this neurotransmitter receptor did not prevent IL-1β secretion at any dose (**Figure 14**). Therefore, we concluded that NMDA receptor inhibition does not block NLRP3 inflammasome activation and is unlikely to be involved in the pathway of ethanol's inhibition of this system.

Different Chain Length Alcohols can Inhibit NLRP3 Inflammasome Activation

To test whether inhibition of the NLRP3 inflammasome is specific to ethanol, we treated cells with the hydroxyl group containing organic compounds: methanol, 1-propanol, isopropanol, glycerol, lactate, and isoamyl alcohol, which are both shorter and longer in carbon chain length than ethanol. We found that when administered to J774 cells these alcohols had inhibitory actions on NLRP3 inflammasome stimulation parallel to those of ethanol (**Figure 15A**).

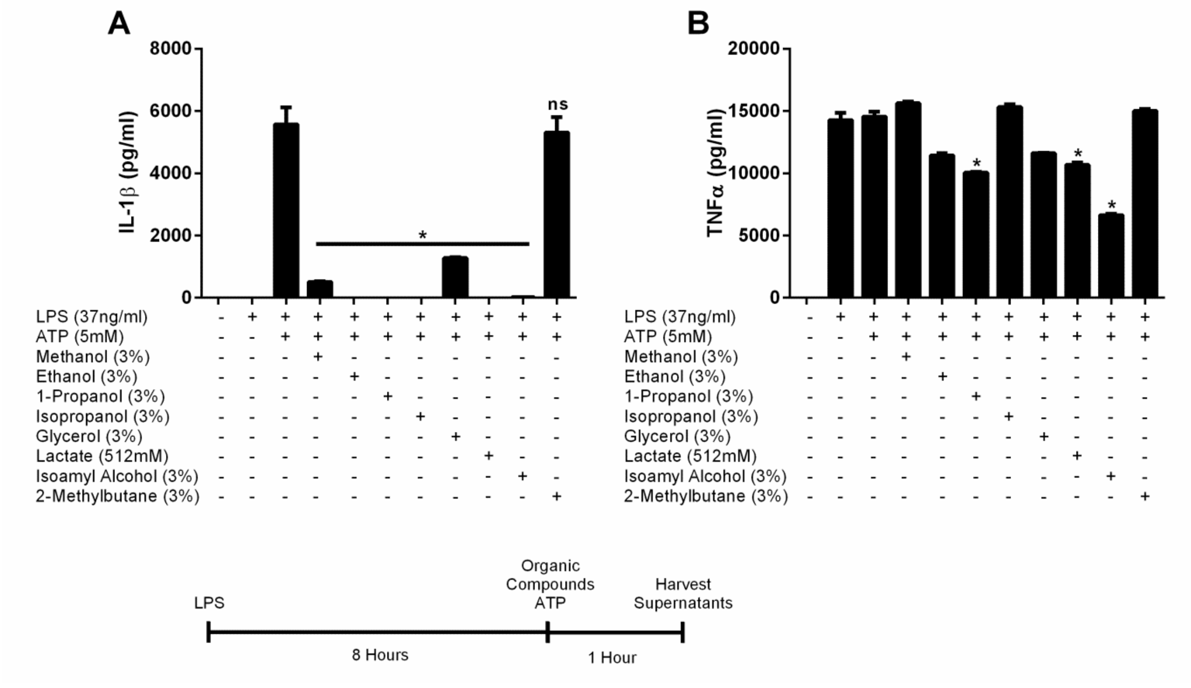

Figure 15. IL-1β (**A**) and TNFα (**B**) ELISAs on supernatants from J774 cells treated with LPS, ATP, methanol, ethanol, 1-propanol, isopropanol, glycerol, lactate, isoamyl alcohol and 2-methylbutane as indicated. *****<0.0001 by one-way ANOVA relative to the LPS+ATP treated group.

However, 2-methylbutane, the non-alcohol analog of isoamyl alcohol, lacked the ability to antagonize IL-1β secretion (**Figure 15B**). These data indicate that several types of alcohols are capable of inhibiting the NLRP3 inflammasome and that this inhibition might be dependent upon the presence of a hydroxyl group.

