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*J Immunol* 2007; 178:1243-1249; doi: 10.4049/jimmunol.178.3.1243

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**TLR4, Ethanol, and Lipid Rafts: A New Mechanism of Ethanol Action with Implications for other Receptor-Mediated Effects**

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Ethanol (EtOH) is the most widely abused substance in the United States, and it contributes to well-documented harmful (at high dosages) and beneficial (at low dosages) changes in inflammatory and immune responses. Lipid rafts have been implicated in the regulation and activation of several important receptor complexes in the immune system, including the TLR4 complex. Many questions remain about the precise mechanisms by which rafts regulate the assembly of these receptor complexes. Results summarized in this review indicate that EtOH acts by altering the LPS-induced redistribution of components of the TLR4 complex within the lipid raft and that this is related to changes in actin cytoskeleton rearrangement, receptor clustering, and subsequent signaling. EtOH provides an example of an immunomodulatory drug that acts at least in part by modifying lipid rafts, and it could represent a model to probe the relationships between rafts, receptor complexes, and signaling. The Journal of Immunology, 2007, 178: 1243–1249.

suppression of innate immunity to infection by ethanol (EtOH)3 was reported by Benjamin Rush in 1785, many years before scientific studies on the subject were conducted (1). When such studies were done, they demonstrated a strong association between excessive EtOH consumption and an increased risk of infections, such as pneumonia and tuberculosis (1). Experimental studies with human subjects given EtOH in a controlled setting and with animals have consistently revealed suppression of innate immunity and inflammation (2–5). However, the proximate molecular mechanism by which EtOH exerts these effects is not known.

Because EtOH per se is relatively unreactive under physiological conditions, attention initially focused on its physical properties. Over 100 years ago, a correlation was reported between the lipid solubility of a series of compounds and their ability to induce anesthesia (6–8). This suggested a mechanism in which EtOH acts by “dissolving” into the cell membrane and altering its fluidity and therefore its function. This idea has been refined and alterations in membrane volume or curvature, membrane disordering, and changes in lipid phase transitions have all been suggested as mechanisms by which EtOH could affect the function of membrane proteins (9). However, objections have emerged to all of these proposed mechanisms (9). For example, the effects of anesthetics, including EtOH, at relevant concentrations on the temperature of phase transition are exceedingly small. Similarly the amount of increase in membrane fluidity caused by a relevant concentration of EtOH is very small and can be produced alternatively by an increase in temperature of just a few tenths of a degree Celsius, a temperature change that certainly does not produce the same changes in the nervous or immune systems as EtOH.

It is not surprising that interest began to shift to direct action of EtOH on proteins to explain its effects in the CNS. Direct, consistent evidence has been difficult to obtain (10), but recent results with γ-aminobutyric acid (GABA)A receptor subunits (inhibitory receptors whose function is enhanced by EtOH) suggest that EtOH can bind to a water-filled pocket in some subunits (11), thereby altering the subunit conformation and increasing the sensitivity of the receptor to GABA. Similar results have been reported for N-methyl-D-aspartate and 2-amino-5-hydroxy-5-methyl-4-isoxazolepropion acid receptors (stimulatory receptors that are inhibited by EtOH). However, the EtOH concentrations required to cause significant changes in receptor function are often greater than attainable in vivo, and effects mediated by lower concentrations have sometimes not been replicated. This suggests a situation in which simple, pharmacological binding of EtOH to protein in a receptor-ligand manner may not be fully applicable (10). Thus, it is interesting that results have recently been reported indicating that GABA, N-methyl-D-aspartate, and 2-amino-5-hydroxy-5-methyl-4-isoxazolepropion acid receptors are associated with lipid rafts (12–15).

