THE ROLE OF CERAMIDE IN OXIDANT-MEDIATED PRIMING OF MACROPHAGES FOR LPS SIGNALING

Patrick S. Tawadros, M.D.

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University of Toronto

ABSTRACT

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Patrick S. Tawadros, Institute of Medical Science, University of Toronto, © 2009
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Introduction: Civilian trauma remains a significant health care problem in North American society. Hemorrhagic shock and resuscitation (S/R) have been shown to prime the immune system for an exaggerated response to subsequent otherwise innocuous inflammatory stimuli such as lipopolysaccharide (LPS), resulting in multiple organ failure or death. Using a rodent model of lung injury, we previously demonstrated that antecedent S/R leads to augmented LPS-induced lung injury by way of heightened NF-κB nuclear translocation, resulting in increased elaboration of pro-inflammatory cytokines in alveolar macrophages. Further studies revealed that oxidative stress generated during S/R is responsible for this priming phenomenon. Our group recently identified two significant alterations to LPS signaling under oxidative stress conditions in macrophages: 1) the rapid recruitment of the LPS receptor Toll-like receptor 4 (TLR4) to membrane lipid rafts, and 2) the reprogramming of LPS signaling to a Src-dependent pathway involving phosphatidylinositol 3-kinase (PI3K).

Major Objective and Hypothesis: The objective of this thesis is to elucidate the molecular mechanisms underlying the augmented cellular responsiveness observed in macrophages following oxidative stress. The central hypothesis is that oxidative stress regulates LPS signaling by altering the activation and assembly of TLR4 receptor signaling components through generation of the lipid ceramide.

Summary of Findings: In the first paper, we demonstrate that the antioxidant stilbazulenyl nitrone (STAZN), a novel second-generation azulenyl nitrone, is protective in a rodent two-

hit model of lung injury involving hemorrhagic S/R and subsequent intra-tracheal LPS injection. Resultant oxidative stress and lung injury *in vivo* were significantly reduced by STAZN following S/R and LPS. In the second paper, we explore the mechanism underlying oxidant-induced surface up-regulation of TLR4 in macrophages. Using immunofluorescence microscopy and flow cytometry techniques, hydrogen peroxide *in vitro* and hemorrhagic S/R *in vivo* are shown to induce TLR4 translocation in macrophages in a ceramide and Src-dependent manner, and the enzyme acid sphingomyelinase (ASM) is shown to mediate ceramide generation. In the third paper, the role of ceramide in oxidant-induced macrophage priming for LPS signaling is investigated. Ceramide generation via ASM is shown to have a prominent upstream role in oxidant activation of the PI3K/Akt pathway via Src kinases in macrophages. Furthermore, oxidative stress is shown to reprogram LPS signaling to a ceramide dependent pathway.

Conclusion: Together, these findings highlight the role of oxidative stress in mediating augmented cellular responsiveness following S/R, and describe the role of ceramide as a central upstream mediator of oxidant priming in macrophages. The hierarchy of signaling molecules and interactions described herein represent novel targets for modulating oxidative stress in the treatment of critical illness and organ injury.

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TABLE OF CONTENTS

DISSEMINATION OF THESIS CONTENTS

Publications arising from this work

1. **Tawadros P.S.**, Powers K.A., Yang I., Becker D.A., Ginsberg M.D., Szaszi K., Kapus A., Rotstein O.D. Stilbazulenyl nitrone (STAZN) decreases oxidative stress and reduces lung injury following hemorrhagic shock/resuscitation and LPS. *Antioxid. Redox Signal. 2007; 9: 1971-1977.*
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3. **Tawadros P.S.**, Wang Z., Ailenberg M., Jung J., Birch S., Marshall J.C., Masszi A., Szaszi K., Kapus A., Rotstein O.D. Oxidant priming of macrophages for increased LPS responsiveness is mediated by ceramide in a src-dependent manner. *Manuscript in preparation.*

Additional publications during research

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3. **Tawadros P.S.**, Jung J.J., Birch S., Szaszi K., Kapus A., Rotstein O.D. Oxidant-induced activation of ERK in RAW 264.7 macrophages is mediated by sphingosine-1-phosphate. *J Surg Res 2008; 144(2), 417.*
4. **Tawadros P.S.**, Wang Z., Birch S., Szaszi K., Rotstein O.D. Oxidant-induced Akt phosphorylation in murine macrophages requires generation of ceramide through sphingosine kinase. *J Am Coll Surg 2007; 205(3): S27.*
5. **Tawadros P.S.**, Wang Z., Birch S., Szaszi K., Kapus A., Marshall J.C., Rotstein O.D. Oxidant-induced TLR4 translocation in murine macrophages is Src kinase dependent. *Critical Care 2007; 11(Suppl 4): P44.*
6. **Tawadros P.S.**, Wang Z., Birch S., Szaszi K., Kapus A., Rotstein O.D. TLR4 translocation following oxidative stress in mouse macrophages requires sphingolipid metabolism through the ASM pathway. *Can J Surg 2007; 50(supp): 8.*
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12. **Tawadros P.S.**, Powers K.A., Yang I., Kapus A., Rotstein O.D. Stilbazulenyl nitrone (STAZN) decreases oxidative stress and reduces lung injury following hemorrhagic shock and resuscitation. *Surg Infections 2005; 6(1): 136.*

Scientific presentations

1. Oxidant-induced priming of macrophages is mediated by acid sphingomyelinase and ceramide. *Poster Presentation at the Canadian Association of General Surgeons Annual Meeting, Halifax, Nova Scotia, Sep 2008.*
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4. The role of ceramide in macrophage priming following oxidative stress. *Podium Presentation for U of T Visiting Professor in Trauma Surgery-Dr. Schwab, Toronto, Ontario, June 2008.*
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8. Oxidant-induced Akt phosphorylation in murine macrophages requires generation of ceramide through sphingosine kinase. *Podium Presentation at the American College of Surgeons, New Orleans, Louisianna, Oct 2007.*
9. Oxidant-induced TLR4 translocation in murine macrophages is Src kinase dependent. *Poster Presentation at the International Sepsis Forum, Paris, France, Sep 2007.*
10. TLR4 translocation following oxidative stress in mouse macrophages requires

sphingolipid metabolism through the acid sphingomyelinase pathway. *Podium Presentation at the Canadian Association of General Surgeons Annual Meeting, Toronto, Ontario, Sep 2007.*

11. Oxidant-induced TLR4 translocation in mouse macrophages is mediated by ceramide in a Src-dependent manner. *Podium Presentation at the American College of Surgeons Trauma Paper Competition for Region XII, Toronto, Ontario, Sep 2007.*
12. Oxidant-induced TLR4 translocation in mouse macrophages involve sphingolipid metabolism through the acid sphingomyelinase pathway. *Podium Presentation at the U of T Critical Care Medicine Research Day, Toronto, Ontario, June 2007.*
13. Oxidant-induced TLR4 translocation in murine macrophages involve sphingolipid metabolism through the acid sphingomyelinase pathway. *Poster Presentation at the U of T Annual Assembly of General Surgeons and Residents, Toronto, Ontario, June 2007.*
14. Oxidant-induced TLR4 translocation in murine macrophages involve sphingolipid metabolism through the acid sphingomyelinase pathway. *Poster Presentation at the U of T Institute of Medical Science Scientific Day, Toronto, Ontario, May 2007.*
15. Oxidant-induced TLR4 translocation in murine macrophages involve sphingolipid metabolism through the acid sphingomyelinase pathway. *Poster Presentation at the Canadian Oxidative Stress Consortium Annual Meeting, Montreal, Quebec, May 2007.*
16. A central role for ceramide in oxidant-induced mechanisms of macrophage priming. *Podium Presentation at the U of T Dept of Surgery Gallie Day, Toronto, Ontario, May 2007.*
17. Oxidant-induced TLR4 translocation to the cell surface in murine macrophages may involve sphingolipid metabolism through the acid sphingomyelinase pathway. *Podium Presentation at the Surgical Infection Society Annual Meeting, Toronto, Ontario, Apr 2007.*
18. Oxidant-induced Akt phosphorylation in murine macrophages involves ceramide generation through the acid sphingomyelinase pathway. *Podium Presentation at the American College of Surgeons, Chicago, Illinois, Oct 2006.*
19. Oxidant-induced Akt phosphorylation in RAW 264.7 cells involves ceramide generation and is dependent on activation of phosphatidylinositol-3-kinase (PI3K). *Poster Presentation at the Canadian Society for Clinical Investigation/CIHR/RCPSC Annual Meeting, Ottawa, Ontario, Sep 2006.*
20. Oxidative stress in RAW 264.7 murine macrophages in vitro and shock/resuscitation in rat alveolar macrophages in vivo lead to generation of the lipid signaling molecule ceramide. *Podium Presentation at the Canadian Association of General Surgeons Annual Meeting, Calgary, Alberta, Sep 2006.*
21. Oxidant-induced Akt phosphorylation in macrophages involves ceramide generation through the acid sphingomyelinase pathway. *Podium Presentation at the U of T Critical Care Medicine Research Day, Toronto, Ontario, June 2006.*
22. Oxidant-induced priming of macrophages involves generation of the lipid ceramide. *Podium Presentation at the U of T Annual Assembly of General Surgeons and Residents, Toronto, Ontario, June 2006.*
23. Oxidant-induced Akt phosphorylation in RAW 264.7 cells involves ceramide generation and is dependent on activation of phosphatidylinositol-3-kinase (PI3K). *Poster Presentation at the U of T Institute of Medical Science Scientific Day, Toronto, Ontario, May 2006.*

24. Oxidant-induced Akt phosphorylation in RAW 264.7 cells involves ceramide generation and is dependent on activation of phosphatidylinositol-3-kinase (PI3K). *Poster Presentation at the U of T Annual Gallie Day, Toronto, Ontario, May 2006.*
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28. Stilbazulenyl nitrone (STAZN) decreases oxidative stress and reduces lung injury following hemorrhagic shock and resuscitation. *Podium Presentation at the Surgical Infection Society Annual Meeting, Miami, Florida, May 2005.*
29. Stilbazulenyl nitrone (STAZN) decreases oxidative stress and reduces lung injury following hemorrhagic shock and resuscitation. *Poster Presentation at the U of T Institute of Medical Science Scientific Day, Toronto, Ontario, May 2005.*

INTRODUCTION

TRAUMA EPIDEMIOLOGY: SEPSIS AND MULTI-ORGAN FAILURE

Civilian trauma continues to be a significant health care problem in North American society [1, 2], ranking 1st in years of potential life lost [3] and 5th in overall mortality [4]. Since the 1980 seminal report on trauma deaths in San Francisco [5], multi-organ failure (MOF) and sepsis have been recognized as leading causes of mortality in the late post-traumatic period, which extends from days to weeks following major trauma [6, 7]. This is in contrast to most early post-traumatic deaths which are due to uncontrolled blood loss and neurological injury. The development of acute respiratory distress syndrome (ARDS) after resuscitated hemorrhagic shock has been shown to be an important contributor to this late morbidity and mortality [8, 9]. While more recent reports have identified an overall decrease in the incidence and mortality associated with post-injury multi-organ failure due to improvements over the last decade in trauma management and early supportive care [10-13], the largest most recent prospective study still reports a MOF incidence of 25% and MOF-related mortality of 26% in patients with an Injurity Severity Score greater than 15 [14]. The clinical context of this thesis is that while early post-traumatic deaths are largely untreatable, delayed organ dysfunction and its consequences may be prevented by using new strategies, such as modified resuscitation fluids, in the early post-trauma period.

HEMORRHAGIC SHOCK AND THE TWO-HIT MODEL OF ORGAN INJURY

Several models have been proposed to explain the clinical course of patients sustaining major trauma and developing organ dysfunction during their hospitalization. Among these, the "two-hit" model has evolved as a paradigm of human disease to explain the development of late organ injury following survival of an initial sub-lethal trauma insult. Our understanding of the pathophysiology of MOF has evolved from a model of infectious etiology [15], to a far more complex model of uncontrolled systemic hyper-inflammation in response to infectious and non-infectious stimuli [8, 16]. Studies have shown that the global ischemia/reperfusion resulting from resuscitated hemorrhagic shock can prime the innate immune system for an exaggerated response to a subsequent otherwise innocuous stimulus which can precipitate dysfunctional hyper-inflammation and MOF. This synergistic effect of sequential stimuli has been coined the "two-hit" model for the development of organ injury in trauma patients [16, 17]. Increased adherence of neutrophils to the endothelium of target organs such as the lungs and excessive release of superoxide anions by primed circulating neutrophils in response to a second stimulus can precipitate the development of MOF [18], and studies have shown that neutrophils are already primed for excessive generation of superoxide anions within 3-6 hours of major trauma and resuscitation [19].

Several groups, including our own, have modeled the "two-hit" phenomenon *in vivo* and *in vitro* to gain insight into the molecular mechanisms underlying these priming events. The lungs are important target organs of the systemic inflammatory response observed after major trauma, affecting up to 50% of patients [20]. Using a rodent model of LPS-induced lung injury, we previously demonstrated that the combination of shock/resuscitation (S/R) followed by intra-tracheal administration of LPS leads to a marked increase in lung

neutrophil accumulation, ^{125}I-albumin trans-pulmonary flux, and heightened NF-κB nuclear translocation in alveolar macrophages (AMs) as compared to LPS alone. The earlier and stronger NF-κB nuclear translocation observed following the combination of S/R and LPS led to increased transcription of the pro-inflammatory genes CINC (cytokine induced neutrophil chemoattractant - the rodent ortholog of IL-8) and TNF-α, which in turn promote lung injury by instigating an excessive influx of neutrophils into the lungs, a hallmark feature of ARDS, and highlight the central role of primed AMs in augmented lung injury following S/R [21, 22].

Further studies revealed that oxidative stress, which is generated by ischemia/reperfusion of the GI tract during S/R [23, 24], plays a central role in this priming phenomenon as various antioxidant strategies such as the use of N-acetylcysteine (NAC) during the resuscitation phase were markedly effective in inhibiting the augmented lung injury caused by sequential S/R and LPS [21, 25, 26].

The mechanisms underlying the ability of oxidative stress to prime macrophages for increased LPS responsiveness remain the focus of much investigation. Our group has recently identified two significant alterations to LPS signaling under oxidative stress conditions in macrophages: 1) the rapid recruitment of the LPS receptor Toll-like receptor 4 (TLR4) to membrane lipid rafts [27], and 2) the reprogramming of LPS signaling to a Src-dependent pathway involving phosphatidylinositol 3-kinase (PI3K) and Akt [28]. These observations led us to study the upstream mechanisms by which oxidative stress might cause macrophage priming for increased LPS responsiveness. Specifically, we examined the role of candidate molecules including Src family kinases and the bioactive lipid ceramide in

oxidant-induced up-regulation of macrophage surface TLR4 expression and re-programming of the subsequent LPS signaling cascade.

LIPOPOLYSACCHARIDE (LPS) AND TOLL-LIKE RECEPTOR SIGNALING

LPS, the prototypical TLR4 ligand, is the principal activating component of the Gram-negative bacterial cell wall and is able to induce a broad range of responses in various cell types [29]. It contains an acylated diglucosamine head group (Lipid A) linked to a chain of repeating disacharrides, and it is this head group (Lipid A) which mediates the biological activity of LPS [30]. In the context of inflammation, its potent ability to activate the mitogen activated protein (MAP) kinase signaling cascade and induce nuclear translocation of NF-κB are central to its ability to regulate gene expression and propagate cell survival signals.

Toll-like receptors cast a wide net for innate immunity

Work performed during the past decade has identified a family of molecules, termed Toll-like receptors (TLRs), critically involved in triggering the innate immune response to invading microbes through the recognition of distinct pathogen-associated molecular patterns (PAMPs) such as lipopolysacharride (LPS) [31]. Each TLR recognizes a distinct repertoire of microbial molecules, such that together the host is innately equipped to respond to a broad array of microbial pathogens [32]. Indeed, there are to date eleven distinct TLRs that have been described in humans with over 40 different ligands [32, 33]. As mediators of the innate immune system, TLRs are found on macrophages, monocytes and neutrophils. However, they can also be found on dendritic and T cells, as well as a host of other parenchymal and epithelial cell types, casting a wider net than previously thought and offering the opportunity for cross-talk between the innate and adaptive immune systems [33, 34]. The discovery of TLR4 as the primary upstream sensor for LPS in 1998 lead to improved understanding of downstream signaling pathways and inflammation related to endotoxic shock [35, 36]. Critical studies since then revealed that cells lacking TLR4 or expressing a mutant receptor

exhibit marked hyporesponsiveness to LPS [35], while overexpression of surface TLR4 leads to augmented LPS responsiveness [37, 38]. These observations founded the rationale for suspecting that alterations in TLR4 surface expression *in vivo* may regulate LPS responsiveness, and hence offered an opportunity for therapeutic intervention.

