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- Toll-Mediated Signaling
- Trauma Induced Mononuclear Cell Reprogramming
- Proposed Mechanism of Lipid Raft Clustering and Reprogramming



## AWARDS

### American College of Surgeons

- Committee on Trauma Region X Competition,  
Best Basic Science Paper

### Michael S. Benninger, M.D. Outstanding Resident Award

### Helen & John Schilling Resident Research Symposium, First Place

### Seattle Surgical Society Award

### Shock Society Travel Award

## FUNDING

### National Institutes of Health

- National Institute of General Medicine Sciences

Severe injury results in the activation of the innate immune system characterized by the systemic inflammatory response syndrome (SIRS). Although this state may persist, resulting in early development of multiple organ dysfunction syndrome (MODS), the majority of injured patients develop a compensatory response that is characterized by a state of dysregulated immune responsiveness. During this state of dysregulated responsiveness, patients are at increased risk for the development of opportunistic or nosocomial infections. If invasive infection occurs during this state, an exaggerated inflammatory response ensues, leading to the development of MODS.

The mechanism responsible for this dysregulated innate immune activation remains poorly understood. This state has been modeled and characterized by the "two-hit" hypothesis. According to this hypothesis, severe injury results in the reprogramming of innate immune cells and during subsequent infection an exaggerated host response occurs, resulting in tissue injury. Both the peripheral blood monocyte and tissue-fixed macrophage appear to play critical roles during this state. The primary mechanism in which these cells interact with invading organisms is through the Toll-like receptors (TLRs), a family of pattern recognition proteins. Activation of these receptors by inflammatory factors, such as lipopolysaccharide (LPS), leads to the liberation of various cytokines and chemokines that are in part responsible for eradication of invading organisms. However, when exaggerated, as is the case following severe injury, liberation of the factors leads to subsequent tissue injury and the development of MODS.

The mechanism in which the TLRs are activated and affected by severe injury remains an area of intense investigation. Recently, we have demonstrated that activation of the TLRs, in particular TLR4, requires the formation of a receptor complex with CD14 and other constituents on lipid rafts. In particular, attenuation and augmentation of this receptor complex formation results in dysregulated inflammatory mediator activation. My laboratory efforts, therefore, are to elucidate the cellular mechanisms involved in mononuclear cell reprogramming in patients suffering from MODS and acute respiratory distress syndrome (ARDS) following trauma. If this is accomplished, it would provide the foundation for the development of novel early therapeutic interventions that could be used during the resuscitative period.

## Toll-Mediated Signaling

The peripheral blood monocyte and tissue fixed macrophage are activated by pathogen-associated molecular patterns. These are structures that are characteristic of large groups of microorganisms, such as bacterial cell wall components and nucleic acid motifs. Unlike the adaptive immune response which requires antigen-specific antibodies, innate immune cells are able to respond rapidly to invading organisms without the need for prior exposure.

In mammalian cells, the key component to this response is the family of TLRs. These receptors are responsible for the recognition of the pathogen-associated molecular patterns and lead to the subsequent activation of the monocyte and macrophage. The founding member of the TLR family is the *Drosophila* protein, Toll, which was initially identified through its ability to control dorsoventral patterning in fruit fly

embryos. Recognition of the importance of Toll in the *Drosophila* innate response prompted exploration for a possible mammalian counterpart.

Currently, a total of 10 human TLRs have been identified that share structural homology and signaling components. Each of the described TLRs, except for TLR9, are all transmembrane molecules. The extracellular amino termini have variable leucine-rich repeat domains, which are involved in the recognition of pathogen-associate molecular patterns. The intracellular domains contain a conserved Toll/interleukin-1 (IL-1) receptor (TIR) domain. The TIR

present within the plasma membrane via a glycerophosphate inositol (GPI) anchor. CD14 is essential as both a functional receptor and scavenger for LPS. The functional role of CD14 leading to LPS-induced cell activation was initially established using neutralizing antibodies to CD14. Transfection of CD14-negative cells with CD14 greatly enhances sensitivity to LPS. Similarly, mice with a disrupted CD14 gene do not respond to low doses of LPS. Under physiological conditions, LPS-induced cell activation involves the formation of a ternary complex with LBP and CD14 within lipid rafts on the monocytic cell surface lead-

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*Limited recruitment of receptor complexes to the lipid raft receptor platform may underlie the increased risk associated with a subgroup of injured patients at risk for devastating infections.*

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domain, a defining characteristic of the Toll/IL-1 receptor superfamily, is involved in the association with downstream signaling molecules that mediate the response to TLR stimulation.