Lipid rafts and alcohol

Lipid rafts, also called detergent-resistant domains, are plasma membrane microdomains characterized by insolubility in non-ionic detergents (at cold temperatures) and enrichment in

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3 Abbreviations used in this paper: EtOH, ethanol; GABA, γ-aminobutyric acid; TIR, Toll/IL-1 receptor.
cholesterol and sphingomyelin (16, 17). They range from a few nanometers to a few hundred nanometers in diameter and represent ~50% of cellular membrane (16, 18). Certain proteins reside preferentially in rafts, whereas others are recruited or excluded from the rafts upon cell activation (16–21). By protein composition, the lipid rafts are heterogeneous; some are enriched in caveolin and others concentrate clathrin and GPI-anchored proteins or dynamin (16, 17). It is generally believed that lipid rafts provide platforms for formation of receptor complexes, their internalization and triggering of signaling pathways, thus facilitating cell activation (19, 21). Lipid rafts had not yet been characterized when most of the experimental evidence was obtained to evaluate the hypothesis that EtOH produces biological effects primarily by interacting with membrane lipids. The identification of lipid rafts and their important role in cellular signaling provides ample rationale to revisit this hypothesis. Any direct interaction of EtOH with lipids would probably not occur in the lipid core but within the more hydrophilic inner and outer margins (9). It is not clear whether this would alter protein conformation to a sufficient degree to explain the actions of EtOH on the innate immune system, but this should be a productive line of investigation.

We propose that a comprehensive hypothesis regarding the mechanisms of EtOH action on cellular signaling should include possible direct action on proteins, nonspecific interactions with lipid components, and disruption of lipid protein interactions that alter protein conformation. Any of these mechanisms could potentially limit entry or exit of receptor components to and from lipid rafts or among different raft types. This could prevent assembly of an optimum receptor complex. Conformational change in a single receptor component (e.g., by direct binding of EtOH or alteration of protein-lipid interactions) could yield similar effects.

It has been suggested previously that EtOH may act on membrane proteins by disrupting protein-lipid interactions (22). If this occurred relatively specifically in lipid rafts, the movement of proteins into or out of rafts or among different raft subpopulations, which is normally part of signaling through several receptor types, could be adversely affected. There is evidence that neurological adaptation (tolerance) to chronic administration of EtOH is associated with increased cholesterol in cell membranes (23), and lipid rafts are enriched in cholesterol compared with the remainder of the membrane. Thus, additional raft microdomains permitted by the presence of additional cholesterol in the membrane may represent additional "targets" for EtOH, so that increasing cholesterol concentration may prevent the effects of EtOH by providing more target sites than EtOH can act to disrupt. This is a quantitatively feasible proposal because relevant concentrations of EtOH provide roughly one EtOH molecule in the membrane for every 200 phospholipid molecules (24). Thus, an increase in cholesterol could provide enough additional target sites to diminish the action of EtOH. Recent evidence from our labs supports an important role for lipid rafts in the actions of EtOH on macrophage activation in humans and mice (25–28).

It is possible that some form of membrane adaptation to chronic EtOH exposure (such as increased cholesterol incorporation) could explain the disparate effects reported for chronic and acute EtOH exposure with regard to susceptibility to infectious diseases and inflammatory responses. Chronic excessive exposure to EtOH is typically associated with increased risk of infection but paradoxically also to increased risk of inflammatory processes such as alcoholic hepatitis in the liver. In part, this may reflect a balance between anti-inflammatory effects of EtOH and proinflammatory effects of bacteria that enter the circulation due to decreased barrier function in the gastrointestinal tract (29). However, there is also evidence that cells obtained from animals that have been exposed to chronic EtOH treatment are more sensitive to inflammatory stimuli such as LPS than cells isolated from untreated animals (30). We observed that macrophages from mice treated with EtOH for 1 mo were less affected by acute EtOH administration than untreated mice with regard to production of cytokines (31). This is consistent with the idea that adaptation is induced by chronic EtOH exposure. It should also be noted that effects of chronic EtOH exposure on the immune system can be relatively persistent, and mechanisms such as increased production of reactive oxygen species and cellular damage caused by the reactive EtOH metabolite acetaldehyde have been implicated in chronic effects of EtOH on the immune system (32, 33). In addition, the effects of acute EtOH exposure on lipid rafts discussed later in this review are relatively short-lived. In contrast, some effects of chronic EtOH exposure on cells of the immune system last much longer (34), indicating that these effects are unlikely to be mediated by short-term alterations in lipid rafts. All of these findings suggest that inhibition of signaling by EtOH is probably a more important mechanism in acute EtOH exposure than chronic exposure.