Although TLRs serve an important role in host defense to invading pathogens, they have also more recently been implicated in the pathogenesis of various human inflammatory diseases resulting from tissue injury and oxidative stress as in hemorrhagic shock and ischemia-reperfusion. Because TLRs also appear to recognize various endogenous danger-associated molecular patterns (DAMPs) such as HMG-B1, heparin sulfate, fibrinogen, hyaluronan, fibronectin, HSP70 and others that are released by sterile tissue injury, many studies have explored the role of TLRs in conditions such as multi-organ failure, myocardial infarctions, cerebral strokes, and hepatic, renal or intestinal ischemia [39, 40]. Indeed, the emergence of TLR4 as a potential common mediator of the systemic inflammatory response syndrome from both microbial and endogenous sources has sparked a growing interest in its therapeutic possiblities.

TLR4 and LPS signaling

TLRs, including TLR4, are a family of closely related transmembrane receptors known as Type 1 integral membrane glycoproteins. Their extracellular component contains leucine rich repeats and the intracellular domain is homologous with members of a larger superfamily of receptors that include the interleukin 1 receptor (IL1-R). The cytoplasmic tail of these receptors is called Toll/IL1-R (TIR) domain and is central to initiation of signaling [32].

At the cell surface, the proposed scenario for LPS signaling first involves binding of LPS by LPS binding protein (LBP) in the serum. LBP/LPS form a ternary complex with CD14 on the cell surface of monocytes/macrophages [41]. CD14 is then thought to deliver LPS to the TLR4 receptor complex [42]. CD14 was originally thought to be the actual LPS receptor. However, its lack of a transmembrane domain suggested that LPS signaling must be initiated by interaction with other receptor components. CD14 exits in soluble and membrane bound forms. The soluble form helps mediate LPS signaling in cell types lacking membrane anchored CD14 such as endothelial and epithelial cells. The membrane bound form of CD14 is found on cells of myeloid lineage and is anchored to cell membrane via a glycosylphosphatidylinositol (GPI) tail [43]. As part of the TLR4 receptor complex, the secretory glycoprotein MD-2 acts as an extracellular adaptor protein for activation by LPS. While some studies have reported residual LPS signaling in cells lacking CD14 [44], the lack of MD-2 has been clearly shown to completely inhibit LPS signaling [45].

These interactions result in receptor dimerization and the formation of a TLR4 "receptosome" which promotes the recruitment of adaptor molecules and the initiation of intracellular signaling. Several factors appear to regulate the magnitude and nature of TLR4 signaling from the cell surface. One of these already mentioned is the total number of functional TLR4 molecules on the cell surface and their integration into a receptosome complex [37, 38]. The mechanisms regulating these events are incompletely understood. In human monocytes, LPS as well as calcium ionophore A23187 have been shown to induce a rapid increase in surface TLR4 [46]. More prolonged LPS exposure in murine macrophages appears to cause downregulation of TLR4 on the cell surface and has been proposed as a mechanism for LPS tolerance [47]. Moreover, distinct from upregulation and

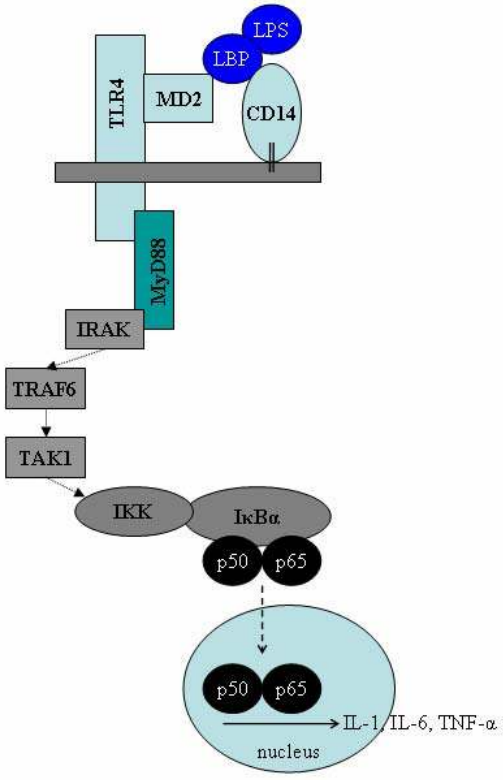
downregulation, it has been suggested that LPS induces lateral aggregation of TLR4 receptors suggesting that the spatial arrangement of the receptors on the cell surface is also relevant [48].

Other factors that regulate TLR4 signaling are the adaptor molecules that are recruited for signal transduction. These include myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal, also known as TIRAP), TIR-containing adaptor molecule (TRIF), and TRIF-related adaptor molecule (TRAM). Studies in MyD88-/- mice revealed that there are two broad categories of LPS-mediated TLR4 signaling: an early MyD88-dependent and a late MyD88-independent pathway [49]. The former has been associated with rapid transcription of NF-κB dependent pro-inflammatory genes, while the later mediates the interferon response and to a lesser extent, a late NF-κB response. MyD88 has a C-terminal TIR domain which mediates its interaction with TLR4, and an N-terminal death domain (DD) which mediates its protein-protein interactions [50]. MyD88 has emerged as an adaptor molecule for all TLRs except TLR3. A second adaptor, Mal (also called TIRAP), is necessary for the propagation of MyD88-dependent signaling [51]. Studies in Mal-/- mice, similar to studies in MyD88-/- mice, revealed resistance to the toxic effects of LPS and a lack of LPS-induced activation of mitogen-associated protein kinase (MAPK), NF-κB and cytokine production [52, 53].

Binding of MyD88 to the TLR4 receptor complex enables the association of IL1-R associated kinases, IRAK1 and IRAK 4. Studies in humans lacking functional IRAK4 have demonstrated extreme sensitivity to bacterial infections [54]. During formation of the complex with MyD88, IRAK4 is phosphorylated and activated, and then hyperphosphorylates IRAK1, which in turn induces activation of TRAF6 [55, 56]. TRAF6 is

a critical molecule for downstream signal transduction to NF-κB as it activates a heterotrimer consisting of TAK1 and the adaptor molecules TAB1 and TAB2. TAK1 is a member of the MAP kinase kinase kinase family and is required for NF-κB activation. IκB kinase (IKK), which is paired with its regulatory partner NEMO, is activated by TAK1, allowing it to phosphorylate IκB and thus mark it for proteosomal degradation. The release of NF-κB from IκB allows it to translocate into the nucleus. TAK1 is also capable of mediating MAPK activation [32, 57]. These recruiting and phosphorylating events are summarized in **Figure 1** and describe the "traditional" path of LPS-induced NF-κB activation.

Figure 1: LPS signaling via TLR4



As mentioned, recent studies have also identified MyD88-independent pathways. Although there remains some overlap between the pathways, different adaptors, namely TRIF and TRAM, have been implicated. Engagement of these adaptors results in the activation of a complex containing two related protein kinases TANK binding kinase 1 (TBK1) and IKKε, along with their adaptor TANK [58]. A recent report suggests that TRIF interacts with the TBK1/ IKKε/TANK complex via TRAF3, a homolog of TRAF6 [59]. The active TBK1/ IKKε kinases then phosphorylate interferon responsive factors (IRF) -3 and -7, leading to activation of a number of interferon (IFN)-inducible genes [60]. The mechanism for the induction of inflammatory cytokines through late activation of NF-κB is unclear, although some suggest that IRF-7 may be able to activate TRAF6 [61].

Negative regulators of TLR signaling have also been reported. IRAK-M inhibits TLR4 signaling in monocytes by impairing dissociation of the IRAK1/IRAK4 from MyD88 [62]. Other negative regulators include Bruton's tyrosine kinase (BTK) and SOCS 1, and act at the level of Mal recruitment [63, 64].

Other LPS-stimulated pathways have been described. Phosphatidylinositol 3-kinase (PI3K) has been shown to participate in TLR4 signaling leading to NF-κB translocation in cells of monocyte/macrophage lineage. Most studies reveal a positive effect of PI3K in LPS signaling, although negative influence has also been reported [65-67]. The precise mechanism of the positive signaling effects of PI3K on TLR4-mediated LPS signaling is incompletely understood since TLR4 lacks the binding motif required for the association with the regulatory p85 subunit of PI3K. Interestingly, MyD88 is known to have the putative PI3K binding site and represents a candidate site of interaction. A recent report showed that LPS treatment of RAW 264.7 cells induced tyrosine phosphorylation of MyD88 causing

association of PI3K with MyD88 and subsequent downstream signaling [68]. Consistent with a role for PI3K in LPS signaling, work from our group previously demonstrated that oxidative stress causes activation of Src family kinases and reprogramming of LPS signaling into a Src and PI3K-dependent pathway [28]. One of the aims of this thesis will be to examine the mechanism underlying this event.

TLR4 trafficking and membrane lipid rafts

Much of the mechanistic investigation regarding TLR4 traffic has been performed in HEK 293 epithelial cells transfected with CD14/TLR4 to give them a phagocytic phenotype [48, 69-71]. Using these cells, TLR4 was shown to reside predominately on the plasma membrane and in the Golgi apparatus, with some evidence of its presence in the early endosome [72-74]. TLR4 receptor trafficking involves shuttling back and forth from the cell surface to the Golgi. In human monocytes, the perinuclear localization of TLR4 has been suggested as providing evidence for its presence in the Golgi, although no formal localization studies have been performed. In RAW 264.7 cells as well as in rat alveolar macrophages, the intracellular localization of TLR4 appears more diffuse, consistent with its presence in preformed vesicles.

Assembly and organization of the TLR4 signaling complex may also dictate signaling. Movement of TLR4 to membrane lipid microdomains in the plasma membrane, called lipid rafts, is necessary for LPS signaling as it not only brings TLR4 receptors together into close proximity to each other and to CD14, but it also allows for the recruitment of various intracellular molecules, including MyD88. Other receptor molecules are also brought into rafts following LPS stimulation including Hsp70, Hsp90, CXCR4 and growth differentiation factor (GDF)-5 [75]. One important recent report indicates that the

composition of the receptor cluster may dictate the magnitude and the nature of the response. Specifically, Triantafilou and colleagues reported that LPS and lipid IVa recruit different sets of molecules to the TLR4 lipid raft complex and mediate different signaling pathways [27].

TLR4 and oxidative stress in the context of hemorrhagic shock

Oxidative stress caused by hemorrhagic shock and resuscitation incurred during major trauma has been shown to prime cells of the innate immune system, macrophages in particular, for an exaggerated inflammatory response when faced with a subsequent stimulus such as LPS. Our group has studied this phenomenon *in vivo* using a rodent model of lung injury where hemorrhagic shock and resuscitation (S/R) are induced and followed by intra-tracheal administration of LPS. Various parameters of lung injury have demonstrated that the combination of intra-tracheal LPS with antecedent S/R markedly augment lung injury [21].

As a potential mechanism of oxidant-induced macrophage priming, we recently demonstrated, both *in vitro* and *in vivo*, that oxidative stress generated during S/R causes upregulation of TLR4 in membrane lipid rafts through exocytosis [76]. Although the mechanism underlying this process remains undefined, it may involve alterations in the recruitment of adaptor molecules to the TLR4 lipid raft complex. Nakahira et al recently demonstrated that carbon monoxide (CO) can inhibit TLR translocation to lipid rafts by suppressing NADPH oxidase-dependent reactive oxygen species (ROS) generation, further validating the role of oxidative stress in TLR trafficking to lipid rafts [77-79]. Furthermore, there is evidence to suggest that ROS can selectively affect the recruitment of associated adaptor molecules in TLR4 signaling such as Mal/MyD88 and the activation of related

transduction molecules such as TRAF6 [28, 80, 81]. Oxidative stress may thus alter subsequent LPS signaling by altering the molecules recruited to the TLR4-lipid raft complex.

SRC FAMILY KINASES

Relevant to our research objectives is the Src family of tyrosine kinases. As mentioned, we have previously reported that oxidative stress redirects subsequent LPS signaling in macrophages to a Src dependent pathway. Elucidating the mechanism by which this occurs is one of the aims of this thesis. Src kinases are an important group of tyrosine kinases that have been extensively studied both in relation to acute inflammatory responses and tumorigenesis, with numerous chemical inhibitors having been developed as potential cancer therapeutic agents [82-84]. A role for Src kinases has been described in an inordinate number of signaling cascades including innate immune signaling, responses to cytokines and growth factors, regulators of apoptosis, regulators of antigen signaling, phagocytosis, response to adhesive stimulation/integrin signaling, G protein couple receptor (GPCR) signaling, and T or B cell development. Interestingly, in most of these examples, Src inhibition results in signal diminution. While Src kinases also impart their effects in non-hematopoetic cells, their role in immune cells is such that they were recently referred to as "rheostats of immune cell signaling" by an authority in the field [82].

Src kinase structure and regulation

There are nine described members in the Src-family of kinases: Src, Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, and Lyn. They are roughly 60kD in molecular weight and have a common structure which consists of an N-terminal unique domain followed by SH3, SH2, and tyrosine kinase domains. Src activation is an indirect process which requires dephosphorylation of the inactivation tyrosine residue (Y527 on c-Src). This causes the Src kinase to unfold and autophosphorylate its activation tyrosine residue (Y416 on c-Src). Negative regulation of Src kinases is mediated mainly by Csk kinase, which phosphorylates

the inactivation site. Src activation is mainly carried out by the CD45 phosphatase, which dephosphorylates the inactivation residue, thereby allowing the Src kinase to activate itself [82]. Interestingly, Csk is regulated by a Csk binding protein (Cbp) which localizes to membrane lipid rafts [85]. Recruitment of Csk by Cbp is a mechanism by which Src kinases may be regulated in lipid rafts.

Src kinase and oxidative stress

Of the nine Src kinases described above, three are active in the monocyte/macrophage lineage: Hck, Fgr and Lyn [82, 86-89]. While there is data to implicate Src kinases in various biological responses of macrophages to LPS [90], it has been shown using $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple knockout mice that Src kinases are not obligatory for LPS-induced signal transduction in macrophages to induce NF-κB nuclear translocation and generation of TNFα, IL-1 and IL-6 [28, 90, 91]. After oxidative stress, however, we have demonstrated that there is reprogramming of subsequent LPS signaling to a Src and PI3K dependent pathway. While Src kinases are known to be activated by oxidative stress [82], the upstream mechanisms responsible for the switch in LPS signaling are not well understood. Several lines of evidence suggest that there may be a relationship between oxidant-induced Src activation and TLR4 translocation to membrane lipid rafts. First, Src kinases have been demonstrated to move into membrane lipid rafts to facilitate signaling [92]. Yasuda et al described a role for the Src kinase Fyn in orchestrating Cbp phosphorylation in lipid rafts and thereby recruiting Csk to the raft domain [93]. Filipp et al showed that upon T-cell receptor (TCR) and CD4 co-aggregation, the Src kinase Lck is activated and translocates into lipid raft domains where it induces activation of another Src kinase, Fyn, thus triggering proximal TCR signaling [94-96]. Recruitment of Src kinases to

lipid rafts following oxidative stress may therefore represent a possible step in the re-programming of LPS signaling. Second, Src tyrosine kinases are known to be involved in receptor sorting and cellular secretion [97]. Sandilands et al demonstrated that Src modulates the activation, transport and signaling dynamics of fibroblast growth factor receptors upon ligand binding [98]. They demonstrated very rapid Src activation (within 5mins of ligand stimulation), and an essential role as part of the Src kinase/RhoB-dependent shuttle of receptor containing endosome delivery to the plasma membrane. Src inhibition by pharmacologic and genetic strategies was shown not only to impair receptor exocytosis, but also to decrease downstream activation of Akt. In a separate earlier study, Mocsai et al had demonstrated a role for the Src kinases Fgr and Hck in mediating adhesion-dependent degranulation in neutrophils [99]. Src kinases could thereby presumably contribute to macrophage priming by mediating oxidant-induced TLR4 surface upregulation. Third, several studies have implicated important interactions between Src kinases and TLR4 signaling molecules. The Src kinase Lyn has been shown to interact with CD14, the LPS co-receptor [88]. Others have suggested that Src and TRAF6 may collaborate in mediating PI3K activation [100, 101], a molecule which we previously implicated as part of the oxidant-primed LPS signaling cascade to NF-κB activation [28]. Finally, Src kinases can be directly activated by oxidative stress and mediate NF-κB translocation through tyrosine phosphorylation of IκBα [102]. This is unlike the usual canonical pathways of NF-κB activation which lead to serine phosphrylation of IκBα by the IKK complex.