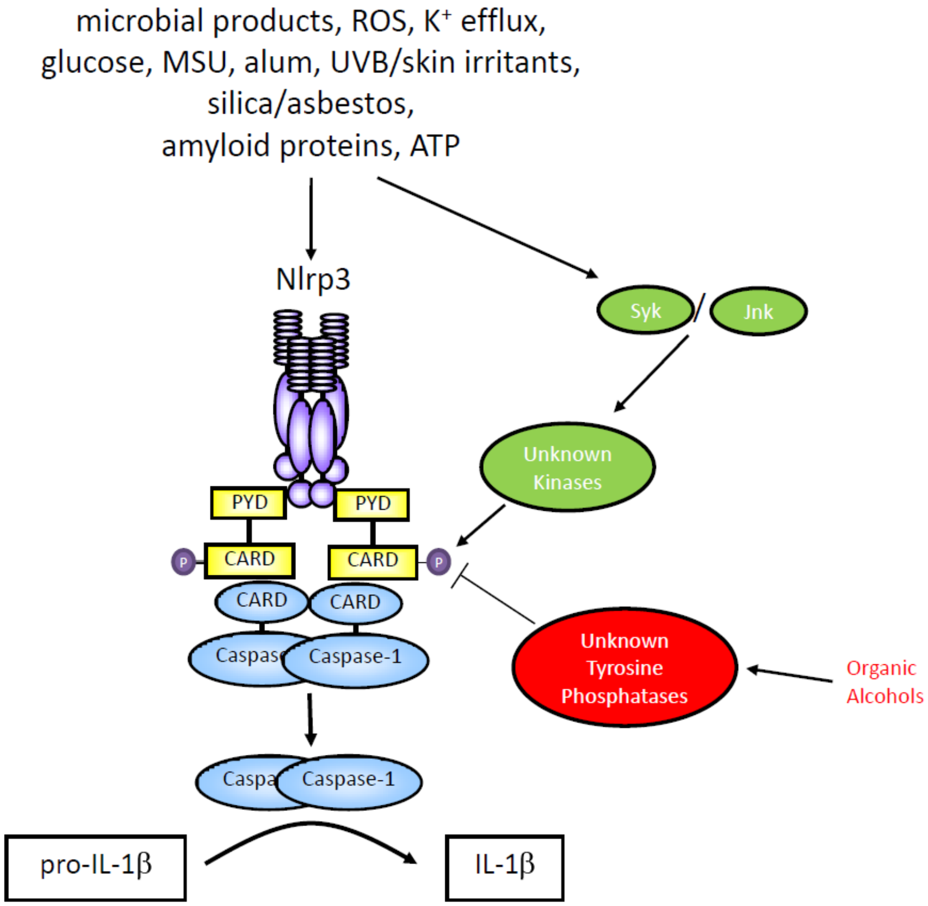

Figure 16. A summary of our proposed mechanism of NLRP3 inflammasome inhibition mediated by organic alcohols.

Discussion

There has been a long noted association between alcohol abuse and susceptibility to opportunistic infections. Alcoholics have globally disturbed immune function, including decreased macrophage phagocytosis, disrupted T-cell signaling, diminished levels of circulating pro-inflammatory cytokines, and paradoxically, symptoms of chronic inflammation (25, 37-39). In these studies, we show that in the setting of acute exposure to ethanol macrophages display markedly attenuated activation of the NLRP3 inflammasome and production of the pro-inflammatory cytokine IL-1β, thus providing a potential site of action for ethanol in the complex syndrome of alcohol induced immunosuppression. The doses of ethanol used in this experiment were high (0.38-3% or 64-512mM). However, considering that concentrations of alcohol in the blood, brain, and upper gastrointestinal tract can reach 100, 200, and 3400mM, respectively, following binge drinking (17, 40, 41), and that our highest dose of ethanol used did not induce measurable cytotoxicity, we feel that our chosen doses have physiologic relevance (**Figure 2**).

We have demonstrated that ethanol is capable of preventing IL-1β secretion after sufficient priming with the bacterially derived TLR4 agonist lipopolysaccharide (LPS) without impacting TNFα release (Figure 4). TNFα production occurs as a result of NF-κB and step 1 activation. The lack of a decline in the production of TNFα subsequent to ethanol treatment validates that the object of the alcohol's inhibition in these experiments is likely (directly or indirectly) assembly of the NLRP3 inflammasome itself (step 2), or secretion of the leaderless protein (step 3).

