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- Toll-Mediated Signaling
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- Trauma-Induced Phenotypic Alterations
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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 following this state, an exaggerated inflammatory response ensues, leading to the development of MODS (Figure 1).

The mechanism responsible for this dysregulated 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 so that 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 specialized membrane components termed lipid rafts. In particular, attenuation and augmentation of this receptor complex formation on these membrane platforms results in dysregulated inflammatory mediator liberation. 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. All of the described TLRs, except for TLR9, are transmembrane molecules. The extracellular amino termini have variable leucine-rich repeat domains, which are involved in the recognition of pathogen-associated molecular patterns. The intracellular domains contain a conserved Toll/interleukin-1 (IL-1) receptor (TIR) domain. The TIR 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 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 leading 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 (Figure 2). 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

FIGURE 1. The development of multiple organ dysfunction syndrome (MODS) after severe injury
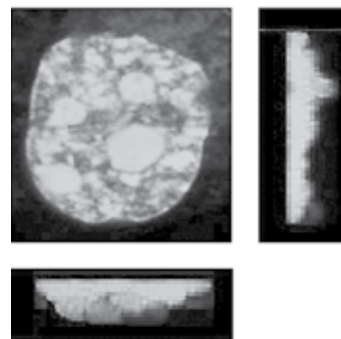

FIGURE 2. Distinct microdomains in the cell membrane that appear to serve as signaling platforms

Severe injury is associated with increased susceptibility to life–threatening infections and sepsis, leading to the development of MODS.

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. Thus, appropriate receptor complex forms to the presenting inciting stimulus resulting in cellular activation.

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, but 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. Recently, we have been able to shed some light on this mechanism by demonstrating that activation of the PKC isoform, PKC-z, is involved. Although the full effects of PKC-z 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 microenvironment 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-z 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. Each of these factors plays an important role in external signal recognition and cellular activation. A coordinated pattern occurs, with counter-regulatory components activated to lead to cellular deactivation. 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. Sustained activation had been previously demonstrated in a number of cell types during sepsis, including cardiac myocytes and smooth muscle cells. Recently, we have demonstrated a similar sustained activation of CaMK II in bronchoalveolar macrophages obtained from injured patients that have gone on to develop ARDS. This is the first example of increased activation of CaMK II following injury, and provides support that cellular alteration of calcium may be an important event in immune cell reprogramming.

In addition to the activation of the regulatory kinase, CaMK II, recent evidence has suggested that sphingo-myelinase activation and ceramide production may play additional regulatory roles. In fact, intracellular ceramide levels, along with serum TNF-□, have been demonstrated to be elevated in patients suffering from severe sepsis. This strong correlation between cell-associated ceramide and serum TNF-□ supports the hypothesis that ceramide, along with sphingomyelinase, plays a role in sepsis and subsequent organ dysfunction. Although sphingomyelinase activation and ceramide production may prove to be important following acute injury, this exploration has only just begun.

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-kb and the MAPK. Recently, we have demonstrated that endotoxin tolerance does in fact affect recruitment and formation of the TLR4 complex on lipid rafts. 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.

Putting these data together, we have just begun to demonstrate that cellular reprogramming following trauma is associated with marked alterations in raft protein and lipid composition. These changes in composition place
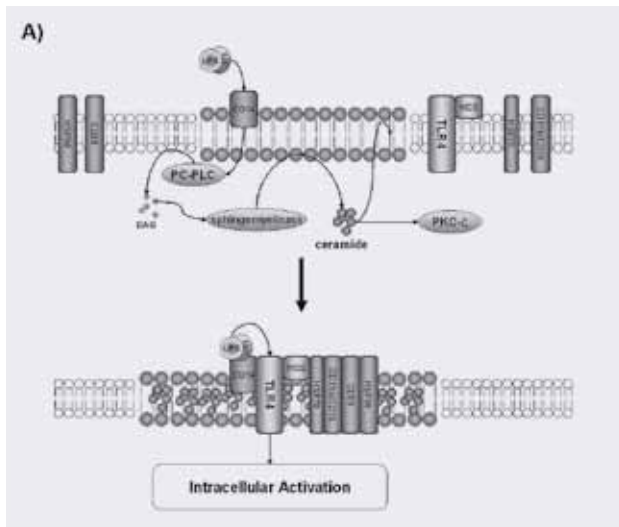
FIGURE 3A. Activation of adapter proteins, leading to cytoskeletal reorganization, protein reorganization, and recruitment of TLR components

FIGURE 3B. Reprogramming following injury, enabled by clustering and pre-assembly of kinases and scaffolding proteins

various regulatory proteins in association, leading to either enhanced or attenuated activation. Due to these changes, immune cells following injury may predispose these patients to either nosocomial infections or the development of MODS. It is therefore our current goal to evaluate these changes, using various high throughput proteomic and HPLC techniques to categorize them.