THE PI3K/AKT SURVIVAL PATHWAY

Activation of the PI3K/Akt pathway plays an important role in cell survival, but also in cell growth, proliferation, differentiation, motility, and intracellular trafficking [103]. Its relevance to our work is made clear by the finding that oxidant priming redirected LPS signaling in macrophages to a Src and PI3K-dependent pathway leading to NF-κB activation.

PI3K structure and signaling

The phosphatidylinositol 3-kinase (PI3K) family consists of three classes of enzymes. Class I and II enzymes are primarily involved in transmitting signals from membrane receptors, whereas Class III enzymes are primarily involved in intracellular trafficking [103]. PI3K is a heterodimeric molecule composed of a regulatory and catalytic subunit. It is further classified as Class IA PI3K if it contains one of the following regulatory subunits: p85α, p55α, p50α, p85β or p55γ, and one of the following catalytic subunits: p110α, p110β or p110δ. The most highly expressed regulatory subunit is p85α and it contains SH2 and SH3 domains. PI3K can be activated by GPCRs or tyrosine kinases. The activation process requires that the p85 subunit first bind to a phosphorylated tyrosine within a YXXM motif of another protein. This causes a conformational change which allows partial activation of the p110 catalytic subunit. Phosphorylation of the p85 subunit by a tyrosine kinase (such as Src) is then required for full activation of p110 [104].

Signaling by PI3K causes generation of specific phosphorylated phosphatidylinositols (PIs). Activated PI3K catalyzes the transfer of ATP to the inositol ring of membrane bound PIs, resulting in generation of phosphatidylinositol phosphate (PIP), phosphatidylinositol (3,4)-bisphosphate (PIP_2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). The

production of PIP_3 in particular results in the recruitment of 3-phosphoinositide-dependent kinase (PDK-1), the upstream activator of Akt [105].

Akt and PDK-1 are both attracted to membrane-bound PIs by their Pleckstrin homology (PH) domains, triggering recruitment from the cytosol to the plasma membrane. Akt binding to phosphorylated membrane PIs causes a conformational change which allows PDK-1 to phosphorylate it on Threonine 308. This allows Akt to auto-phosphorylate its activation residue at Serine 473 [106]. In practice, phosphorylation of Akt on Ser 473 is used as a surrogate marker for PI3K activity [107]. Once activated, Akt is a serine/threonine kinase with numerous pro-survival downstream targets. Akt inactivates the following pro-apoptotic factors: caspase 9, Bad, GSK3, Forkhead family of transcription factors; and activates the following pro-survival ones: IKK and iNOS [30]. Interestingly, a recent report suggests that membrane lipid raft domains play a crucial role in mediating PI3K/Akt activation by facilitating Akt recruitment and activation upon PIP_3 accumulation in the plasma membrane [108].

PI3K and LPS signaling

The role of PI3K in LPS signaling remains controversial. Some studies have shown a positive signaling effect as a dominant-negative p85 construct led to diminished NF-κB activation following LPS [109]. Other studies have demonstrated the opposite, namely that PI3K inhibition leads to increased activation of NF-κB and MAPK, and induction of COX-2 and iNOS [67, 110, 111]. While p85 has been reported to associate with TLR2, TLR3, TLR4, and TLR5 in cells, only TLR2, TLR3 and TLR5 have YXXM motifs in their cytosolic domains, and mutations in those motifs have been shown to prevent binding and activation of PI3K with TLR2 and TLR3 [112, 113]. In the case of TLR4, as mentioned before, LPS

stimulation was shown to cause PI3K association and activity via interaction via MyD88 [68]. A more recent study suggests that MyD88, p85 and TLR4 may require multiple protein-protein interactions to associate and may function by way of a "signaling platform" effect, as mutation of the YXXM motif on MyD88 did not prevent LPS-induced p85 association. Surprisingly, this mutation led to a stronger association of PI3K but decreased activity [107]. On balance, the role of PI3K in LPS signaling remains controversial, but offers rationale for our observations in the setting of oxidative stress.

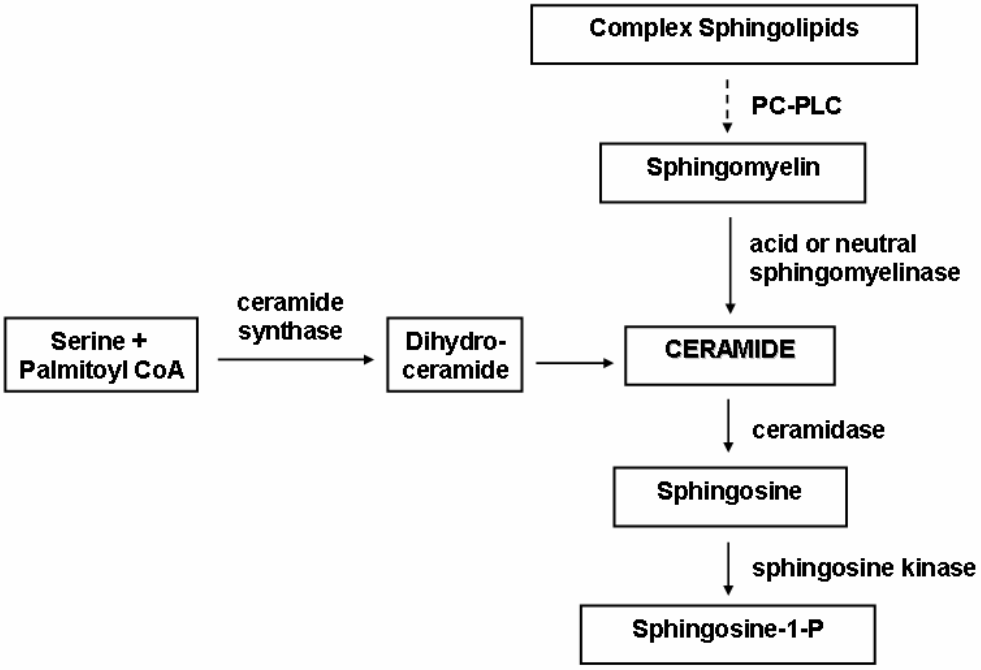
CERAMIDE AND RELATED SPHINGOLIPIDS

The sphingolipid ceramide has emerged as a powerful second messenger molecule in a variety of inflammatory signaling pathways including oxidative stress, and an important structural component of membrane signaling platforms known as lipid rafts [114], making it an appealing candidate as upstream regulator of oxidant-primed TLR4 signaling.

Sphingolipid metabolism

Sphingolipids are amphipathic molecules with a hydrophobic region consisting of a sphingoid long chain base and a hydrophilic long chain fatty-acid region of varying complexity [115]. As depicted in **Figure 2,** ceramide can be generated by *de novo* synthesis from serine and palmitoyl CoA via ceramide synthase, or by rapid degradation from complex sphingolipids that are converted to sphingomyelin by phosphatidylcholine-phospholipase C (PC-PLC), and subsequently hydrolyzed to ceramide via one of five sphingomyelinases. Ceramide may then be degraded to sphingosine by ceramidase, which in turn is converted to sphingosine-1-phosphate (S1P) by sphingosine kinase (SphK). The degradation pathway of ceramide generation is favored upon stress stimuli such as heat, UV light, or oxidative stress, and the acid sphingomyelinase (ASM) in particular has emerged as the mediator of rapid ceramide generation in various models of ischemia-reperfusion injury [116, 117] and lung injury [118]. Ceramide has been labeled "a coordinator of eukaryotic stress responses" by an authority in the field [119]; known inducers of ceramide generation include environmental stresses (heat, UV radiation, hypoxia/reperfusion), chemotherapeutic agents (Ara-C, doxorubicin, etoposide, etc), and other agents such as LPS [118].

Figure 2: Ceramide metabolism



Mammalian plasma membranes are chiefly composed of phospholipids, sphingolipids, and cholesterol. Lipid rafts are distinct membrane domains with a high concentration of sphingolipids stabilized by hydrophobic interactions with cholesterol. Cholesterol disrupting agents are known to cause raft destruction. In addition to the selective lipid composition, lipid rafts also favor selective proteins, such as GPI anchored-proteins like CD14 [120], thereby conferring their role in various signaling cascades.

ASM, which is found on the membrane of lysosomal vesicles, operates at an optimum pH of 4.5-5, and was therefore originally thought confined to that location. Recent evidence, however, has shown that various stimuli including oxidative stress can trigger ASM translocation to the outer leaflet of cell membranes, where sphingomyelin is

concentrated, and become activated to generate ceramide [121]. Jin et al demonstrated that lysosomal vesicular delivery of ASM to surface membrane upon Fas ligand stimulation in endothelial cells causes formation of membrane lipid rafts, ROS generation, and ROS-mediated amplification of ASM function [122, 123].

Sphingolipids in cell signaling

Ceramide is generally shown to inhibit cellular proliferation and promote apoptosis, while S1P exhibits opposite effects, i.e. stimulating growth and suppressing apoptosis [124, 125]. The precise mechanisms whereby these molecules exert their effects are incompletely elucidated and involve a number of downstream signaling pathways [65]. Sphingolipid levels and their function are thought to be regulated by cytokines and oxidative stress which modulate enzyme activity, by cellular localization of the enzymes, and by adaptor molecules such as FAN [118, 126]. Exogenous ceramide as well as that induced endogenously by LPS has been shown to activate PI3K and Akt phosphorylation in macrophages with downstream induction of NF-κB translocation [116]. In a murine model, ASM-induced ceramide generation was shown to contribute to lung edema following treatment with platelet activating factor, LPS or acid instillation [127]. In addition, TNF-induced NF-κB translocation as well as prostaglandin synthesis have been shown to be mediated via the S1P/Sph kinase pathway [128]. S1P contributes to C5a receptor mediated calcium transients, migration and cytokine production [129]. One recent study demonstrated the ability of a Sph kinase inhibitor to lessen lung injury after shock/resuscitation, but the mechanisms of this protection were not evaluated [130]. Only a few studies have evaluated the potential role of sphingolipid metabolites in the priming process. Balsinde and colleagues reported that LPS-primed cells treated with PAF (but not PAF or LPS alone)

induced a 40% rise in ceramide and induced arachidonic acid release in a ceramide-dependent manner in $P388D_1$ macrophages [131]. In neutrophils, sphingolipids were shown to prime cells for FMLP-induced superoxide production by TNF-α, PAF, and substance P [132] [133].

In addition to its effects on intracellular signaling pathways, ceramide has also been implicated in contributing to cell signaling via its effects on raft formation. Activation of sphingomyelinases with generation of ceramide by a number of stimuli including oxidative stress, receptor cross-linking, UVA light, bacterial pathogens and chemotherapeutics has been shown to cause fusion of lipid rafts into large ceramide-enriched membrane platforms [121, 134-137]. These platforms appear essential for signaling following stimulation with CD95 and CD40, and contribute to bacterial ingestion and downstream signaling [138, 139]. A recent study demonstrated that the generation of ceramide through acid sphingomyelinase and the formation of ceramide-enriched membrane platforms induced by TRAIL (a TNF-related apoptosis inducing factor) were both inhibited by the antioxidant NAC, supporting the role of reactive oxidant species in mediating these events [140]. The mechanism by which ceramide-enriched platforms exert these effects is not known, although one possibility is that these platforms may alter protein composition in the raft. This mechanism may prove central to the altered signaling pathway observed in the setting of macrophage priming by oxidative stress.

THESIS AIMS AND HYPOTHESES

Chapter 1: The aim was to study the ability of a novel antioxidant, stilbazulenyl nitrone (STAZN), to confer protection against lung injury following S/R and LPS. We hypothesized that STAZN may confer protection against lung injury in this setting by reducing oxidative stress and lowering the production of NF-κB-dependent pro-inflammatory cytokines.

Having reaffirmed the role of oxidative stress as the mediator of priming for augmented organ injury following S/R in Chapter 1, the subsequent studies were focused on elucidating the molecular mechanisms underlying the augmented cellular responsiveness observed in macrophages following oxidative stress. The central hypothesis is that oxidative stress regulates LPS signaling by altering the activation and assembly of TLR4 receptor signaling components through generation of the lipid ceramide.

Chapters 2: The central aim was to explore the role of Src kinases and ceramide in oxidant-induced TLR4 translocation. The detailed hypotheses were that:

1) oxidant-induced TLR4 translocation to membrane lipid rafts is Src-dependent
2) oxidative stress leads to ceramide generation
3) oxidant-induced ceramide generation occurs via ASM
4) oxidant-induced ceramide generation mediates Src activation
5) oxidant-induced TLR4 translocation to membrane lipid rafts is also ceramide-dependent.

Chapters 3: The central aim was to explore the role of ceramide in oxidant-induced reprogramming of macrophage signaling to a Src and PI3K-dependent pathway. The detailed hypotheses were that:

1) oxidative stress activates the PI3K/Akt pathway in a Src-dependent manner
2) oxidant-induced activation of Akt is also dependent on ceramide generation via ASM
3) ceramide is upstream of Src in oxidant-induced activation of the PI3K/Akt pathway
4) oxidant priming redirects LPS signaling to a ceramide-dependent pathway
5) sphingosine-1-phosphate mediates the activation of Akt.

CHAPTER 1

Stilbazulenyl nitrone decreases oxidative stress and reduces lung injury following hemorrhagic shock/resuscitation and LPS.

Antioxid. Redox Signal. 2007; 9: 1971-1977.

ABSTRACT

Multi-organ failure is a major cause of late morbidity and mortality following trauma. Reactive oxygen species generated during shock/resuscitation contribute to tissue injury by priming the immune system for an exaggerated response to subsequent inflammatory stimuli such as LPS. Stilbazulenyl nitrone (STAZN) is a novel second-generation azulenyl nitrone that has been shown to have potent antioxidant properties in a rat model of brain ischemia. We hypothesized that STAZN may confer protection against lung injury following shock/resuscitation and LPS by reducing oxidative stress and lowering the production of NF-κB-dependent pro-inflammatory cytokines. Sprague-Dawley rats were submitted to a two-hit model of lung injury involving hemorrhagic shock/resuscitation and subsequent intra-tracheal LPS injection, +/- intra-peritoneal injections of STAZN. STAZN reduced overall lung injury in response to LPS alone, and also following shock/resuscitation plus LPS. STAZN also reduced plasma levels of 8-isoprostane, a proxy measure of oxidative stress, indicating its antioxidant activity *in vivo*. The effect of STAZN was, at least in part, related to its effect on nuclear translocation of NF-κB and generation of the pro-inflammatory cytokine TNF-α. Azulenyl nitrones such as STAZN represent a promising novel class of antioxidants for treating organ injury.

INTRODUCTION

Civilian trauma continues to represent a significant health care problem. In the United States, it ranks first in terms of hospital days and years of life lost, and fourth in overall disease related mortality [2]. While the majority of early deaths caused by trauma occur as a result of hemorrhage or central nervous system injury, almost two thirds of late deaths (>1 week post-trauma) occur due to the development of multi-organ failure [6]. The lungs are among the earliest and most commonly affected organs in critically ill patients following hemorrhagic shock, affecting up to 50% of patients [8]. The prevailing paradigm for the development of multi-organ failure in this context is the so-called "two-hit hypothesis", whereby the global ischemia/reperfusion caused by shock/resuscitation primes cells of the immune system, including neutrophils and macrophages, for an exaggerated response when faced with a late subsequent inflammatory stimulus such as infection [141]. In this state, these inflammatory cells are poised to initiate tissue inflammation and injury out of keeping with the magnitude of the delayed stimulus. This scenario is well described in experimental settings whereby sequential shock/resuscitation followed by delayed stimulus leads to synergistic organ injury. In man, Botha and colleagues showed that neutrophils recovered from trauma patients were "primed" and exhibited augmented superoxide production, compared to neutrophils from healthy volunteers, when exposed to the agonist formyl-methionyl-leucyl-phenylalanine [19].

Oxidants can be generated during critical illness by activated inflammatory cells as above, or by distant tissues that have undergone ischemia/reperfusion. The gut is a well known source of such oxidants as ischemic intestinal mucosa produces xanthine oxidase, an enzyme which upon re-oxygenation converts hypoxanthine and xanthine into superoxide and

peroxide [142]. Work by several research groups, including our own, has implicated oxidative stress generated during ischemia/reperfusion as being central to the priming process. For example, using a rodent model of lung injury, we have previously shown that shock/resuscitation induces a systemic oxidative stress and that generation of these oxidants renders alveolar macrophages more responsive to intra-tracheal LPS. Alveolar macrophage priming was characterized by heightened nuclear translocation of the oxidant-sensitive transcription factor NF-κB and increased expression of NF-κB-dependent genes such as TNF-α, cytokine-induced neutrophil chemoattractant (the rat orthologue of IL-8) and tissue factor [21]. The observation that antioxidant supplements in the resuscitation fluid prevented macrophage priming and resultant lung injury provided support for the role of oxidants in this process. Similarly, in man, the use of an antioxidant "cocktail" in trauma patients was shown to reduce alveolar cytokines, to impact positively on ventilation parameters and to lessen the incidence of multiple organ failure [143].