Evidence that acute EtOH exposure can be immunosuppressive in humans is accumulating. For example, people with detectable EtOH in the blood at the time of admission for trauma or burn injury have increased morbidity (35), and ~25% of such patients do not have a history of chronic EtOH use (36). A convincing case can also be made that moderate EtOH consumption exerts beneficial effects by inhibiting inflammation that contributes to the development of cardiovascular disease (37). Thus, inhibition of signaling by the mechanism discussed in this review could be quite relevant with regard to both harmful and beneficial effects of acute EtOH exposure.

**TLRs and lipid rafts**

The innate immune system, macrophages, and TLRs are critically involved in the initial phase of microbial detection (38, 39). TLRs recognize pathogen-associated molecular patterns that induce expression of proinflammatory cytokines and initiate pathogen-specific immune responses (38, 39). Each TLR has a unique extracellular domain that allows specific ligand recognition. The intracellular Toll/IL-1 receptor (TIR) domains share considerable homology, but there are sufficient differences to cause different adapter molecules to be used by some TLRs (38–41). Upon ligand-induced stimulation, the TIR domain of TLRs associates with the TIR domain of their respective adaptor molecules to initiate intracellular signaling. MyD88 is a common TLR adaptor used by all TLRs, except for TLR3, which uses TIR-domain-containing adapter-inducing IFN-β (41). Uniquely, TLR4 can associate with both of these adaptor molecules (38–41). CD14, a membrane-associated receptor that has a GPI-linked transmembrane but no intracellular domain, transduces signals by interacting with other signaling molecules, including TLRs (41). CD14, similar to other
GPI-linked proteins, resides in lipid rafts (42). Upon stimulation with specific ligand the recruitment of membrane-associated TLRs, such as TLR2 and TLR4, and other components of TLR complex occurs into the lipid rafts (19, 25, 42–44). Lipid rafts appear to provide a platform for interaction of endosomal TLRs, such as TLR3, TLR7, and TLR9, with their ligands in macrophages (45–47). Of interest, it remains to be determined whether CD14 has a role in TLR association with lipid rafts. The mechanisms by which CD14 amplifies TLR signaling remains to be explored; however, multiple lines of evidence suggest that CD14 association with lipid rafts may be a common element where the CD14-lipid raft association provides a platform for recruitment of not only TLRs but also key signaling molecules for potent interaction and signal initiation.

Recent results

In previous independent studies in our labs, we noted that EtOH inhibits TLR-mediated signaling in macrophages and that both early and late events are inhibited (25–28, 31, 48–50). This led both of us to evaluate the possibility that EtOH acted at the earliest points in signal transduction, possibly by altering lipid rafts. We recently described results indicating that acute EtOH exposure alters LPS-induced redistribution of TLR4 receptor components to lipid rafts, that LPS-induced TLR4 signaling is dependent on normal raft structure, that EtOH also alters the reorganization of the actin cytoskeleton (which is required for full cellular activation by LPS), that EtOH decreases TLR4 and CD14 clustering and colocalization, and that these changes are associated with decreased production of TNF-α, a key proinflammatory cytokine (27, 28). Results were similar for a mouse macrophage like cell line, mouse peritoneal macrophages, and human monocytes.