Toll-like receptor 4 is part of a complex that recognizes LPS. Lipopolysaccharide is an abundant glycolipid present on the outer membrane of gram-negative bacteria. During gram-negative infections, the highly conserved lipid A component of LPS activates the immune system, leading to generalized inflammation, manifested clinically as sepsis and septic shock.

Lipopolysaccharide released from Gram-negative bacteria is present as an aggregate due to the amphiphilic structure of the molecule. Spontaneous diffusion of LPS monomers from these aggregates to CD14 occurs at a very low rate. However, LPS is transformed into monomers through the action of plasmatic LBP. LBP is a lipid transfer molecule catalyzing movement of phospholipids, in particular LPS monomers from LPS aggregates to CD14. This process results in either cell activation through CD14 or neutralization of LPS. Thus, the rate of either process will determine the response of the host to LPS. Kinetic studies have shown that LPS/LBP complexes bind to CD14 before LPS is transferred to HDL. This suggests that normally LPS first activates immune cells before it is neutralized to prevent overstimulation of the immune system.

Membrane bound CD14 is a 53-kDa glycoprotein

ing to cellular activation.

The classical fluid mosaic model proposed by Singer and Nicolson in 1972 has been modified in recent years to accommodate a role for distinct microdomains in the cell membrane, which appear to serve as signaling platforms. The cell membrane is mainly composed of glycerophospholipids, sphingolipids and cholesterol. The headgroups of sphingolipids trigger a lateral association of lipids of this class with one another, which is further enhanced by hydrophobic interactions between the saturated side chains. Cholesterol seems to fill voids between the large glycerosphingolipids, and tightly interacts with sphingolipids, in particular sphingomyelin, by hydrogen bonding. The tight interaction of sphingolipids with one another and with cholesterol results in the segregation of these lipids into discrete membrane structures characterized by a gel-like phase, while glycerophospholipids in the bulk of the cell membrane reside in a more fluid liquid-disordered phase.

These distinct sphingolipid- and cholesterol-enriched membrane microdomains are considered to be floating in an "ocean" of phospholipids, and hence have been termed lipid rafts. In addition to the selective lipid composition, selected proteins are preferentially targeted or constitutively found within the lipid raft. Within mononuclear cells, these modified proteins are composed of saturated acyl-chain proteins, including GPI-anchored proteins, such as CD14, and double acylated proteins. Other receptor

proteins, such as the TLRs, are not constitutively found on rafts, but during activation these proteins are recruited into rafts through a mechanism that remains unclear, resulting in the formation of receptor complexes and the presentation of the inciting stimulus.

Rafts appear more prominent and more central to the function during activation of the monocyte and macrophage. In resting cells, rafts appear small and unstable, and consensus now suggests that they are smaller than the optical diffraction limit (250 nm). Upon stimulation, the raft-preferring receptors are clustered through a poorly defined mechanism leading to the generation of lipid raft macrodomains, allowing LPS to be briefly released into the lipid bilayer where it finally interacts with the complex of receptors, including TLR4. Due to the abundance of sphingolipids within the raft membrane, it is our hypothesis that sphingomyelinase activation resulting in degradation of lipid raft sphingolipids into the secondary messenger ceramide is the likely candidate involved in lipid raft reorganization within mononuclear cells.

The sphingomyelin pathway is initiated by the rapid hydrolysis of plasma membrane sphingomyelin to the second messenger ceramide via the action of sphingomyelinase. This is believed to result in the reorganization of lipid rafts. Ceramide, which has the unique property of fusing membranes, appears to drive the coalescence of raft microdomains to form large, ceramide-enriched membrane platforms, which exclude cholesterol. Recently, we have been able to demonstrate the formation of these lipid raft ceramide fused macrodomains following LPS stimulation.