Stilbazulenyl nitrone **(**STAZN) is a novel second generation azulenyl nitrone with significantly enhanced potency as a chain breaking antioxidant compared to other members of the α-phenyl nitrone family [144]. It was found to have great promise in a study by Ginsberg et al as a lipophilic neuroprotective agent in a rat model of focal brain ischemia [145, 146]. In that study, STAZN conferred a 64-97% reduction in mean cortical infarct volume, a reduction far greater than that achieved by any other antioxidant used for that purpose. Based on these observations and the putative role of antioxidants in lung injury, we hypothesized that STAZN may confer protection against lung injury following shock/resuscitation and LPS by reducing oxidative stress and lowering the production of NF-κB-dependent pro-inflammatory cytokines. The following study reveals that STAZN

reduces overall lung injury as measured by transpulmonary lung leak, leukocyte infiltration and neutrophil sequestration in the lungs following shock/ resuscitation and LPS. STAZN also reduces plasma levels of 8-isoprostane, a proxy measure of oxidative stress, and dampens the activation of the critical transcription factor NF-κB and the generation of the pro-inflammatory cytokine TNF-α.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (E. coli O111:B4), sterile normal saline (NS), and sodium citrate were obtained from Sigma-Aldrich Co. (St Louis, MO). Sodium citrate was prepared as 3.8% solution using sterile water, stored at 4°C, and filtered before use in resuscitation. Endotoxin-free Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen Co. (Carlsbad, CA), phosphate buffered saline (PBS) and EDTA were obtained from Gibco BRL (Burlington, Ontario), Ringer's Lactate (RL) from Baxter Co (Mississauga, Ontario), and dimethylsulfoxide (DMSO) from Sigma-Aldrich Co. (St Louis, MO). The anesthetic drugs were xylazine from Bayer Inc (Toronto, Ontario), ketamine and pentobarbital from Bimeda-MTC Pharmaceuticals (Cambridge, Ontario). STAZN was synthesized in the laboratory of Professor David Becker according to the published method of Becker et al [144], and was dissolved in the lipophilic vehicle DMSO (0.2 mg/mL).

Animal Model

Animals were cared for in accordance with the guidelines set forth by the Canadian Council on Animal Care. Male Sprague-Dawley rats weighing 300g to 350g (Charles River, St Constant, Quebec) were anaesthetized with intra-peritoneal ketamine (80mg/kg) and xylazine (8mg/kg). The right carotid artery was cannulated with a 22 gauge angiocath (Becton Dickinson, Franklin Lakes, NJ) for monitoring of mean arterial pressure (MAP), blood sampling, and resuscitation. Hemorrhagic shock was initiated by blood withdrawal leading to a reduction of the MAP to 40 mmHg within 15 mins. This blood pressure was maintained by further blood withdrawal if the MAP > 45 mm Hg, and by infusion of 0.5 ml of RL if the MAP was < 35 mm Hg. Shed blood was collected into 0.1 mL Na citrate/mL

blood to prevent clotting. After a hypotensive period of 60 mins, animals were resuscitated by transfusion of the shed blood plus an equal volume of RL. Total resuscitation time was standardized to two hours. After resuscitation, a tracheostomy was performed with a 14 gauge angiocath and either LPS (300 μg/kg in 200 μl NS) or NS alone was administered intra-tracheally followed by 10 mechanically ventilated breaths using a rodent ventilator. The animals received their first dose of STAZN (0.6mg/kg in one ml DMSO or the vehicle DMSO) by intra-peritoneal injection at the beginning of resuscitation, and their second dose two hours later, just before receiving LPS. The animals were assigned to one of the following groups: sham, shock, LPS, LPS/STAZN, shock/LPS, shock/LPS/STAZN, and shock/LPS/DMSO. Sham animals were instrumented but not bled, and NS alone was instilled intra-tracheally; shock animals were bled and resuscitated, but received only intra-tracheal NS; and LPS animals were instrumented but not bled, and received intra-tracheal LPS. Animals were sacrificed by pentobarbital overdose at various time points depending on the specific study. For the animals undergoing histological assessment, the right lung was fixed in 10% formalin, later stained with hematoxylin/eosin and examined using an optical microscope.

Bronchoalveolar Lavage (BAL)

Immediately following sacrifice, the lungs were perfused via the tracheostomy cannula with cold PBS (containing 0.1 mM EDTA) in 10 mL aliquots and gently withdrawn to a total volume of 50 mL [147]. For cell counts and differential, BAL fluid was centrifuged at 300g (1200 rpm) for 10 mins. After discarding the supernatant, the pelleted cells were re-suspended in serum-free DMEM. Total cell counts were determined on a grid hemocytometer, and differential cell counts were measured on a cytospin-prepared slide

stained with Wright-Giemsa (Fisher Scientific, Middleton, VA). A total of 300-500 cells were counted in cross-section per sample, and the number of neutrophils and alveolar macrophages was calculated as the total cell count multiplied by the percentage of the respective cell type in the BAL fluid sample.

Transpulmonary Albumin Flux

Transpulmonary albumin flux was assessed by injecting 1 mCi of ^{125}I-albumin in a total volume of 0.2 mL NS into the tail vein immediately following intra-tracheal LPS or NS [21, 147]. Six hours later, 1 mL of blood was withdrawn by cardiac puncture for counting counts per minute (CPM). At the same time, the lungs were perfused with 10 mL of PBS (in a manner similar to the BAL described above). The perfused PBS was withdrawn gently and 1 mL was used to calculate CPM. CPM was determined using a gamma counter machine (Perkin Elmer Life Sciences), and the transpulmonary albumin flux was normalized to blood CPM as follows: transpulmonary albumin flux = BAL fluid CPM /blood CPM.

8-Isoprostane Assay

8-Isoprostane levels in plasma were determined using an eicosanoid immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). 500 µl of plasma sample was vortexed with 1 mL of 100% ethanol, allowed to stand at 4°C for 5 mins, and then centrifuged at 1500g for 10 mins. The supernatant containing 8-isoprostanes was then decanted, an equal volume of 15% KOH was added, and samples were incubated at 40°C for 1 hr. The assay was performed as per the manufacturer's specifications, and the developed plate was read by a microplate reader (Diamed, Mississauga, ON, Canada) at 405 nm. The concentration was calculated by comparing to a standard curve.

Tissue collection and analysis of TNF-α and NF-κB

Lungs were retrieved at 1 hr, 2 hrs, and 4 hrs post-LPS, flash frozen in liquid nitrogen, and stored at -80°C. Lung tissue levels of the pro-inflammatory cytokine TNF-α and the activated nuclear transcription factor NF-κB were measured by ELISA analysis (Biosource, Camarillo, CA and Active Motif, Carlsbad, CA, respectively) according to specifications. Aliquots of 200-500 mg of frozen lung samples were homogenized in 1 mL of the following buffer: 25 mM TRIS/HCl (adjusted to pH 7), 2 mM EGTA, 1 mM benzamidine, and 1 mM phenyl methyl sulfonyl fluoride (PMSF) added immediately before use. The homogenized lung tissue was centrifuged at 12000 rpm for 20 mins and the supernatant (extracellular fluid) was used for TNF-α ELISA analysis. To standardize the samples, total protein concentration was determined by adding 1 μL of sample to diluted Protein Assay Dye Reagent (BioRad, Hercules, CA), and measured by spectrophotometry at 595 nm against a bovine serum albumin (BSA) standard curve. The leftover pellets were washed once in cold PBS, and then sonicated for 10 secs following resuspension in 150 μL of the following buffer: 50 mM TRIS/HCL (adjusted to pH 7), 250 mM sucrose, and 1 mM PMSF added immediately before use. The sonicated product was centrifuged at 12000 rpm for 10 mins and the supernatant (cytoplasm and nucleoplasm) was used for activated NF-κB analysis. The primary antibody used in this ELISA analysis is specific for an epitope of the p65 subunit that is only accessible when NK-κB is activated and bound to target DNA. To standardize the samples, protein concentration was determined as described above.

Statistical Analysis

The data are presented as mean ± standard error of n determinations as indicated in the figure legends. Data were analyzed by one-way analysis of variance, and post-hoc

testing was performed using the Newman-Keuls Multiple Comparison Test. In the figures presented, significance is indicated with an asterisk (*) and signifies that the mean difference between indicated treatment groups is significant at the 0.05 level.

RESULTS

Effect of STAZN on oxidative stress following shock/resuscitation and LPS

STAZN has been shown to exhibit antioxidant properties *in vitro* and to prevent neurological injury in a rat model of focal ischemia, an injury considered to be related to oxygen free radical generation. However, its ability to quench oxidative stress following shock/resuscitation and hence potentially prevent cellular priming in this scenario is unknown. 8-Isoprostane is a by-product of lipid peroxidation, a consequence of direct cell membrane damage caused by ROS and has been used as a measure of oxidative stress in the blood. As shown in Figure 1, there is a steady rise in the level of 8-isoprostanes during the 2 hour resuscitation phase. Consistent with its antioxidant properties, treatment with STAZN significantly reduced plasma levels of 8-isoprostane at all time points. This effect persisted over the several hours following LPS administration.

Effect of STAZN on lung injury following shock/resuscitation and LPS

Animals undergoing shock/resuscitation followed by intra-tracheal injection of LPS sustained a significant rise in transpulmonary albumin leak, a measure of lung damage, while neither shock nor LPS alone exerted such an effect (Figure 2). This is consistent with our previous data using this model [21]. As shown, STAZN significantly reduced transpulmonary albumin leak in animals exposed to both shock plus LPS. The diluent DMSO is known to have some antioxidant properties. To rule out a role of the diluent in the effect, we administered DMSO vehicle alone. DMSO had no effect on transpulmonary albumin flux.

Figure 3 illustrates the effect of STAZN on lung leukocyte infiltration, an alternate marker of lung inflammation. Animals receiving LPS alone had a significant rise in their

BAL total leukocyte count at 4 hrs post-LPS as compared to sham. This effect was accentuated when animals were subjected to antecedent shock/resuscitation. In both cases, STAZN treatment reduced leukocyte infiltration. In a separate group, the vehicle DMSO was again shown to have no effect (Figure 3).

As neutrophil sequestration represents one of the hallmarks of ARDS, we evaluated the proportion of cells in the BAL total leukocyte count that were neutrophils. We found that both LPS alone and shock/LPS caused a significant rise in the percentage of neutrophils to approximately 80% from a baseline sham value of less than 10%. Animals treated with STAZN experienced a significant reduction in neutrophil sequestration in both the LPS alone and shock LPS groups. The vehicle DMSO is again shown to have no effect (Figure 4). Taken together, these data support the notion that the antioxidant STAZN reduces lung injury following shock/resuscitation and LPS.

Effect of STAZN on generation of pro-inflammatory markers NF-κB and TNF-α

A common target of antioxidant therapy on cell signaling is nuclear translocation of the transcription factor NF-κB. Using an ELISA kit specific for the DNA-bound/ active form of NF-κB, we first measured the concentration of active NF-κB in whole lungs from LPS-treated animals (Figure 5A). There was a progressive increase in the level of activated NF-κB following intra-tracheal LPS, reaching statistical significance by 4 hours after LPS injection. As shown, this increase was attenuated by treatment with STAZN. In parallel, STAZN was observed to prevent the rise in whole lung TNF-α by 4 hours after LPS treatment (Figure 5B). These findings indicate that STAZN exerted anti-inflammatory effects in non-primed animals receiving LPS, suggesting a mechanism for the protective effects of STAZN in this model.

We also performed studies to discern whether comparable mechanisms were operating in LPS treatment of animals primed by antecedent shock/resuscitation. As anticipated, shock plus LPS increased both whole lung NF-κB and TNF-α (Figures 6A and 6B, respectively) compared to control animals. STAZN caused a modest, but not significant reduction in NF-κB at 4 hours following LPS treatment and had no effect on whole lung TNF-α levels.

DISCUSSION

Electron spin resonance spectrometry using various nitrone spin traps is an established technique for measuring oxygen free radical generation in both *in vitro* and *in vivo* settings. The ability of these agents to trap oxygen free radicals has also led to their use as antioxidants in a number of experimental models, where oxidative stress is known to cause injury. This is best studied in neurological diseases such as ischemic stroke, where these agents have been shown to mitigate neurological injury. STAZN is a second-generation azulenyl nitrone with superior antioxidant properties compared to other nitrones. Prior work with this compound has demonstrated its ability to be neuro-protective in models of focal ischemic stroke and traumatic brain injury. The present studies are the first to demonstrate that an azulenyl nitrone compound such as STAZN is able to prevent lung inflammation in LPS-induced lung injury with or without antecedent shock/resuscitation. In addition, our investigations demonstrate the ability of this compound to prevent oxidative stress as demonstrated by reduced 8-isoprostanes in the plasma, thereby confirming the antioxidant efficacy of this compound *in vivo* and suggesting a mechanism for its effect.

Several laboratories, including our own, have previously investigated antioxidant strategies in the treatment of lung inflammation induced by LPS administration in rodent models. The rationale for this approach has been two-fold. First, since reactive oxygen species are known to contribute to tissue damage, quenching of these molecules by various compounds has been considered a reasonable strategy for tissue protection. Second, *in vitro* investigations have implicated intracellular induction of oxidative stress as a key mechanism leading to activation and nuclear translocation of the transcription factor NF-κB and downstream induction of pro-inflammatory genes [148, 149]. The ability of antioxidants to

prevent these events has been repeatedly demonstrated in cell systems. Interestingly, the efficacy of antioxidants as an anti-inflammatory strategy *in vivo* has been quite variable, depending on the specific agent tested and also the model of lung injury. For example, N-acetylcysteine (NAC), a substrate for the synthesis of intracellular reduced glutathione, was shown to prevent lung neutrophil sequestration and permeability when LPS was given intra-peritoneally [150], yet was without effect when LPS was administered intra-tracheally [21]. By contrast, in the present work, STAZN was highly effective in protecting against lung neutrophil infiltration when LPS was given intra-tracheally. We attribute this difference to the high lipid solubility of STAZN and the potential that its effect may be more pronounced and prolonged than that of NAC. In this regard, P-tert-butyl nitrone (PBN) was shown to be effective in reducing shock-induced lipid peroxidation in the lung when PBN was given intra-peritoneally [151], suggesting the ability of this class of compounds to penetrate into the lung. We did not examine oxidative stress in lung tissue per se. However, the finding that STAZN reduced whole lung NF-κB translocation and TNF-α levels following LPS treatment is consistent with its ability to exert antioxidant properties in the lung. It also suggests that the effect is, at least in part, mediated by inhibition of pro-inflammatory signaling pathways.

The two-hit model of shock/resuscitation followed by LPS administration has been favored as one which recapitulates the events following trauma and leading to delayed lung inflammation. In this model, shock/resuscitation is presumed to prime for increased cellular responsiveness to a subsequent stimulus leading to exaggerated inflammation and injury. These findings were recapitulated in the present studies. The important role of oxidants as priming agents in this model has been demonstrated by several groups. In the present

studies, we demonstrate that STAZN reduces oxidative stress in the systemic circulation, presumably generated by xanthine oxidase elaborated by the gastrointestinal tract following ischemia/reperfusion. Concomitantly, STAZN reduced both lung neutrophilia as well as lung albumin permeability back to baseline levels when administered *in vivo.* Fan and colleagues implicated infiltrating neutrophils as the source of reactive oxygen species involved in priming alveolar macrophages, demonstrating that a membrane permeant form of catalase prevented the effect of LPS on macrophage activation [21]. While STAZN was effective in reducing inflammation and injury in the shock/resuscitation plus LPS group, we were unable to attribute its effects to its ability to inhibit cell signaling, since neither NF-κB translocation nor TNF-α was reduced by STAZN treatment. These findings suggest that STAZN may be exerting its effect on some other signaling pathway. In this regard, we have previously reported that oxidative stress reprograms LPS signaling in inflammatory cells via a Src-dependent pathway [28]. Alternatively, STAZN may have exerted its effect through a direct antioxidant effect in the lung tissue as its primary action, as has been reported with P-tert-butyl nitrone.

In summary, the present studies are the first to show that STAZN, a novel second generation azulenyl nitrone, is able to prevent lung injury in response to hemorrhagic shock and LPS. Future studies should investigate the optimal dosing and timing of this agent and its relative effectiveness compared to existing antioxidant compounds.