Proposed Mechanism of Lipid Raft Clustering and Reprogramming

Based upon our 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-z. Activation of PKC-z 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,

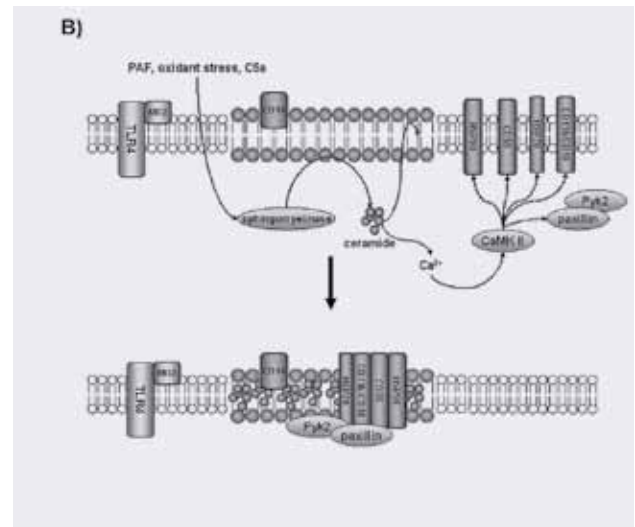these adapter proteins are phosphorylated and activated, leading to cytoskeletal reorganization and protein reorganization and recruitment of TLR components (Figure 3A).

Reprogramming following injury is associated with changes in both protein and lipid content within rafts. 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 (Figure 3B).

Trauma-Induced Phenotypic Alterations

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.

As a result, we have begun to explore changes in the phenotypic makeup of monocytes following injury. We have been able to consistently demonstrate an increase in the number of CD14+CD16+ monocytes. Sustained elevation in the expression of this phenotype following injury is associated with the subsequent development of ARDS and MODS. Although causality has not been examined, these cells do liberate increased levels of pro-inflammatory chemokines and cytokines that may in part be responsible for the development of ARDS and MODS.

The mechanism responsible for the development of this phenotype has, however, remained poorly elucidated. Recently, we have demonstrated that circulating monocytes subjected to reprogramming factors, such as oxidant stress, results in the surface expression of CD16. This increased expression of CD16 appears to be cytoskeletally regulated. Therefore, minimizing changes in cellular architecture following injury by therapeutic interventions, such as hypertonic saline, may become a means leading to improved outcome following injury.

Class Prediction Based on Cytokine Profiles

In addition to the alterations in immune cells following injury, we have recently begun to explore the relative changes in cytokine expression profiles following injury. As a result of our multicenter collaboration with the Host Response to Injury and Inflammation Consortium, we have examined the early and sustained changes in cytokine expressions following severe injury. To date, we have demonstrated that early elevation in IL-6 to 350 pg/ml within the first 24 hours is predictive of the development of MODS. Although mortality was not predicted by this cytokine profile, patients with elevation in IL-6 were demonstrated to have prolonged ventilator requirements, intensive care unit length of stay (LOS), hospital LOS, and risk for infection (Table 1).

Similar effects appear to occur with other mediators in a time-dependent fashion. These alterations following initial injury may serve to be predictive of poor outcome, and potentially more importantly serve to distinguish future therapies based on innate immunity. Specific therapies targeted at different immune responses would lead to directed individual therapy, rather than to non-specific disease-based therapy.

Nosocomial Infections in the ICU

The overall effects of this dysregulated immunity following injury clearly predispose patients to increased risk for the development of nosocomial infections and eventual organ dysfunction. Ventilator-associated pneumonia remains the most common infection in the critically injured patient. Recently, we have been able to demonstrate that these infections, which occur at a rate of 15-20 infections/1000 ventilator days, are associated with severe chest injury and the patient's nutritional status. Although the severity of chest injury cannot be changed post-injury, the nutritional status of the patient can be optimized to diminish this risk. Recently, we have investigated the effect of immediate enteral nutrition on a severely injured cohort of patients with trophic feeds initiated within 36 hours of injury. In this cohort of patients, immediate enteral nutrition was associated with a diminished risk of ventilator-associated pneumonia, nearly reducing the risk in half.