The formation of these ceramide-enriched membrane platforms serves to trap and cluster receptor molecules, and potentially exclude other receptor complexes. We have been able to demonstrate that initial binding of LPS to CD14 results in the activation of acid sphingomyelinase resulting in the liberation of ceramide, and the formation of TLR4 raft associated complexes. The mechanism responsible for sphingomyelinase activity, however, remains unresolved but may occur through the activation of phosphatidylcholine (PC)-specific phospholipase C (PC-PLC).

Once this membrane platform is formed, the signaling pathways leading from LPS/CD14 binding to TLR4 complex assembly are not well understood and are important because of the potential for early and selective pharmacological intervention. Although PC-

PLC and sphingomyelinase may play a role through the induction of ceramide, the subsequent events leading to TLR4 complex assembly remain for the most part uncertain. However, we have recently been able to shed some light on this mechanism by demonstrating that activation of the PKC isoform, PKC- $\zeta$ , is involved. Although the full effects of PKC- $\zeta$  remain to be elucidated, it appears that the mechanism is ceramide dependent and results in the engagement of integrins and the recruitment of various raft associated proteins.

The high degree of organization observed within lipid raft structures, coupled with their dynamic nature, appears to be important in modulating and integrating signals by providing a signaling micro-environment that is tailored to produce specific biological responses. Changes in protein or lipid composition, size, structure, number, or membrane localization of lipid rafts could potentially affect the functional capabilities of these domains in signaling with important physiological consequences.

Thus, the clustering of lipid rafts and receptor proteins appears to be an efficient means in regulating cell signaling during activation. Additionally, pre-assembly of these factors could be induced following injury and may result in amplification or modulation of signals in a spatially regulated manner. This alteration, induced in part by ceramide content and PKC- $\zeta$  activation, may be involved in not only augmenting signaling but could also negatively regulate signaling by sequestering or excluding signaling components in an inactive state.

Among the proteins that are targeted to form clusters within rafts are those that are anchored in part on the outer leaflet of the membrane and can covalently attach to the GPI-protein, CD14. Examples of such proteins include TLR4, HSP70, HSP90, CXCR4 and CD55. Other proteins that are linked to saturated acyl chains, such as the SRC family of kinases in particular Lyn, and various integrins, such as Cdc42, CD11b and CD18, are also targeted to rafts and may additionally affect raft morphology and function. The formation of these complexes is induced by factors such as LPS, but the effects of severe injury remain unknown.

### Trauma Induced Mononuclear Cell Reprogramming

Severe injury is associated with increased susceptibility to life-threatening infections and sepsis, leading to the development of MODS. Severely injured patients appear to have a dysregulated innate immune

response following injury which appears to be central to the development of these clinical syndromes. The effect of trauma on mononuclear cell phagocytosis, killing of microorganisms, antigen presentation, cytokine production, and induction of cytotoxic effector cells has been characterized. However, the mechanisms responsible remain unknown due to both exaggerated pro- and anti-inflammatory responses. Insight into the mechanisms involved, however, can be determined through *in vitro* modeling of factors induced by severe injury, including PAF, oxidant stress and C5a, and through the induction of tolerance.

Treatment of mononuclear cells with various agents, including PAF, oxidant stress and C5a, results in a heightened responsiveness to subsequently encountered stimuli such as LPS. Critical to this reprogramming is cellular adherence. This is fortunate, since it is difficult to envision an *in vivo* situation where local tissue injury might occur from stimulation of suspension phase cells.

Common to these various agents is the mobilization of calcium and subsequent activation of CaMK II that we have demonstrated to occur following exposure to each of the reprogramming conditions. Although the cellular source of calcium varies, each factor results in the autophosphorylation and sustained activation of CaMK II. Although sustained activation of CaMK has not previously been studied in mononuclear cells obtained from injured and septic patients, enhanced activation of CaMK has been demonstrated in a number of other cell types, including cardiac myocytes and smooth muscle cells during sepsis.