FIGURES

Figure 1. The effect of STAZN on plasma 8-isoprostane levels. Rats were subjected to hemorrhagic shock, resuscitation, and intra-tracheal LPS, with or without intra-peritoneal STAZN as indicated. Plasma samples were withdrawn at various time points and 8-isoprostane ELISA measurements were performed as a proxy measure of oxidative stress. STAZN caused a significant reduction in plasma 8-isoprostane levels at all time points. Data are represented as mean +/- SEM of 3 animals per group. (Data marked * are statistically significant at $p<0.05$ compared to Before Shock value and corresponding data marked **)

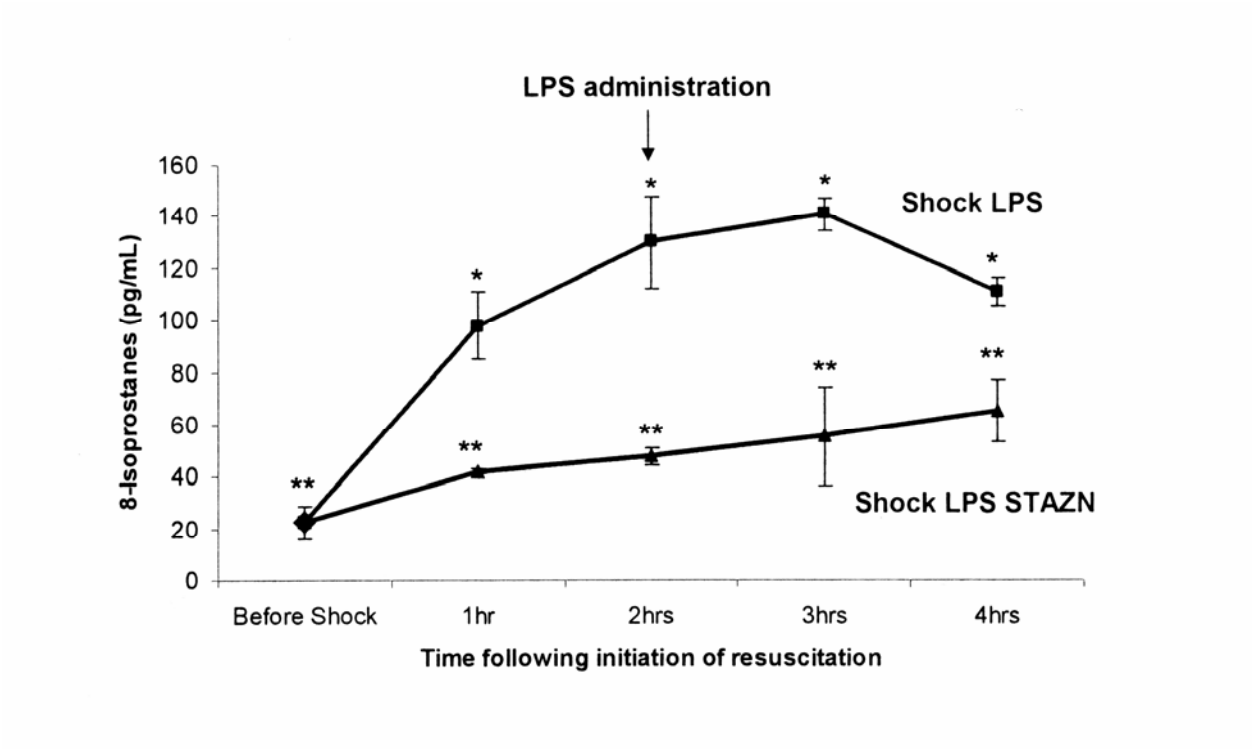


Figure 2. The effect of STAZN on transpulmonary albumin flux. Rats were subjected to all or part of the two hit model including hemorrhagic shock, resuscitation, and/or intra-tracheal LPS, with or without intra-peritoneal STAZN as indicated. 1mCi of 125I-albumin was injected in the rat tail vein immediately following LPS. BAL and blood samples were withdrawn for CPM analysis 6 hrs following LPS. Shock + LPS caused a significant increase in transpulmonary albumin flux, which was prevented by treatment with STAZN. The STAZN vehicle DMSO had no effect on its own. Data are represented as mean +/- SEM of 3 animals per group. (The data point marked * is statistically significant at $p<0.05$ compared to data marked **)

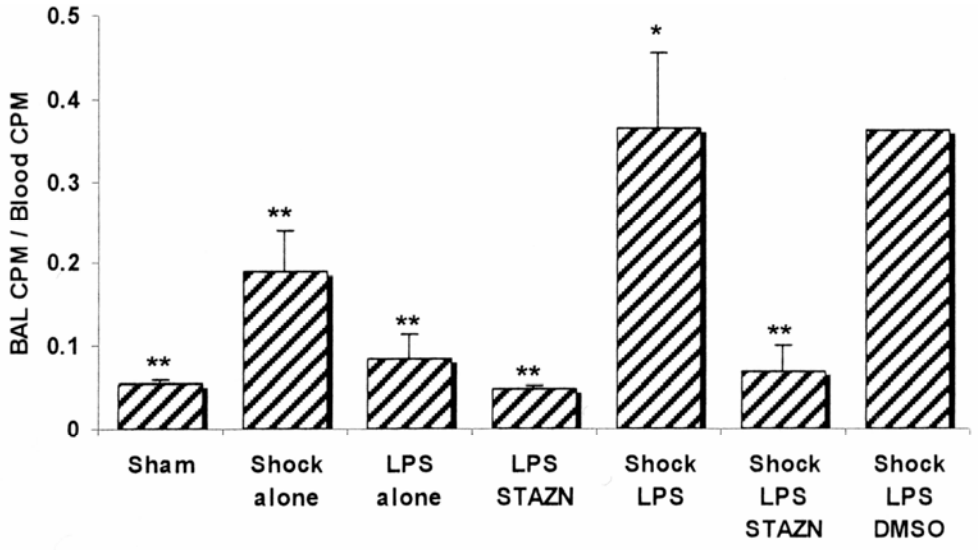


Figure 3. The effect of STAZN on leukocyte lung infiltration. Rats were subjected to all or part of the two hit model including hemorrhagic shock, resuscitation, and/or intra-tracheal LPS, with or without intra-peritoneal STAZN as indicated. Four hours post-LPS, BAL was performed and cell counts were determined. LPS alone and Shock + LPS both caused a significant increase in leukocyte infiltration, which was prevented in both cases by treatment with STAZN. The STAZN vehicle DMSO had no effect on its own. Data are represented as mean +/- SEM of 3 animals per group. (Data marked * are statistically significant at $p<0.05$ compared to data marked **)

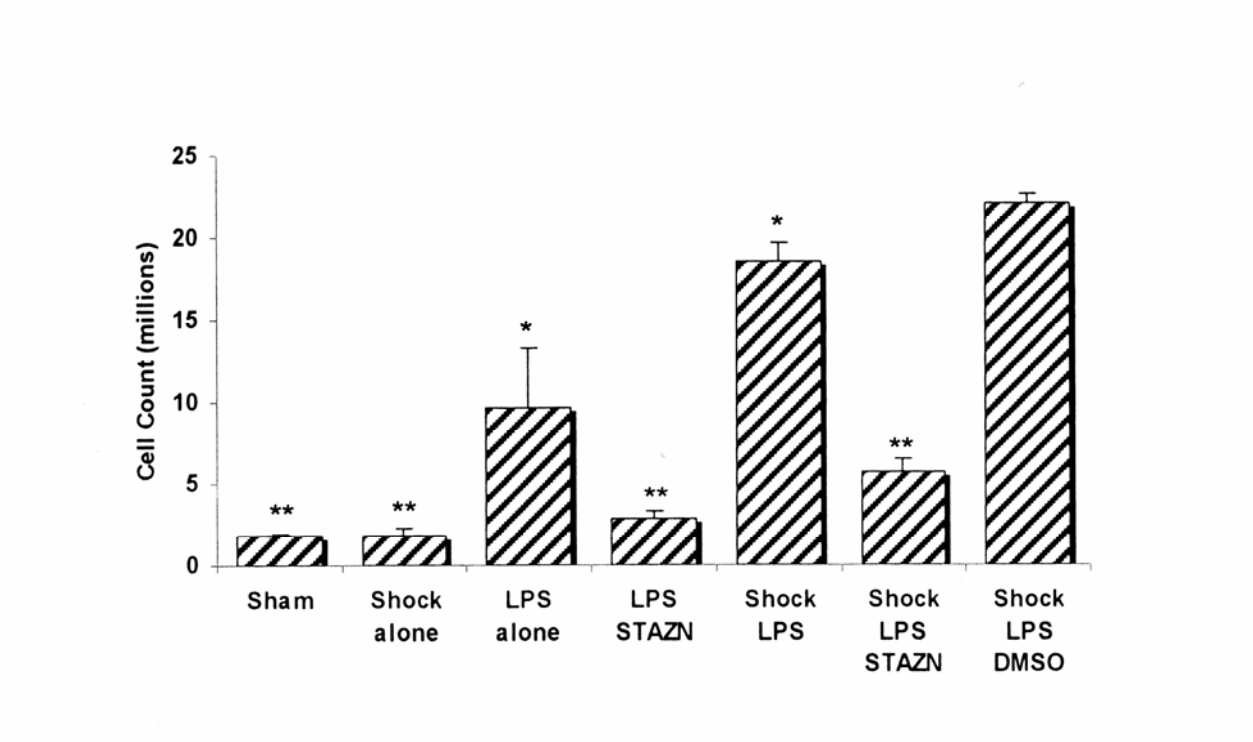


Figure 4. The effect of STAZN on neutrophil percentage in the lung. Rats were subjected to all or part of the two hit model including hemorrhagic shock, resuscitation, and/or intra-tracheal LPS, with or without intra-peritoneal STAZN as indicated. Four hours post-LPS, BAL was performed and cell counts were determined. Slides were further analysed to determine the neutrophil percentage of the total BAL leukocyte population. LPS alone and Shock + LPS both caused a significant increase in neutrophil percentage, which was prevented in both cases by treatment with STAZN. The STAZN vehicle DMSO had no effect on its own. Data are represented as mean +/- SEM of 3 animals per group. (Data marked * are statistically significant at $p<0.05$ compared to data marked **)

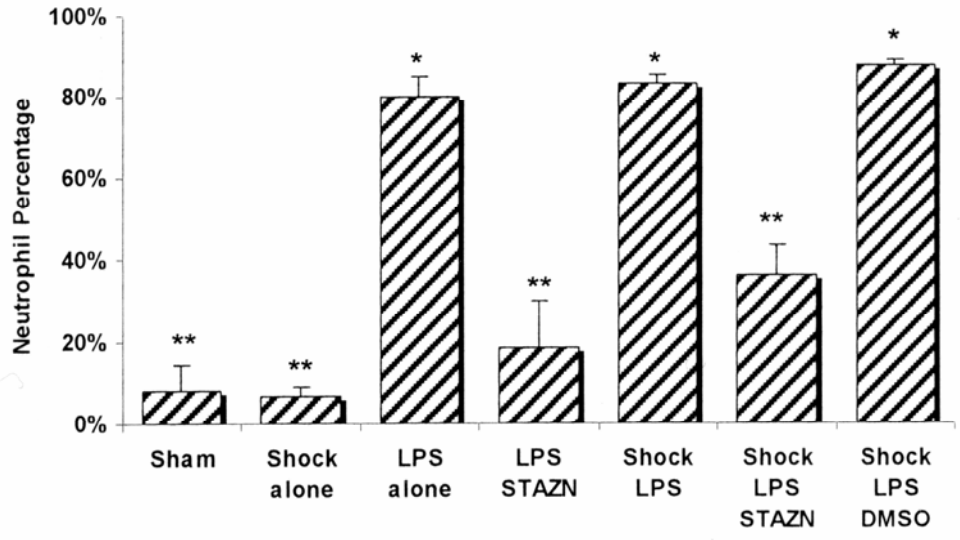
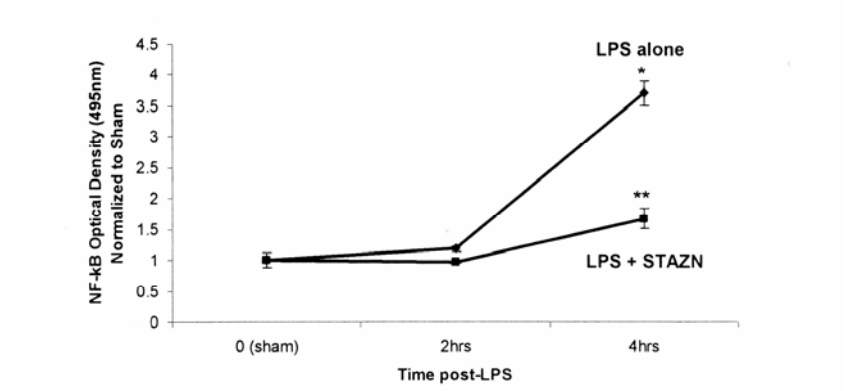


Figure 5. The effect of STAZN on generation of activated NF-κB and TNF-α in animals treated with LPS. Rats were given intra-tracheal LPS, with or without intra-peritoneal STAZN as indicated. Two and four hours post-LPS, rat lungs were retrieved and ELISA measurements of activated transcription factor NF-κB were made in Fig 5a, and ELISA measurements of pro-inflammatory cytokine TNF-α were made in Fig 5b. STAZN caused a significant reduction in the generation of activated NF-κB and TNF-α at four hours post-LPS. Data are represented as mean +/- SEM of 3 animals per group. (Data marked * are statistically significant at $p<0.05$ compared to corresponding data marked **)



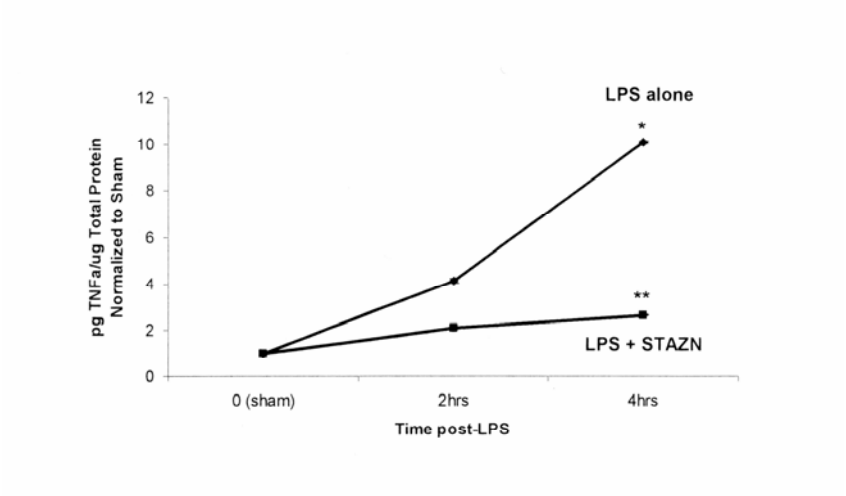
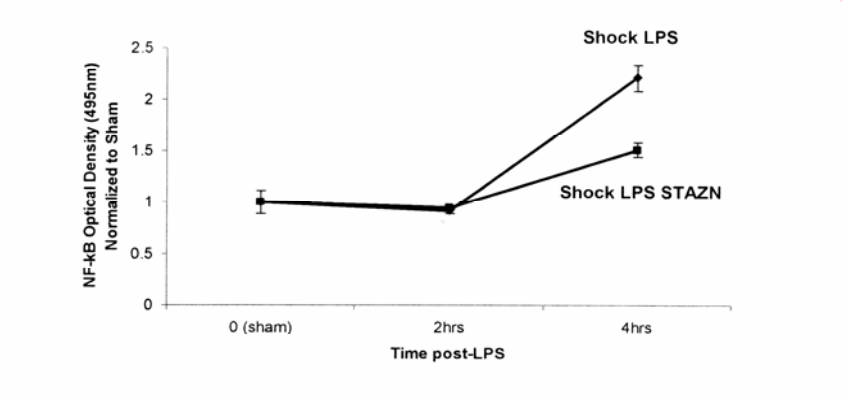
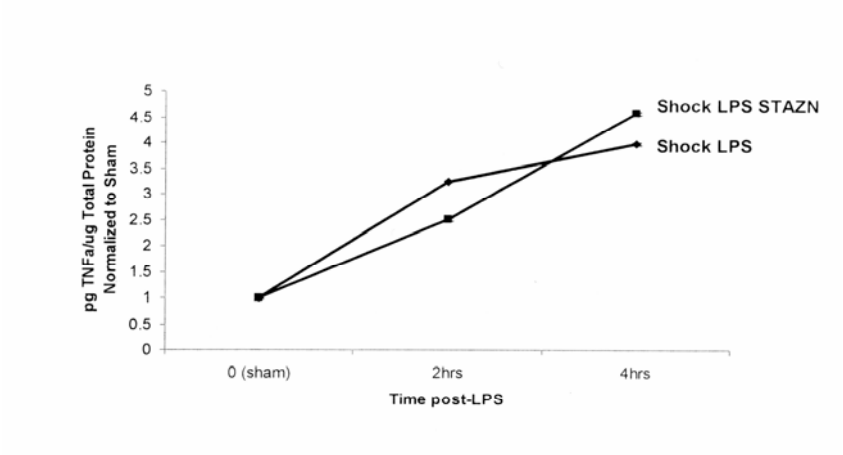


Figure 6. The effect of STAZN on generation of activated NF-κB and TNF-α in animals exposed to shock/resuscitation followed by LPS. Rats underwent hemorrhagic shock, resuscitation, and intra-tracheal LPS, with or without intra-peritoneal STAZN as indicated. Two and four hours post-LPS, rat lungs were retrieved and ELISA measurements of activated transcription factor NF-κB were made in Fig 6a, and ELISA measurements of pro-inflammatory cytokine TNF-α were made in Fig 6b. Although STAZN did not have a significant impact on activated NF-κB or TNF-α generation, there is a strong trend toward reduction in activated NF-κB at four hours post-LPS. Data are represented as mean +/- SEM of 3 animals per group.