In a recent report from one of our labs (S. B. Puett), it was demonstrated that acute EtOH treatment of cells or mice almost completely prevented the redistribution of CD14 (a component of the TLR4 receptor complex) between different lipid raft fractions that is normally induced by LPS (28). This is associated with decreased production of TNF-α, and agents that inhibit lipid raft function and rearrangement of the actin cytoskeleton similarly inhibited TNF-α production, suggesting that lipid raft-mediated events and rearrangement of the actin cytoskeleton are required for optimum signaling and TNF-α expression. In a subsequent study, this relationship was confirmed, and the involvement of TLR4 and CD14 colocalization and clustering were also implicated in activation of macrophages for TNF-α production (27). Confocal microscopy revealed that TLR4 and CD14 colocalization and clustering occurred rapidly (3 min) and persisted for at least 1 h. Inhibiting this clustering with cholesterol sequestering agents also inhibited TNF-α production. Interestingly, increased expression of membrane-associated TNF-α (from which the secreted form is derived) was observed almost exclusively in the same cells that had TLR4 and CD14 colocalization and clustering (~5% of total cells). EtOH altered actin reorganization in LPS-treated cells, and alteration of actin reorganization by cytochalasin D inhibited expression of TNF-α as did EtOH. These results suggest a mechanism of LPS-mediated macrophage activation and a mechanism of inhibition of this activation by EtOH consistent with that depicted in Fig. 1. Three other groups have reported rapid clustering of the TLR4 receptor complex induced by LPS (19, 42–44), but our studies are the only ones to date that provide evidence for the involvement of the actin cytoskeleton in this process or in signaling (27, 28).

Studies from one of our labs (G. Szabo) in human monocytes demonstrated that both TLR2 and TLR4 are localized outside of lipid rafts, whereas CD14, a coreceptor of TLR4, is distributed both in and outside of rafts in resting cells (25). TLR4 ligand activation with LPS resulted in concentration of both TLR4 and CD14 in lipid rafts. Acute EtOH treatment interfered with TLR4 recruitment to lipid rafts at doses that occur in humans after consumption of three to four standard drinks. Attenuation of TLR4 and CD14 recruitment to lipid rafts by EtOH was associated with reduction in LPS-induced TLR4 downstream signaling events and inflammatory cell function. Our studies found reduced NF-κB activation and attenuation of NF-κB-driven gene activation of TNF-α by acute EtOH administration in vitro in monocytes (25, 26, 51).

It remains to be explored whether EtOH would affect similarly other TLRs that may associate with lipid rafts. Our studies demonstrated that TLR2 is also associated with lipid rafts after receptor ligation; however, TLR2 recruitment to the raft and/or NF-κB activation was not prevented by EtOH administration (25). This was consistent with the lack of modulation of TLR2-induced TNF-α production by acute EtOH in monocytes (51). It remains to be investigated whether these results reflect a selective effect of EtOH on TLR4-induced signaling or, for example, on specific lipid rafts involved in TLR4 signaling. Previous studies have demonstrated the presence of different lipid rafts (16, 17), and at this time, it is unknown whether TLR2 and TLR4 would associate with the same types of rafts. In other systems, TLR2-mediated murine macrophage activation was inhibited by acute alcohol and involved impaired p38 and ERK1/2 MAPK activation (52, 53). Alcohol administration in vitro also attenuated TLR9-induced macrophage production of IL-6 and TNF-α (52). While TLR9 intracellularly is localized to the endoplasmic reticulum and is translocated to the lysosomes upon ligand activation, association of TLR9 with lipid rafts has been also demonstrated recently (47, 54). Interestingly, TLR3, unlike TLR2, 4, and 9, was found in association with lipid rafts by some investigators (45) but not by others (47). Thus, it remains to be evaluated whether inhibition of TLR3-induced proinflammatory cytokine production and mRNA levels of the IFN-related amplification loop after in vivo administration of acute alcohol in mice involves lipid rafts (49). Based on these observations and additional evidence that EtOH also modulates intracellular pathways that are not lipid-raft associated, we can conclude that disruption of signaling events associated with lipid rafts may represent just one of the multiple intracellular effects of EtOH.