	Group 1 (IL-6<350pg/ml) N=47	Group 2 (IL-6>350pg/ml) N=32	p value
IL-6 (pg/ml)	160.3 ± 13.45	1450 ± 390.7	0.0028
Age (yr)	34.64 ± 1.58	32.00 ± 1.62	ns
Gender (% Male)	20(42.5)	25 (78.1)	0.0014
Initial Base Deficit	-8.43 ± 0.529	-8.59 ± 0.67	ns
ER Lowest SBP	87.89 ± 3.33	86.06 ± 4.15	ns
APACHE II score	25.13 ± 0.96	29.75 ± 1.11	0.0026
ISS	28.49 ± 1.90	33.69 ± 2.32	ns
RBCs first 24hrs	1987 ± 181.8	4124 ± 605.4	0.0018
ICU LOS	8.25 ± 0.88	16.28 ± 2.11	0.0011
ICU VENT days	5.72 ± 0.83	13.41 ± 1.86	0.0005
Hospital LOS	16.79 ± 1.99	28.66 ± 4.06	0.0120
Mortality	2 (4.2)	2 (6.2)	ns

TABLE 1. **Relationship between cytokine (IL-6) expression levels after injury and characteristics of patients' hospital stays**

Although the risk of infection remains high in patients with severe injury, infections by multi-resistant organisms remains an even higher concern. Infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) or *Acinetobacter baumannii* are common and associated with very poor outcomes compared with other infectious organisms. Thus, an attempt to minimize this risk is essential. Recently, we have begun to use 2% chlorhexidine washes in the ICU. This strategy has led to a significant reduction in the colonization of patients with both MRSA and *Acinetobacter baumannii*. Additionally, initiation of this daily wash has been associated with a reduction in nosocomial infections caused by these organisms.

RELATED PUBLICATIONS

1. Cuschieri J, Gourlay D, Garcia I, Jelacic S, Maier RV. Hypertonic preconditioning inhibits macrophage responsiveness to endotoxin. *J Immunol* 168:1389-1396, 2002.

2. Cuschieri J, Gourlay D, Bulger E, Garcia I, Jelacic S, Maier RV. Platelet activating factor (PAF) priming of inflammatory cell activity requires cellular adherence. *Surgery* 132:157-166, 2002.

3. Cuschieri J, Umanskiy K, Solomkin J. Protein kinase-zeta is essential toward endotoxin-induced macrophage activation. *J Surg Res* 121:76-83, 2004.

4. Cuschieri J. Implications of lipid raft disintegration: enhanced anti-inflammatory macrophage phenotype. *Surgery* 136:169-175, 2004.

5. Cuschieri J, Bulger E, Garcia I, Maier RV. Oxidative induced calcium mobilization is dependent on annexin VI release from lipid rafts. *Surgery* 138:158-164, 2005.

6. Cuschieri J, Billgren J, Maier RV, Phosphatidylcholine-specific phospholipase C (PC-PLC) is required for LPS-mediated macrophage activation through CD14. *J Leukoc Biol* 80:407-414, 2006.

7. Cuschieri J, Bellgren J, Maier RV. Endotoxin tolerance attenuates LPS-induced TLR4 mobilization to lipid rafts: A condition reversed by PKC activation. *J Leukoc Biol* 80:1289-1297, 2006.

8. Cuschieri J, Bulger E, Billgren J, Garcia I, Maier RV. Vitamin E inhibits endotoxin-mediated transport of phosphatases to lipid rafts. *Shock* 27:19-24, 2007.

9. Winfield RD, Delano MJ, Dixon DJ, Schierding WS, Cendan JC, Lottenberg L, Lopez MC, Baker HV, Cobb JP, Moldawer LL, Maier RV, Cuschieri J. Differences in outcome between obese and nonobese patients following severe blunt trauma are not consistent with early inflammatory genomic response. *Crit Care Med* 38:51-58, 2010.

10. Evans H, Dellit T, Nathens A, Chan J, Maier RV, Cuschieri J. Impact of 2% chlorhexadine gluconate whole-body bathing on hospital acquired infections among trauma patients. *Arch Surg*, in press.

11. Cuschieri J, Bulger EM, Schaeffer V, Sakr S, Nathens AB, Hennessy L, Minei J, Moore EE, O'Keefe G, Sperry J, Remick D, Tompkins RT, Maier RV; The Inflammation and the Host Response to Injury Collaborative Research Program. Early elevation in random plasma IL-6 following severe injury is associated with development of organ failure. *Shock* 34:346-351, 2010.

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