CHAPTER 2

Oxidant-induced Toll-like receptor 4 translocation in murine macrophages is mediated by ceramide in a src-dependent manner.

Manuscript not yet submitted for publication

ABSTRACT

Multi-organ failure is a major cause of late mortality following trauma. Oxidative stress generated during shock/resuscitation contributes to tissue injury by priming the immune system for an exaggerated response to subsequent inflammatory stimuli, such as lipopolysaccharide (LPS). Our group recently reported that oxidative stress causes rapid recruitment of the LPS receptor Toll-like receptor 4 (TLR4) to membrane lipid rafts, as a mechanism underlying increased LPS responsiveness and hence cellular priming. The present studies were performed to examine the signaling pathways underlying these events. We hypothesized that activation of Src family kinases by oxidants might mediate TLR4 translocation. In the present studies, TLR4 translocation was examined using both immunofluorescence microscopy and flow cytometry. Using hydrogen peroxide *in vitro* and hemorrhagic shock/resuscitation *in vivo*, oxidant-induced TLR4 translocation in macrophages was found to occur in a Src-dependent manner. Complementary approaches supporting this conclusion included: pharmacologic inhibition of the Src family kinases by PP2, molecular Src inhibition by RAW cell transfection with Csk, and genetic Src inhibition via triple Src ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) knockout (KO) mice. We further hypothesized that oxidant-induced generation of the lipid ceramide may mediate the upstream pathway of Src activation. Oxidants were shown to induce ceramide generation in macrophages both *in vitro* and *in vivo*, an effect that was inhibited by desipramine, a specific inhibitor of acid sphingomyelinase (ASM). Using the sphingolipid degradation inhibitors D609 and desipramine, and ASM KO mice, oxidant-induced TLR4 translocation was shown to be ceramide-dependent. TLR4 translocation was recapitulated by exogenous addition of

ceramide, and was found to be mediated by Src. This study identifies a novel hierarchy of signaling molecules in oxidant-mediated priming of macrophages for LPS signaling.

INTRODUCTION

Civilian trauma continues to be a significant health care problem in North American society [3], [4]. The development of acute respiratory distress syndrome (ARDS) after resuscitated hemorrhagic shock is an important contributor to morbidity and mortality in the late post-traumatic period [8, 9]. Studies have shown that the global ischemia/reperfusion resulting from resuscitated hemorrhagic shock can prime the innate immune system for an exaggerated response to a subsequent otherwise innocuous inflammatory stimulus. The synergistic effect of sequential stimuli has been coined the "two-hit model" for the development of organ injury in trauma patients [16, 17]. Several groups, including our own, have modeled this two-hit phenomenon *in vivo* and *in vitro*. Using a rodent model of LPS-induced lung injury, we previously demonstrated that antecedent shock/resuscitation (S/R) leads to augmented LPS-induced lung injury by way of heightened NF-κB nuclear translocation and increased transcription of pro-inflammatory genes in alveolar macrophages (AMs). Further studies revealed that oxidative stress generated during ischemia/reperfusion is responsible for the priming phenomenon, as various antioxidant strategies such as the use of N-acetylcysteine (NAC) or stylbazulenyl nitrone (STAZN) during the resuscitation phase were markedly effective in inhibiting the augmented lung injury caused by sequential S/R and LPS [21, 25, 26]. The mechanisms underlying the ability of oxidative stress to prime macrophages for increased LPS responsiveness remain the focus of much investigation. Our group has recently identified two significant alterations to LPS signaling under oxidative stress conditions in macrophages: 1) the rapid recruitment of the LPS receptor Toll-like receptor 4 (TLR4) to membrane lipid rafts [27], and 2) the reprogramming of LPS signaling to a Src-dependent pathway involving phosphatidylinositol 3-kinase (PI3K) and Akt [28].

Taken together, these observations led us to hypothesize that oxidative stress might prime macrophages for increased LPS responsiveness by augmenting TLR4 surface expression through a mechanism involving activation of Src kinases.

Furthermore, the sphingolipid ceramide has emerged as a powerful second messenger molecule in a variety of inflammatory signaling pathways including oxidative stress, and an important structural component of membrane signaling platforms known as lipid rafts [114, 115, 118, 125], making it also a candidate upstream mediator of oxidant-induced TLR4 translocation. Ceramide can be generated by *de novo* synthesis from serine and palmitoyl CoA via ceramide synthase, or by rapid degradation from complex sphingolipids that are converted to sphingomyelin by phosphatidylcholine-phospholipase C (PC-PLC), and subsequently hydrolyzed to ceramide via one of five sphingomyelinases. The latter mechanism of ceramide generation is favored upon stress stimuli such as heat, UV light, or oxidative stress, and the acid sphingomyelinase (ASM) in particular has emerged as the mediator of ceramide generation in various models of ischemia-reperfusion injury [117, 152] and lung injury [116].

In these studies, using pharmacologic, molecular and genetic strategies of Src inhibition, we demonstrate that oxidant-induced TLR4 translocation in macrophages occurs in a Src-dependent manner. Further characterization of the pathway using similar strategies aimed at ceramide inhibition reveals a central upstream role for rapid oxidant-induced ceramide generation via ASM as both necessary and sufficient to mediate TLR4 translocation to the plasma membrane in a Src-dependent manner. Using both *in vitro* and *in vivo* models of oxidative stress, this study identifies a hierarchy of signaling molecules

following oxidative stress that might represent novel targets for therapy in critical illness and organ injury.

MATERIALS AND METHODS

Solutions and reagents

All chemicals were purchased from Sigma Aldrich Co. (Oakville, ON), unless otherwise stated. C2-ceramide and dihydroceramide were purchased from Avanti Lipids Inc. (Alabaster, AL) and reconstituted in DMSO. The inhibitor D609 was purchased from Biomol International (Plymouth Meeting, PA), desipramine from Sigma, and PP2 from Calbiochem, Inc. (San Diego, CA).

Cell culture and activation

The RAW 264.7 murine macrophage cell line was purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Burlington, ON) with 10% endotoxin-free fetal calf serum (FCS, Hyclone Lab, Logan, UT) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2. RAW cells were starved in serum-free conditions for at least 2hrs prior to treatments. Experiments with H_2O_2 (100-300μM) were performed in Hank's Balanced Salt Solution (HBSS, Gibco) while those with C2-ceramide (100-200μM) were performed in serum-free DMEM. Where pharmacologic inhibitors were used, cells were pre-incubated with the inhibitor for 15mins prior to treatment.

RAW 264.7 macrophages stably transfected with a Csk plasmid were kindly provided by Dr. Z. Honda (University of Tokyo, Japan) [153]. The cells were maintained in similar growth medium supplemented with the neomycin antibiotic G418 (Invitrogen, Burlington, ON).

Diacylglyceride kinase reaction and thin layer chromatography

RAW 264.7 cells cultured on a 6-well plate (approx. $2x10^6$ cells plated 48hrs prior) were harvested and lipids were extracted using chloroform and methanol according to the Bligh and Dyer method [154]. Lipids were then isolated by evaporating the chloroform under direct N_2 gas flow. The same evaporation technique was applied to a dilution series of ceramide controls ranging from 0 to 600pmol (Avanti Polar Lipids, catalogue #860052). Ceramide levels were evaluated using the diacylglyceride (DG) kinase assay. Briefly, lipids were incubated at 37°C for 30mins with 20uL of micelle solution, consisting of β-octylglucoside/dioleoyl-phosphatidyl glycerol micelles. The DG kinase reaction was then performed by adding 50uL of reaction mixture (100mM imidazole, 100mM LiCl, 25mM $MgCl_2$, 2mM EGTA, 4mM dithiothreitol), 5ug of *Escherichia coli* DG kinase (Calbiochem, catalogue #266726), and 20uL of initiation mixture (10mM imidazole, 1mM diethylenetriaminepentaacetic acid, 5mM ATP with [γ-^{32}P]ATP (spec activity 50000-100000cpm/nmol, 50uCi, Perkin Elmer, Waltham, MA)) for 30mins at 26°C. Lipids were then separated by thin layer chromatography (TLC) on TLC silica glass plates (Sigma) in a chloroform - acetone - methanol - acetic acid - water mixture (10:4:3:2:1 v/v) and visualized by autoradiography for phosphatidic acid and ^{32}P-ceramide.

Immunofluorescence microscopy

Cells plated on coverslips were fixed with 4% paraformaldehyde for 30mins. Cells were then permeabilized in phosphate buffered saline (PBS, Gibco) solution containing 100 mM glycine and 0.2% Triton X-100 for 20mins, blocked with 5% bovine serum albumin in PBS for 1hr, and incubated with a TLR4 primary antibody (H-80, Santa Cruz Biotechnology, Santa Cruz, CA) for 1hr followed by a Cy3-labeled secondary antibody (Jackson

ImmunoResearch Laboratories, West Grove, PA). Samples were analyzed by an Olympus IX81 microscope (60x or 100x objectives, Melville, NY) coupled to an Evolution QEi Monochrome camera controlled by the QED InVivo Imaging software (Media Cybernetics, Silver Spring, MD). Images were processed by the ImagePro Plus 3DS 5.1 software (Media Cybernetics).

Flow cytometry

Cell surface expression of TLR4 was detected by flow cytometry on non-permeabilized cells fixed with 4% paraformaldehyde for 30mins. Cells were blocked with 5% bovine serum albumin in PBS for 1hr, and incubated with a TLR4 primary antibody (L-14, Santa Cruz) for 1hr followed by a FITC-labeled secondary antibody (Jackson) for 1hr. Each sample represents 10,000 cells analyzed in a BDFACSCanto instrument using the BDFACSDiva software (BD Bioscience, San Jose, CA) at the FITC band detector excitation wavelength of 488nm. Results were expressed as mean channel fluorescence (MCF).

siRNA transfections

Sparsely plated RAW cells were transfected with 40pmols of ASM siRNA construct (catalogue sc-41651, Santa Cruz) or a negative control siRNA (Ambion, Austin TX) in the presence of OptiMEM medium (Gibco) and Lipofectamine RNAiMax (Invitrogen) in a total volume of 0.5mL. ASM knockdown was verified 24hrs later by RT-PCR (BioRad iQ5 iCycler, BioRad Laboratories, Hercules, CA), using the following primers: sense 5'-ACAGTCTCGCCAAGATCAGC and anti-sense 5'-AGAAGCCCCCAATTCTTAGG. Data were normalized to glycerol aldehyde phosphate dehydrogenase (GAPDH) expression in the same samples using the following primers: sense 5'-TCACCACCATGGAGAAGGC and

anti-sense 5'-GCTAAGCAGTTGGTGGTGCA. Experiments on RAW cells using this ASM knockdown system were performed 24hrs after transfection.

Western blotting

Proteins were detected using Western blotting as described previously [28]. Briefly, following treatment cells were lysed in Triton-based lysis buffer (Cell Signaling Technologies, Danvers, MA) and equal amount of protein was subjected to SDS-PAGE. Membranes were incubated with phospho-Src family Y416 antibody (Cell Signaling). To verify equal loading, membranes were reprobed with Akt or Lyn antibodies (Cell Signaling). Bands were visualized by Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech).

Animal Model

Animals were cared for in accordance with the guidelines set forth by the Canadian Council on Animal Care. The following mouse strains were used: C57BL/6 mice weighing 20 to 30g (Charles River, St Constant, Quebec), triple Src KO ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) mice obtained from Dr. Clifford Lowell (University of California, San Francisco, CA) and ASM WT/KO mice obtained from Dr. Richard Kolesnick (Memorial Sloan-Kettering Cancer Center, New York, NY). Mice were anaesthetized with intra-peritoneal ketamine (200mg/kg) and xylazine (10mg/kg). The right femoral artery was cannulated for hemorrhagic shock and resuscitation. Hemorrhagic shock was initiated by blood withdrawal equivalent to 20% of blood volume (22.5mL of blood/kg) over 15mins as previously reported. To prevent clotting, shed blood was collected in the presence of 3.8% Na-citrate. After a hypotensive period of 60mins, animals were resuscitated by transfusion of the shed blood plus an equal volume of Ringer's Lactate using a timed-delivery pump system. Total resuscitation time

was standardized to two hours. In certain experiments, the animals received a total dose of D609 (40mg/kg) *in vivo* divided into three intra-peritoneal injections given at 0, 30, and 60mins into the two hour resuscitation phase. Sham animals were instrumented but not bled. Finally, animals were sacrificed at end resuscitation by pentobarbital overdose. At this time, a tracheostomy was performed and alveolar macrophages were recovered by bronchoalveolar lavage (BAL).

BAL was achieved by perfusing the lungs via the tracheostomy cannula with cold PBS (containing 0.1mM EDTA) in 1mL aliquots and gently withdrawing to a total volume of 5mL. BAL fluid was centrifuged at 300g (1200 rpm) for 5mins. For experiments where AMs were being recovered for *in vitro* experiments, the pelleted cells were re-suspended in DMEM with 2% FCS and plated in 6-well tissue culture dishes for 24hrs. For experiments where animals had already undergone shock and resuscitation, the pelleted cells were re-suspended in serum-free DMEM and processed for immunofluorescence staining or flow cytometry analysis.

Statistical Analysis

The data are presented as mean ± standard error of n determinations as indicated in the figure legends. Data were analyzed by one-way analysis of variance, and Newman-Keuls Multiple Comparison Test post-hoc using the Prism software (GraphPad, San Diego, CA). Results were considered significant when $p \leq 0.05$. In the figures, significance is indicated with an asterisk.

RESULTS

H_2O_2 induces TLR4 translocation *in vitro* in a Src-dependent manner

H_2O_2 has been previously shown to induce a dose-dependent increase in surface TLR4 expression [27]. To explore the possible role of Src kinases in oxidant-induced TLR4 translocation, RAW cells were stimulated with H_2O_2 in the presence or absence of the Src kinase inhibitor PP2 (10uM). TLR4 translocation was assessed by flow cytometry (Fig 1A). The 50% increase in TLR4 surface expression caused by oxidative stress was completely abolished by the Src inhibitor PP2.

To further support this finding, a molecular strategy of Src inhibition was used, whereby RAW cells stably transfected with a plasmid expressing the natural Src inhibitor Csk were stimulated with H_2O_2 *in vitro*. TLR4 translocation was assessed by flow cytometry (Fig 1B). In this case, a nearly twofold increase in H_2O_2-induced TLR4 translocation by 60mins was also abolished by Src inhibition.

A genetic approach to Src inhibition was also pursued and H_2O_2-induced TLR4 translocation was assessed by flow cytometry in AMs from triple Src KO mice ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) (Fig 1C). Once again, the near doubling in TLR4 translocation seen in WT AMs was almost completely inhibited in AMs from Src KO mice. Together, these results indicate that Src kinase activity is necessary for oxidant-induced TLR4 translocation to occur.

H_2O_2 induces ceramide generation in macrophages through acid sphingomyelinase

To explore the hypothesis that ceramide might also be involved in TLR4 translocation, we first determined whether oxidative stress induces ceramide generation in macrophages by exposing RAW cells *in vitro* to H_2O_2 (300uM) for 0 to 30mins. Cellular lipids were extracted according to the Bligh and Dyer method and then subjected to

diglyceride kinase reaction. The radio-labeled ^{32}P-ceramide product was visualized by thin layer chromatography (TLC) and autoradiography (Fig 2A). The results from three independent experiments were averaged and revealed that H_2O_2-induced ceramide generation in RAW cells was greatest at 10mins with 197% increase over basal (Fig 2B). To determine whether this H_2O_2-induced doubling of ceramide was predominantly the product of sphingolipid degradation versus *de novo* ceramide synthesis, two independent pharmacologic inhibitors of the sphingolipid degradation pathway were used. First, it was found that peak ceramide generation at 10mins was reduced to 129% of basal in the presence of D609 (100uM), an upstream inhibitor of the sphingolipid degradation pathway that blocks the action of PC-PLC. Second, peak ceramide generation at 10mins was reduced to 97% of basal in the presence of desipramine (5uM), a specific inhibitor of ASM which converts sphingomyelin to ceramide (Fig 2C). Together these results suggest that H_2O_2-induced ceramide generation is primarily the product of rapid sphingomyelin degradation through the ASM pathway.