It is unclear whether alteration of lipid rafts by EtOH is a major mechanism by which EtOH alters TLR signaling in cell types that do not express CD14. Some types of cells (e.g., epithelial cells) do not express CD14 on the cell membrane but rely on soluble CD14 for LPS-induced signaling (55). It is not yet known whether lipid rafts are involved in TLR signaling under these circumstances. However, if TLR4 mobilization to rafts is important in these cells, as it is in monocytes (which express attached CD14), it would not be surprising to find the EtOH alters TLR4 signaling in cells that do not express CD14 on the cell membrane.
On the basis of recent findings for TLRs and by analogy with other receptors (such as the BCR for Ag), a sequence of events and mechanism of EtOH action with regard to TLR4 signaling is proposed in Fig. 1. Although this figure is simplified (e.g., LPS-binding protein, MD-2, and other components of the receptor complex are not shown), it illustrates the following key points: 1) LPS induces conformational changes causing TLR4 to enter lipid raft microdomains to interact with CD14 and LPS; 2) this causes initial signaling leading to rearrangement of the actin cytoskeleton; and 3) actin mediates massive clustering of the CD14 and TLR4 complex and possibly the addition of other components leading to optimal cellular signaling. As indicated in Fig. 1, we hypothesize that EtOH alters this series of events by interfering with step 1 above. This hypothesis leads to the testable conclusion that some cellular signaling should be detectable in cells in which step 1 is inhibited, for example, by cholesterol-sequestering agents, but this signaling will be quantitatively and/or qualitatively different and less effective than signaling induced by the same stimulus in normal cells.

It is not clear whether ligand-induced alterations in the distribution of receptor components are mediated by an initial (perhaps weak) cellular signal induced by interaction of the ligand with its nominal receptor or by direct physical changes in the receptor and its components mediated by ligand binding and causing conformational changes that lead to migration from, to, or among rafts. There are indications that in some cases initial transmembrane signaling events are induced by binding of ligand and that this induces rapid rearrangement of the actin cytoskeleton, which is required for assembly of the fully functional receptor complex. This sequence of events has been best documented in the case of signaling through the BCRs for Ag (56, 57). There are important commonalities between BCR and TLR signaling such as multicomponent receptor complexes, involvement of lipid rafts, and early involvement of the actin cytoskeleton. Therefore, it seems reasonable to suggest a sequence of events in TLR4 signaling similar to that reported for BCR signaling.

**Lipid rafts: implications for the effect of alcohol on microbial defense**

While lipid rafts are used by cellular receptors, including TLRs, to amplify efficient signaling in microbe recognition, microbes can independently alter the dynamics of lipid rafts to modulate these host defense mechanisms. Pathogens have evolved strategies to ensure their own survival in some cases by “hijacking” lipid rafts. Thus, in addition to modification of host receptors involved in pathogen recognition and pathogen-induced immune responses, alcohol may directly affect pathogen-lipid raft interactions. A broad spectrum of bacteria (Legionella, Pseudomonas, Brucella, Salmonella, Shigella, Chlamydia, Streptococcus, Listeria, etc.), viruses (HIV, Ebola, HSV, hepatitis C virus, influenza, EBV, etc.), and some protozoa (toxoplasma, plasmodium) use lipid rafts from host cells for their advantage in entry, intracellular survival, or replication (58). Many of these infections are more frequent in patients with excessive alcohol use (59, 60).
Lipid rafts and immune receptors: relevance to alcohol-induced immune abnormalities