In addition to the activation of the regulatory kinase, CaMK II, recent evidence has suggested that sphingomyelinase activation and ceramide production may play critical roles. In fact, intracellular ceramide levels along with serum TNF- $\alpha$  have been demonstrated to be elevated in patients suffering from severe sepsis. This strong correlation between cell-associated ceramide and serum TNF- $\alpha$  supports the hypothesis that ceramide, along with sphingomyelinase, plays a role in sepsis. Although sphingomyelinase activation and ceramide production may prove to be important following acute injury, this has not been previously explored.

Desensitization or tolerance is characterized by diminished responsiveness due to repeated stimulation. Lipopolysaccharide has been consistently shown to induce desensitization in mononuclear cells.

Cells in the LPS tolerant state respond to a much lesser extent than the initial stimulation resulting in attenuated liberation of chemokines and cytokines. Tolerance has been shown to attenuate several endotoxin mediated components, including IRAK-1, NF- $\kappa$ b and the MAPK. Recently, we have demonstrated that endotoxin tolerance does in fact effect recruitment of the TLR4 complex. In fact, this attenuation in recruitment of TLR4 and HSP70 during tolerance is reversed by non-specific PKC activation with PMA. This finding is consistent with previous observation that demonstrated reversal of tolerance with PMA administration. Thus, limited recruitment of receptor complexes to the lipid raft receptor platform may underlie the increased risk associated with a subgroup of injured patients at risk for devastating infections.

Finally, peripheral blood CD14 positive monocytes have been recently divided into two subpopulations, namely one with CD16 surface expression but with diminished CD14 expression (CD14+CD16+) and one without any CD16 expression (CD14++CD16-). The population of CD14+CD16+ monocytes normally represents about 10% of monocytes in healthy adults. These CD14+CD16+ cells demonstrate features of differentiated monocytes or tissue macrophages such as increased migration into tissues. They have also been described as "pro-inflammatory" in nature, producing high levels of pro-inflammatory cytokines, increased HLA-DR expression and little to no anti-inflammatory cytokines. Although not previously investigated following severe injury, the percentages and absolute number of CD14+CD16+ monocytes have been shown to be significantly increased in patients with monocytosis associated with cancer, septicemia, acquired immunodeficiency syndrome, and chronic renal failure undergoing dialysis. These findings suggest that CD14+CD16+ cells may play a key regulatory role following severe injury and may therefore be prognostic.

### Proposed Mechanism of Lipid Raft Clustering and Reprogramming

Based upon the material presented above and our preliminary findings, we have developed the following model for lipid raft receptor clustering and severe injury induced reprogramming (Figure 3). Activation is initiated by LPS/LBP binding to CD14 on lipid rafts. This ligand specific binding results in the activation of PC-PLC and the generation of DAG. Liberation of DAG results in the membrane recruitment and activation of sphingomyelinase, leading to

lipid raft sphingolipid conversion to ceramide within the lipid raft. Ceramide then results in the clustering of lipid raft proteins through the fusion within lipid rafts leading to increased gel phase fluidity and the activation of various kinases, in particular PKC- $\zeta$ . Activation of PKC- $\zeta$  then potentially leads to the engagement of b2 integrins on lipid rafts, leading to the formation of macrodomains, as well as cytoskeletal changes resulting in lipid raft recruitment of TLR4 components and scaffolding proteins. These cytoskeletal changes are perhaps induced through engagement of b2 integrin intracytoplasmic tails of paxillin, Pyk2 and other adapter and scaffolding molecules and kinases. As a result, these adapter proteins are phosphorylated and activated leading to cytoskeletal reorganization and protein reorganization and

recruitment of TLR components.

Reprogramming following injury is not only associated with constitutive changes in CD16 expression, but also changes within the raft. These changes are due to local generation of ceramide through the activation of sphingomyelinases by reprogramming factors, such as PAF, oxidant stress and C5a. Generation of ceramide leads to calcium mobilization, followed by the sustained activation of CaMK II. Activation of CaMK II, along with lipid raft ceramide fusion, leads to the early mobilization of TLR components, such as HSP70. This clustering and pre-assembly of kinases and scaffolding proteins results in altered signaling induced by subsequent stimuli.

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