H_2O_2 induces TLR4 translocation *in vitro* in a ceramide-dependent manner

The role of ceramide in oxidant-induced TLR4 translocation to the cell surface of macrophages was first assessed with the pharmacologic inhibitor D609. AMs recovered from C57BL/6 mice were stimulated with H_2O_2 (300uM) for 60mins and resultant TLR4 translocation to the cell surface was measured by flow cytometry (Fig 3A). D609 (100uM) caused complete inhibition of the near 50% increase in TLR4 surface expression observed after H_2O_2 alone. This suggests a role for oxidant-induced sphingolipid degradation products such as ceramide in the mechanism of TLR4 surface up-regulation.

To further explore the potential role of ceramide in oxidant-induced TLR4 translocation, we conducted similar *in vitro* studies using AMs from ASM KO mice. In this instance, H_2O_2-induced TLR4 translocation was assessed by immunofluorescence microscopy (Fig 3B) and flow cytometry (Fig 3C). Both approaches revealed that genetic deficiency of ASM completely inhibited oxidant-induced TLR4 translocation. As ASM was shown to be an important mediator of oxidant-induced ceramide generation, these data suggest that ceramide generation is in turn necessary for oxidant-induced TLR4 translocation to the cell surface.

Ceramide induces TLR4 translocation *in vitro*

Having demonstrated that ceramide is necessary for oxidant-induced TLR4 translocation to occur, we sought to determine if it was also sufficient to cause it. The synthetic ceramide analogue C2-ceramide was used to treat RAW cells *in vitro* for 0-30mins, and TLR4 translocation was assessed by immunofluorescence microscopy (Fig 4A) and flow cytometry (Fig 4B). C2-ceramide caused TLR4 peripheralization in RAW cells as detected by immunofluorescence microscopy and revealed a time-dependent increase in TLR4 translocation as determined by flow cytometry, suggesting that it is sufficient to mediate TLR4 translocation. As a control for the C2-ceramide vehicle DMSO, studies were conducted in RAW cells using the inactive analogue of C2-ceramide, dihydroceramide (200uM), and no change in TLR4 surface expression was observed by flow cytometry (data not shown).

To further corroborate the role of ceramide in mediating TLR4 translocation, we treated AMs from ASM KO mice with C2-ceramide for 60mins and assessed TLR4 translocation by flow cytometry (Fig 4C). Ceramide induced over 100% increase in TLR4

surface expression in both WT and ASM KO AMs compared to their respective controls, suggesting that 1) the lack of H_2O_2-induced TLR4 translocation in ASM KO AMs observed in figures 3B and 3C was due to their inability to generate ceramide, and 2) oxidant-induced ceramide generation is primarily mediated through the ASM pathway in macrophages.

H_2O_2 leads to src activation in a ceramide-dependent manner

Given the role of Src kinases in oxidant priming of macrophages for subsequent LPS signaling [28], and the potential ability of ceramide to mediate Src kinase activation, we investigated the role of ceramide in oxidant-induced Src activation. A molecular strategy for ASM inhibition was achieved by transfecting RAW cells with an ASM siRNA. PCR analysis after 24hrs revealed that ASM siRNA transfection caused a 70% reduction in ASM mRNA compared to a scrambled negative siRNA control (Fig 5A). Using this molecular ASM knockdown system, we found that early (5mins) H_2O_2-induced Src family kinase activation was inhibited in cells transfected with ASM siRNA (Fig 5B). Further, C2-ceramide (100uM) added directly to RAW cells recapitulated the early activation of Src family kinases (Fig 5C). Taken together, these data suggest that H_2O_2-induced Src activation in macrophages is mediated by ceramide generated via the ASM pathway.

Ceramide-induced TLR4 translocation is Src-dependent

As both ceramide and Src appear necessary mediators of H_2O_2-induced TLR4 translocation, further studies were conducted to establish the hierarchy of action between these two molecules. Ceramide has been shown capable of activating Src kinases in figure 5C, and H_2O_2-induced Src kinase activation was inhibited by an ASM knockdown in figure 5B, suggesting that ceramide generation is upstream of Src kinase activation. To confirm this hypothesis, RAW cells were treated with C2-ceramide in the presence of the Src

inhibitor PP2 (Fig 6A). While C2 alone caused a 54% increase in TLR4 surface expression, this increase was reduced to 30% by PP2 compared to its respective control, representing a near 50% decrease in C2-induced TLR4 surface up-regulation. To further explore this finding, AMs from Src KO mice were treated with C2-ceramide revealing similar results (Fig 6B). In this case, C2 caused an 80% increase in TLR4 surface expression in WT AMs, which was reduced to 50% in Src KO AMs compared to their respective control. While these studies suggest that direct ceramide-mediated TLR4 translocation is partially Src-dependent, the data in aggregate support the hierarchy that oxidant-induced TLR4 translocation is mediated by ceramide in a Src-dependent manner.

Shock/resuscitation also causes ceramide generation and TLR4 translocation *in vivo*

To corroborate our *in vitro* data with H_2O_2, studies were conducted *in vivo* using a mouse model of hemorrhagic shock and resuscitation to induce oxidative stress. This model has been used extensively by our group to study lung injury in the context of S/R and LPS [21, 26]. Using this model, AMs were recovered by BAL following S/R and analyzed as previously described for ceramide generation (Fig 7). It was observed that oxidative stress generated through this more physiologic *in vivo* model of hemorrhagic shock and resuscitation also caused ceramide generation in alveolar macrophages.

The model was then applied to elucidate the role of ceramide in the S/R-induced TLR4 translocation we have previously described in AMs [27]. The pharmacologic inhibitor of sphingolipid degradation D609 was used *in vivo* during resuscitation of C57BL/6 mice, and TLR4 translocation was assessed by immunofluorescence microscopy (Fig 8A) and flow cytometry (Fig 8B). In both cases, D609 used *in vivo* completely inhibited S/R-induced TLR4 translocation in AMs. To corroborate this finding, S/R-induced TLR4 translocation

was also assessed in ASM KO mice by immunofluorescence (Fig 9A) and flow cytometry (Fig 9B). Again, TLR4 translocation was completely inhibited in ASM deficient mice, supporting the necessary role of ceramide in oxidant-induced TLR4 translocation.

Last, to reaffirm the role of Src kinases *in vivo* in S/R-induced TLR4 translocation, the same experiment was conducted on triple Src knockout (KO) mice ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) (Fig 10A and 10B). Again, these studies corroborated their *in vitro* counterparts and supported the necessary role of Src kinases in oxidant-induced TLR4 translocation.

DISCUSSION

The clinical scenario of resuscitated hemorrhagic shock is known to render patients susceptible to the development of a systemic inflammatory response and possible multiple organ failure. The ability of antecedent S/R to prime cells of the innate immune system for increased responsiveness to a second stimulus, such as Gram-negative bacterial LPS, has provided a mechanistic framework for the investigation of post-resuscitation organ injury. One demonstrated mechanism of oxidant priming for subsequent LPS exposure is the up-regulation of TLR4 on the cell surface of macrophages [27]. These studies focused on characterizing the pathway of oxidant-induced TLR4 translocation to identify upstream mediators and potential targets for modulating this priming mechanism. Src kinases were found to have an essential role in mediating oxidant-induced TLR4 translocation. Further experiments identified a central upstream role in macrophages for rapid oxidant-induced generation of ceramide via ASM, which was found to be both necessary and sufficient to mediate TLR4 translocation to the plasma membrane in a Src-dependent manner.

In order to assess the role of Src kinases in oxidant-induced TLR4 exocytosis, three complementary approaches of Src inhibition were used. First, the pharmacological agent PP2 is a potent and selective inhibitor of the Src family of tyrosine kinases [155]. It was demonstrated to inhibit H_2O_2-induced TLR4 translocation in RAW cells. Second, C-terminal Src kinase (Csk) is a molecular inhibitor of Src kinases known to mediate phosphorylation of Src kinases at their inactivation tyrosine residue (Y527). In this case, we used RAW cells that were transfected with a plasmid leading to constitutive over-expression of Csk and a resultant state of Src inhibition, which also blocked H_2O_2-induced TLR4 translocation. The physiologic role of Csk as negative regulator of Src kinases makes it an interesting molecule

in its own right, especially since it has also been shown to be activated by oxidative stress, although it has mostly been attributed the role of returning Src kinases to their basal state of inactivity after an inciting stimulus [156]. Third, $hck^{-/-}fgr^{-/-}lyn^{-/-}$ triple Src knockout mice obtained from Dr. Clifford Lowell (UCSF, California) were used. Despite that there are at least 9 kinases in the Src family, peritoneal or bone marrow derived macrophages from these mice were shown to have no residual Src kinase activity, suggesting that Hck, Fgr and Lyn are the only functional Src kinases in the monocyte/macrophage lineage [82, 90]. H_2O_2 experiments performed *in vitro* on AMs derived from these mice, and S/R studies performed *in vivo* on these mice both demonstrated complete inhibition of oxidant-induced TLR4 translocation. Together, these findings are the first to demonstrate that Src family kinases are involved in regulating translocation of TLR4 under oxidative stress conditions.

This finding complements our earlier description in macrophages that oxidative stress redirected LPS signaling to a Src dependent pathway [28]. But while oxidative stress is well known to activate Src kinases, the exact mechanism by which Src kinases can mediate oxidant-mediated TLR4 surface upregulation remains undefined. Lipid rafts have recently been demonstrated to be important contributors to membrane recruitment and clustering of the LPS receptor complex in response to LPS stimulation or oxidative stress [27, 75, 157]. Several studies, particularily in T cells, have described various roles for Src kinases in lipid rafts. Yasuda et al describe a role for the Src kinase Fyn in orchestrating Csk-binding protein (Cbp) phosphorylation in lipid rafts and thereby recruiting Csk to the raft domain [93]. Recruitment of Src kinases to lipid rafts by oxidative stress may therefore represent a critical step in organizing TLR4 up regulation at the cell surface.

Alternatively, there is also an established role for Src kinases in mediating receptor traffic and vesicular transport. Sandilands et al demonstrated that Src kinase modulates the activation, transport and signaling dynamics of fibroblast growth factor receptors upon ligand binding [98]. They demonstrated very rapid Src activation (within 5mins of ligand stimulation), and an essential role as part of the Src kinase/RhoB-dependent shuttle of receptor containing endosome delivery to the plasma membrane. Src inhibition by pharmacologic and genetic strategies was shown not only to impair receptor exocytosis, but also to decrease downstream activation of Akt. In a separate earlier study, Mocsai et al had demonstrated a role for the Src kinases Fgr and Hck in mediating adhesion-dependent degranulation in neutrophils [99].

Others have implicated a critical role for ROS in ligand-mediated TLR4 trafficking [158, 159]. Nakahira et al demonstrated that LPS mediated TLR4 assembly in lipid rafts was mediated by NADPH oxidase-dependent ROS generation [77]. Interestingly, CO was shown to both negatively regulate TLR4 trafficking to membrane lipid rafts by inhibiting ROS generation following ligand stimulation, and also to impair TLR4-MyD88 and TLR4-TRIF complex formation which represent signal initiating events. The observation that oxidative stress can also mediate the aggregation of TLR4 adaptor and signaling molecules represents another mechanism whereby Src inhibition may interfere with TLR4 surface up-regulation, as several studies have implicated important interactions between Src kinases and TLR4 signaling molecules such as TRAF6 [100, 101]. In those studies, Src and TRAF6 were shown to collaborate in mediating PI3K activation, a molecule which we previously implicated as part of the oxidant-primed LPS signaling cascade to NF-κB activation [28], and

also a candidate mediator of exocytosis possibly through effects on intracellular calcium stores [160-163].

Further studies were conducted to elucidate the upstream mechanisms by which oxidative stress might lead to lipid raft formation and orchestrate the recruitment of TLR4 through activated Src kinases. Investigation of the lipid ceramide, known to be generated in response to various stimuli including oxidative stress, and known to participate in lipid raft formation was performed. We found that oxidative stress led to a rapid doubling of ceramide by 10mins. This finding is consistent with a previous report in lung epithelial cells which found a 150% increase in ceramide generation after treatment with H_2O_2 (100uM) by 5 to 10mins [135]. We further demonstrated that this early peak was the result of sphingolipid degradation as two independent inhibitors of the degradation pathway, D609 and desipramine, inhibited oxidant-induced ceramide generation. The use of desipramine specifically suggested that oxidant-induced ceramide generation was occurring primarily through ASM as desipramine is specific for this enzyme [164].

The role of ceramide generation in oxidant-induced TLR4 translocation was assessed using both pharmacologic and genetic strategies for inhibiting the sphingolipid degradation pathway. D609, an inhibitor of the upstream enzyme PC-PLC, was found to completely block oxidant-induced TLR4 translocation by flow cytometry. Genetic deficiency of ASM was also found to inhibit oxidant-induced TLR4 translocation by immunofluorescence and flow cytometry. Since ASM deficiency confers a disorder of lysosomal lipid storage known as Newmann-Pick's disease, it was imperative to decipher whether the observed lack of TLR4 up-regulation on the surface of AMs from ASM deficient animals was due to the suggested lack of ceramide generation, rather than an inherent defect in some other cellular

process such as exocytosis. To this end, we found that direct treatment of ASM deficient cells with the synthetic ceramide analogue C2-ceramide completely re-established the TLR4 upregulation that was observed in ASM WT cells treated with C2. Together, these studies demonstrated that ceramide generated via ASM is not only necessary to mediate oxidant-induced TLR4 translocation, but it is also sufficient to mediate this upregulation.

Given the demonstrated role of Src kinases in oxidant-mediated TLR4 translocation, we next sought to establish whether ceramide might act upstream of Src in mediating this phenomenon. Using a molecular strategy of ASM inhibition, H_2O_2-induced early activation of Src family by 5mins was significantly inhibited in RAW cells transfected with ASMsiRNA, suggesting a role for ceramide in mediating Src activation. C2-ceramide added directly to RAW cells did recapitulate Src family kinase activation by 5mins as well. This finding is consistent with a study in colonic smooth muscle cells where ceramide-induced PI3K activation was found to be mediated by tyrosine phosphorylation of pp60src [165], and another study in RAW cells where C2-ceramide was found to cause Hck phosphorylation [166]. The involvement of ASM in mediating ceramide generation and downstream Src activation, however, is novel.

The emerging hierarchy between ceramide and Src was further tested. C2-induced TLR4 upregulation was assessed by flow cytometry with Src-inhibition via PP2 and in triple Src KO AMs. Both strategies revealed partial inhibition of C2-induced TLR4 translocation, suggesting that Src kinases are not the only mechanism underlying TLR4 recruitment to lipid rafts. Indeed, some studies have demonstrated that ceramide can play a role in regulating endocytic and exocytic vesicular processes through associations with Rab proteins [167]. Nevertheless, C2-induced TLR4 translocation was reduced by about half under Src inhibitory

conditions and served to reaffirm the apparent hierarchy involved in oxidant-induced TLR4 translocation.

Last, the role of Src and ceramide in oxidant-mediated translocation of TLR4 were further corroborated by *in vivo* experiments where the oxidative stress was delivered by the more physiologic method of hemorrhagic shock and resuscitation. These studies confirmed that S/R leads to ceramide generation and that S/R-induced upregulation of surface TLR4 seen by immunofluorescence and flow cytometry was inhibited by *in vivo* administration of D609, in ASM deficient mice, and in triple Src KO mice.

This study is the first to demonstrate that oxidant-induced TLR4 translocation in macrophages occurs in a Src-dependent manner. Furthermore, rapid oxidant-induced ceramide generation via ASM was shown to have a central upstream role as both necessary and sufficient to mediate TLR4 translocation to the plasma membrane in a Src-dependent manner. Using both *in vitro* and *in vivo* models of oxidative stress, this study identifies a hierarchy of signaling molecules following oxidative stress that might represent novel targets for therapy in critical illness and organ injury.