The association and dissociation of particular proteins with lipid rafts is necessary for cellular signal transduction mediated through TLRs, the BCRs for Ag, and the TCRs for Ag (20, 21, 25, 28, 56). Thus, lipid rafts are involved in the induction of both innate and acquired immune responses. This involvement has been discerned by several experimental approaches, including: 1) disruption of signaling by agents known to disrupt lipid rafts (e.g., β-methylcyclohexdrin and nystatin) (25, 28); 2) ligand-induced changes in the distribution of receptor components among raft and non-raft fractions (25, 28); and 3) microscopic analysis indicating coalescence (61) of rafts or clustering (27) of membrane proteins that are known to occur in rafts. However, only a few reports have described receptor clustering in the case of TLRs (62, 63), and the relationships between initial signaling events, rearrangement of cytoskeletal elements, receptor clustering, movement of proteins into, out of, or among raft fractions, and assembly of an optimally responsive receptor complex are just beginning to be investigated. As an agent that alters all of these events, EtOH has already been useful in suggesting that they are connected. In addition to defective microvillar clearance and alterations in inflammatory cell functions, alcohol use is also associated with defects in adaptive immune responses. Ag-specific T cell activation, Th1/Th2 cytokine balance and T cell expansion, increased Ig production due to B cell dysfunction, NK cell abnormalities, defects in macrophage phagocytic activity, and Ag-presenting function of dendritic cells have all been described in animal models or human alcohol use conditions (64–67). Importantly, all of these cellular functions involve receptor complexes associated with lipid rafts, including MHC class II, TCRs, BCRs, TNFRs, and FcRs (20, 21, 68–71). While a direct role for lipid raft modulation by alcohol in these receptors and/or cellular defects is yet to be investigated, based on the previously identified role of lipid rafts in all of these immune functions, it is tempting to speculate that lipid raft-associated signaling events may be common targets of EtOH to result in such diversity of immune cell defects. A recent report from Joshi-Barve et al. (72) showed that EtOH treatment interferes with efficient TCR assembly within lipid rafts by down-regulation of Lck, Zap-70, linker for activation of T cells, and phospholipase C phosphorylation and leads to decreased IL-2 gene expression. MHC class II molecules involved in the formation of the immunological synapse between APCs (B cells or monocytes) and T lymphocytes are associated with lipid rafts, and disruption of raft results in decreased Ag presentation that has been previously described (73, 74), similar to deficient Ag presentation after alcohol administration or in vitro treatment (75). It is intriguing that reduced Ag-specific T cell activation by monocytes or dendritic cells was associated with no changes in the levels of surface MHC class II expression in alcohol-treated cells consistent with the possibility that the signaling function rather than the absolute levels of these proteins are affected by EtOH. This is also suggested by inhibition of anti-CD3-induced Ca2+ mobilization, inositol-(1,4,5)-trisphosphate production, and T cell proliferation in the presence of alcohol (76). In addition, alcohol-induced treatment leads to an increase in high m.w. isoforms of CD45, as reported by the same investigators (76). It is interesting that CD45 exclusion from raft fractions is required for formation of an immunological synapse and for optimal T cell signaling (77). Taken together, these observations are suggesting a possible role for lipid raft alterations in the effects of EtOH on cellular receptor signaling and await further investigation.

Conclusions and implications for effects of EtOH in other physiological systems

Recent discoveries on the effects of EtOH on lipid rafts and TLR4 demonstrate that EtOH has significant modulating potential on receptor recruitment to lipid rafts, resulting in functional changes in inflammatory cell functions. Based on our findings related to acute alcohol administration and TLR4 signaling, we proposed a model in which recruitment of TLR4 and subsequent actin polymerization is attenuated by alcohol (Fig. 1). However, our results cannot rule out alternate possibilities that may contribute to alcohol-induced alterations in lipid raft associated receptor functions. First, EtOH may modulate not only the recruitment of the raft-associated molecules but also the interactions and/or the stoichiometry of proteins associated/recruited in the raft, which itself could result in conformational changes potentially modulating the efficiency of downstream signaling. Second, EtOH may differently affect recruitment of receptors and their adaptor molecules, thereby affecting downstream signaling. Third, EtOH may change the lipid composition of the detergent-resistant domains. Finally, based on the observation that EtOH can inhibit actin filament dimerization, it remains to be determined whether EtOH would affect trafficking of lipid rafts to intracellular components. These possibilities for modulation of lipid rafts and associated cellular function are likely to occur beyond TLRs and immune cells. Recent reports (78, 79) indicate that recruitment of different receptor components to rafts and actin polymerization were inhibited in rat adipocytes and mouse neurons by administration of EtOH, suggesting that this mechanism may contribute to the effects of EtOH on signaling through a number of receptor types in which lipid rafts play a role. In conclusion, we propose that the influence of EtOH on lipid raft-mediated dynamics and signaling of TLRs, and possibly other receptors, constitutes a previously unrecognized mechanism that may contribute, along with other mechanisms, to alcohol-induced modulation of the immune cell functions. Future studies should shed light on modulation of lipid raft-associated specific receptor-mediated events by acute or chronic alcohol administration in other systems.

References