The fact that ethanol can prevent IL-1β secretion occurring in response to a variety of step 2 agonists implies that its site of action is probably an apical event in NLRP3 inflammasome stimulation in which each of the four pathways tested converge (**Figure 4**). The endogenous DAMP, ATP, and the bacterially derived PAMP, nigericin, are both believed to activate the NLRP3 inflammsome by inducing K^+ efflux from the cells. This occurs via activation of the P2X7 receptor and the pannexin 1 hemichannel by ATP and through the formation of pores in the plasma membrane by nigericin itself (9, 10). Ethanol does not inhibit the release of cellular potassium induced by nigericin (17), so we do not believe this to be a mechanism to explain the effects of ethanol on NLRP3 inflammasome activation. Aluminum hydroxide, a particulate commonly used as an adjuvant in vaccines, incites inflammasome activation through frustrated phagocytosis, lysosomal rupture, and leakage of cathepsins into the cytosol (11). Apo-SAA is a recombinant protein containing an amino acid sequence that is a hybrid of the endogenous acute phase proteins human SAA1 and 2, and an N-terminal methionine. It is not yet completely understood how apo-SAA stimulates the NLRP3 inflammasome, but due to the protein's ability to form amyloid plaques disrupted phagocytosis is a possibility (42, 43). The complete pathway for each of these stimuli is still unclear, but all lead to the formation of an ASC speck upon inflammasome activation (14). This is one of the first well characterized points of convergence between the pathways of all known NLRP3 inflammasome step 2 agonists.

Also lending credibility to the hypothesis that ethanol inhibits apical events in NLRP3 inflammasome activation is our finding that the alcohol can reduce IL-1β secretion when given up to 15 minutes after stimulation with ATP (**Figure 5**). This additionally implies that ethanol's effects on macrophages is nearly immediate, making ethanol's known ability to alter gene expression an unlikely candidate for its influence on NLRP3 inflammasome activity.

Activation of inflammasomes leads to conventionally and alternatively cleaved 17 and 28kDa mature IL-1β. Consistent with a lack of NLRP3 inflammasome activity, no cleaved IL-1β was detected in primed J774 cells given ethanol alongside ATP, despite abundant production from those cells given LPS and ATP alone (**Figure 6**). There was additionally no cleaved IL-1β present in the lysates of ethanol treated cells, supporting the hypothesis of inhibition of inflammasome activation rather than protein secretion, although it is possible that retained IL-1β might have been degraded before the lysates were collected and therefore went undetected. Furthermore, no cleaved caspase-1 or secreted ASC could be identified in the supernatants of ethanol treated cells, which are other signs of inflammasome activity (**Figure 7**). Taken together, these results support adequate NLRP3 inflammasome stimulation following 8 hours of priming with LPS and 1 hour of stimulation with ATP, which is completely blocked when ethanol is administered alongside ATP. This blockade of mature IL-1β, caspase-1, and ASC secretion is unlikely to be due to inhibition of protein secretion (step 3) by ethanol, as we have shown by Ponceau stain that ethanol treatment does not influence general protein secretion in response to ATP (**Figure 8**).

In an attempt to test whether ethanol may act by preventing ASC speck formation, ethanol was given simultaneously with the addition of the NLRC4 inflammasome agonist flagella or the NLRP1b agonist anthrax LT. Unlike its complete inhibition of IL-1β secretion due to NLRP3 inflammasome activation, ethanol at our highest administered dose of 3% could only partially inhibit IL-1β production subsequent to activation of the NLRC4 inflammasome by flagella (**Figure 9A**) and was incapable of inhibiting NLRP1b reliant IL-1β secretion (**Figure 9B**). NLRC4 and NLRP1b inflammasomes are amplified by, but are not dependent on, ASC speck formation. Therefore, the partial and absent responses to ethanol in these experiments could be due to a lack of a requirement for ASC speck formation by these inflammasomes. If this is the case, this would indicate that a main point of action for ethanol is inhibition of the adaptor protein’s ability to mediate inflammasome assembly. It should be noted that we used flagella for this experiment, while the true NLRC4 agonist is a subunit of flagella, flagellin. It is possible that flagella might exert unanticipated effects our macrophages beyond those of the purified flagellin subunit, making our results more difficult to interpret. As a more direct method of determining whether ethanol can prevent ASC speck formation, we used ASC-EGFP stably transfected THP-1 cells to visualize ASC speck formation in real time. In response to LPS and ATP, the quantity of specks visible per cell per visual field rose above baseline and this increase was completely prevented by ethanol treatment (**Figure 10**). Consistent with ethanol's incomplete inhibition of the NLRC4 inflammasome, this indicates that ethanol does act to prevent NLRP3 inflammasome activation at the level of ASC speck formation.

It has recently been shown that phosphorylation at Tyr146 (human) and Tyr144 (mouse) of ASC is necessary for speck formation to occur (14). Additionally, treatment of neurons with ethanol results in the activation of several tyrosine phosphatases (44, 45). Therefore, we measured total levels of tyrosine phosphorylation in the lysates of J774 cells stimulated to activate the NLRP3 inflammasome, treated with and without ethanol, to determine whether ethanol might decrease tyrosine phosphorylation in macrophages as well. A 3% dose of ethanol was capable of markedly decreasing global tyrosine phosphorylation when measured via western blot (**Figure 11**). In addition, pre-treatment with the phosphatase inhibitor sodium orthovanadate reversed the ability of ethanol to prevent IL-1β secretion (**Figure 12**). As proper functioning of the NLRP3 inflammasome requires the phosphorylation of ASC at these critical tyrosine residues and since the actions of ethanol appear to be phosphatase dependent, it is possible that ethanol could work by activating phosphatases, inducing the dephosphorylation of ASC, and preventing its aggregation. We did attempt to immunoprecipitate ASC and perform an anti-phosphotyrosine western blot to observe whether ethanol treatment can reduce the phosphorylation of ASC. However, due to the insolubility of ASC specks, the protein is difficult to immunoprecipitate and we did not obtain interpretable results.

We next attempted to determine how ethanol first transduces its signal into macrophages. The alcohol can serve as an agonist and antagonist to a number of neurotransmitter receptors, which leads to many of its effects on cerebral functioning (30, 31). Several of these receptors have recently been found to be expressed on leukocytes and their activation can skew immune responses towards pro- or anti-inflammatory states. Activation of $GABA_A$ receptors results in decreased production of TNFα, IL-1β, and several other pro-inflammatory cytokines from macrophages and T-cells, and treatment of mice with $GABA_A$ agonists can improve disease status in models of multiple sclerosis and asthma (35, 46). In contrast, stimulation of NMDA receptors on microglial cells leads to increases in TNFα and IL-1β secretion (36). Ethanol is both an agonist of $GABA_A$ receptors and an antagonist of NMDA receptors, making each of these proteins feasible targets for the alcohol in NLRP3 inflammasome suppression (30, 31). However, our results show that neither the stimulation of $GABA_A$ nor the antagonism of NMDA receptors is sufficient to inhibit IL-1β secretion and mimic the effects of ethanol (**Figures 13 and 14**). These receptors were attractive targets since they are expressed on macrophages, have been shown to alter IL-1β production, and are modified by ethanol. However, there are many other receptors on macrophages, not already associated with inflammasome activity, with which ethanol may interact (47, 48). It is possible that one of these may be the relevant target of the alcohol to prevent inflammasome activation. It is equally likely that ethanol might diffuse across the plasma membrane and directly modify cytosolic proteins. Additional techniques beyond the scope of this thesis work will be required to provide meaningful insight into these interesting possibilities.

Alcohols of similar chain lengths can exert comparable effects on proteins (28). Here we show that several different organic compounds containing alcohol residues can mimic ethanol's antagonism of the NLRP3 inflammasome (**Figure 15**). The parallel actions of these alcohols on the inflammasome could be due to similar chemical properties, or the fact that many of the binding pockets for ethanol within proteins can interact with similarly sized alcohols. Notably, we show here that isoamyl alcohol possesses the ability to inhibit IL-1β secretion subsequent to NLRP3 inflammasome stimulation while 2-methylbutane, containing the same carbon backbone but lacking an alcohol group, does not (**Figure 15**). These findings indicate that the hydroxyl group of a chemical is important in exerting an inhibitory effect on this system.

While the knowledge that the presence of an alcohol group on a chemical plays a key role in inhibiting NLRP3 inflammasome formation could provide significant information about the mechanism of ethanol's action within cells, it also might prove useful in identifying other relevant molecules capable of inhibiting this vital PRR. The ketone metabolite β-hydroxybutyrate (BHB), a four carbon compound containing both a hydroxyl and carboxyl group, is capable of specifically inhibiting the NLRP3 inflammasome by preventing potassium efflux and ASC oligomerization (49). This compound is produced endogenously during fasting and exercise to support ATP production during states of energy deficit, providing a possible explanation for the reduction in inflammation observed with prolonged fasting (49). Similar to our results obtained from isoamyl alcohol and 2-methylbutane, BHB's immunosuppressive activity on the NLRP3 inflammasome is absent in its alcohol free analog, butyrate (49). As we have shown that similarly sized short-chain alcohols exert comparable inhibition on the NLRP3 inflammasome, it is possible that all of these small molecules are acting through the same pathway. Therefore, it is probable that still more endogenous metabolites containing hydroxyl groups could possess inhibitory activity against the NLRP3 inflammasome. Identifying these compounds could afford significant insight into the mechanisms of immune homeostasis during both diseased and resting states.

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