FIGURES

Figure 1. H_2O_2-induced TLR4 translocation in macrophages is Src-dependent. TLR4 surface expression was assessed by flow cytometry on non-permeabilized cells and is reported as mean channel fluorescence (MCF) normalized to control. Three corroborative methods of Src inhibition were used: **A)** Pharmacologic inhibition of Src kinases was achieved by treating serum-starved RAW 264.7 macrophages with H_2O_2 (100uM) *in vitro* for 0-60mins +/- the Src inhibitor PP2 (10uM). Data bars represent mean +/- SEM of 3 experiments per group and * indicates $p<0.05$ compared to control and PP2+ H_2O_2. **B)** Molecular Src inhibition was achieved by using RAW 264.7 macrophages stably transfected with a Csk-expressing plasmid. Wild type and Csk-transfected RAW cells were then stimulated with H_2O_2 (100uM) *in vitro* for 0-60mins (n=3). **C)** Genetic Src inhibition was achieved by recovering alveolar macrophages (AMs) from C57BL6 wild type (WT) and triple Src knockout (KO) mice ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) by bronchoalveolar lavage and treating them with H_2O_2 (300uM) *in vitro* for 0-60mins. Data bars represent mean +/- SEM of 4 experiments per group.

Fig 1A

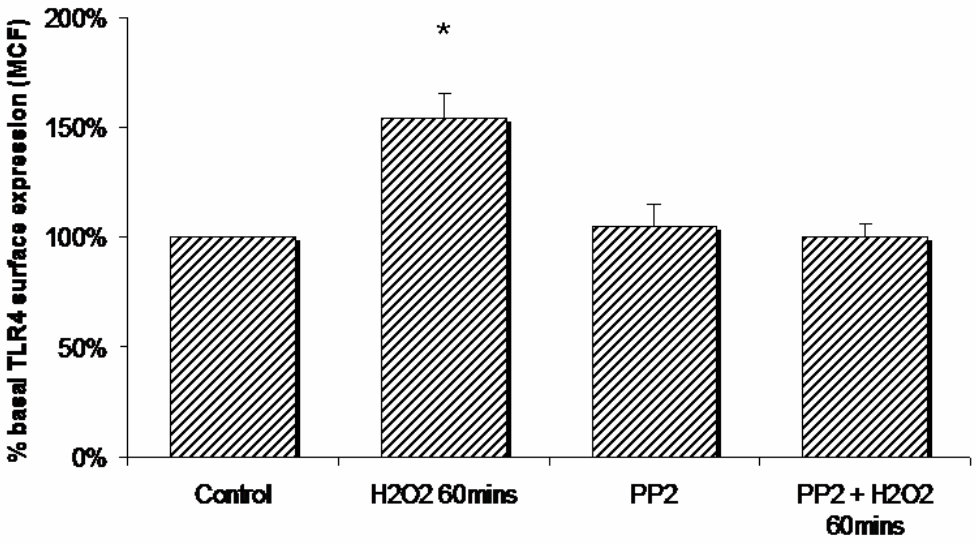

% basal TLR4 surface expression (MCF)
200%
150%
100%
50%
0%
*
Control
H2O2 60mins
PP2
PP2 + H2O2 60mins

Fig 1B

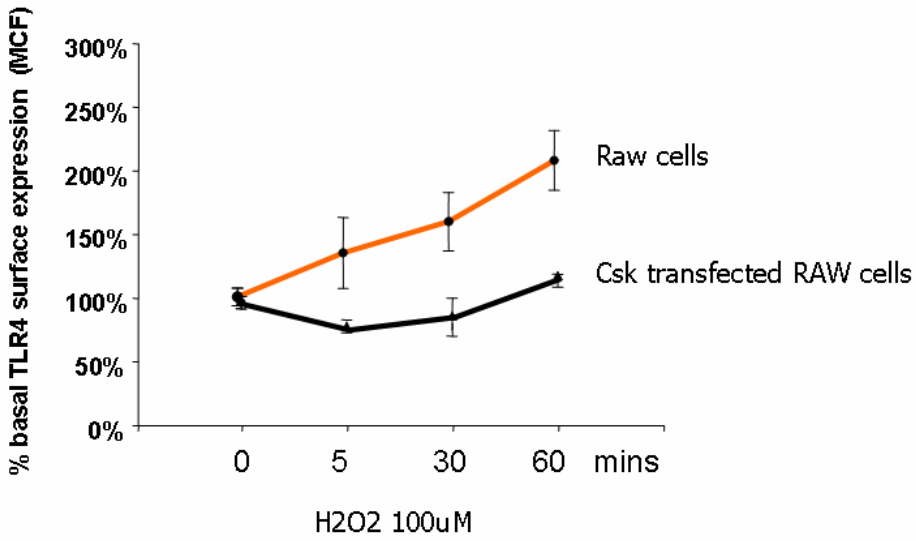

% basal TLR4 surface expression (MCF)
300%
250%
200%
150%
100%
50%
0%
Raw cells
Csk transfected RAW cells
0
5
30
60
mins
H2O2 100uM

Fig 1C

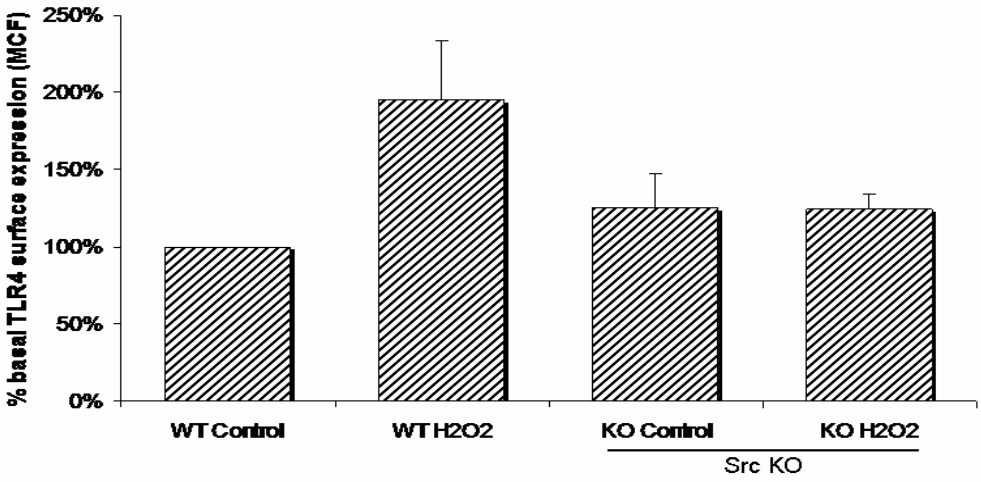

% basal TLR4 surface expression (MCF)
250%
200%
150%
100%
50%
0%
WT Control
WT H2O2
KO Control
KO H2O2
Src KO

Figure 2. H_2O_2-induced ceramide generation in macrophages. A) Serum starved RAW 264.7 cells were stimulated with H_2O_2 (300uM) *in vitro* for 0-30mins. Cellular lipids were extracted according to the Bligh and Dyer method and then subjected to diglyceride kinase reaction. The radio-labeled ^{32}P-ceramide product was visualized by Thin Layer Chromatography (TLC) and autoradiography. A representative blot is shown. **B)** The blots were analyzed by densitometry and H_2O_2-induced ceramide generation was calculated in pmol based on the corresponding standard curve. Ceramide generation was found to be greatest at 10mins with 197% increase over basal (n=3 and * indicates $p<0.05$ compared to control). **C)** The H_2O_2-induced ceramide generation peak at 10mins (197%) was reduced to 129% and 97% over basal when pretreated with D609 (100uM) or desipramine (5uM) for 15mins, respectively. Data bars represent mean +/- SEM of 3 experiments per group, and 2 experiments per group with inhibitors.

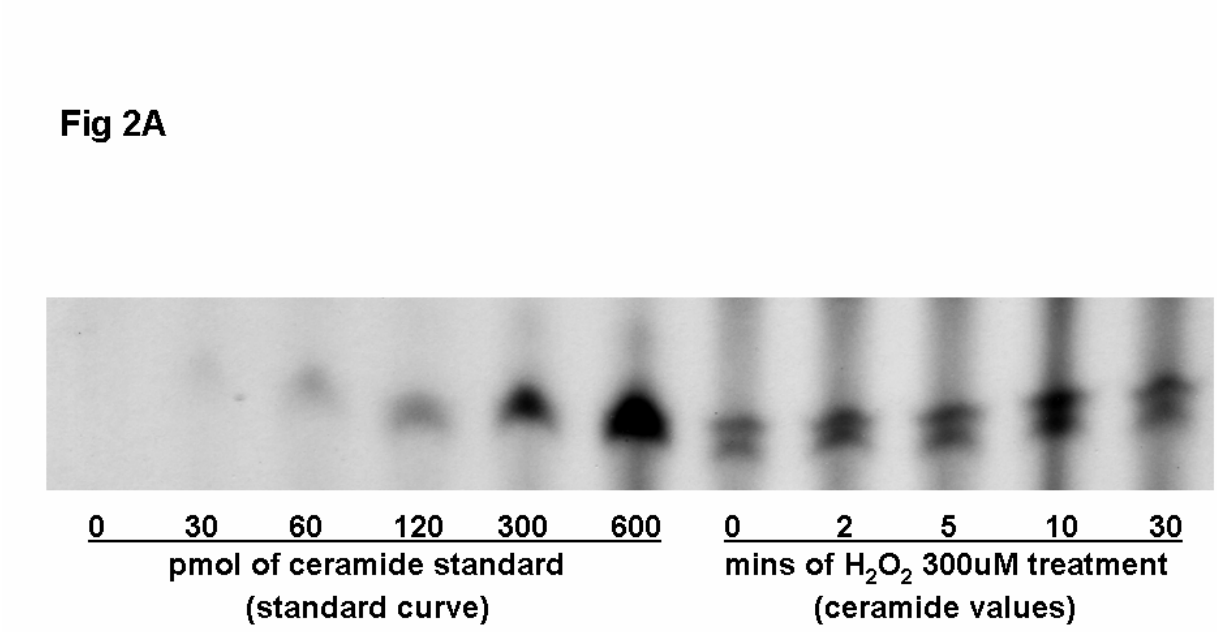


Fig 2B

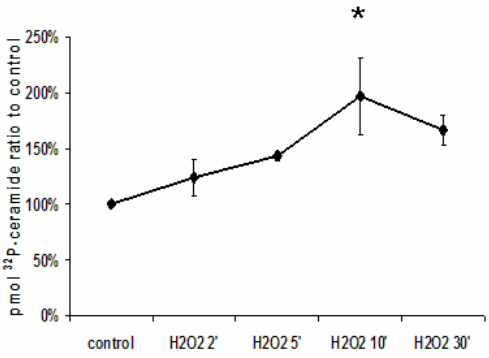

*
250%
200%
150%
100%
50%
0%
pmol 32P-ceramide ratio to control
control
H2O2 2'
H2O2 5'
H2O2 10'
H2O2 30'

Fig 2C

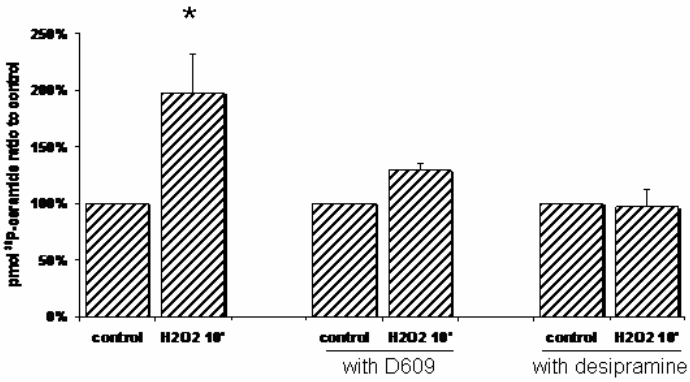

*
250%
200%
150%
100%
50%
0%
pmol 32P-ceramide ratio to control
control
H2O2 10'
control
H2O2 10'
with D609
control
H2O2 10'
with desipramine

Figure 3. H_2O_2-induced TLR4 translocation in macrophages is ceramide-dependent. The role of ceramide was assessed by using pharmacologic and genetic inhibitory strategies along the sphingolipid degradation pathway. **A)** Alveolar macrophages (AMs) from C57BL6 mice were recovered by bronchoalveolar lavage and stimulated with H_2O_2 (300uM) *in vitro* for 0-60mins +/- D609 (100uM). TLR4 surface expression was assessed by flow cytometry on non-permeabilized cells and is reported as mean channel fluorescence (MCF) normalized to control. Data bars represent mean +/- SEM of 3 experiments per group and * indicates $p<0.05$ compared to control and H_2O_2+D609. **B)** Alveolar macrophages (AMs) from ASM wild type (WT) and ASM knockout (KO) mice were recovered by bronchoalveolar lavage and stimulated with H_2O_2 (300uM) *in vitro* for 0-60mins. Immunofluorescence staining for TLR4 expression in permeabilized cells was performed and representative images are shown. **C)** TLR4 surface expression on non-permeabilized ASM WT and KO AMs under the same conditions was also assessed by flow cytometry and is reported as mean channel fluorescence (MCF) normalized to control. Data bars represent mean +/- SEM of 3 experiments per group.

Fig 3A

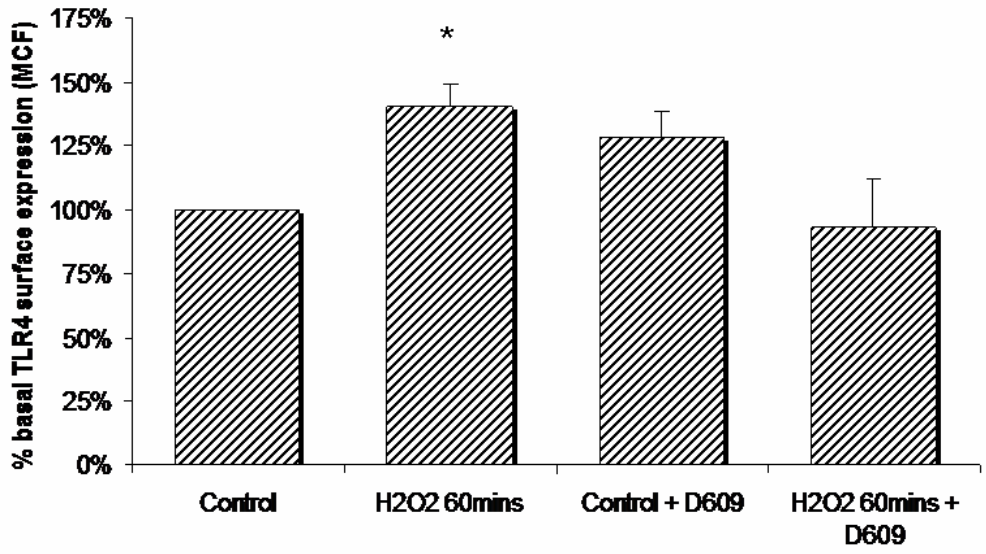

% basal TLR4 surface expression (MCF)
175%
150%
125%
100%
75%
50%
25%
0%
*
Control
H2O2 60mins
Control + D609
H2O2 60mins + D609

Fig 3B

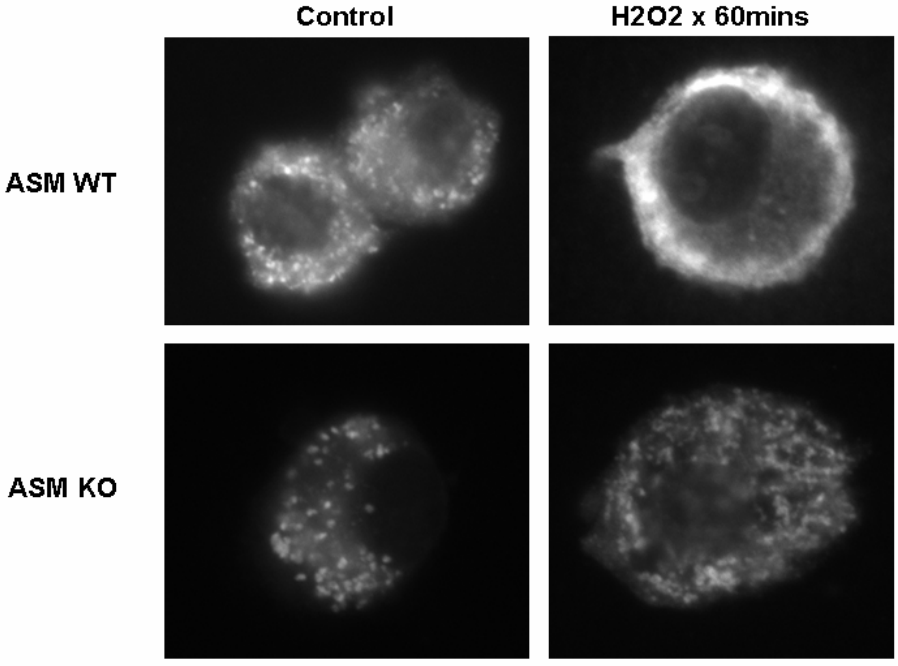

Control
H2O2 x 60mins
ASM WT
ASM KO

Fig 3C

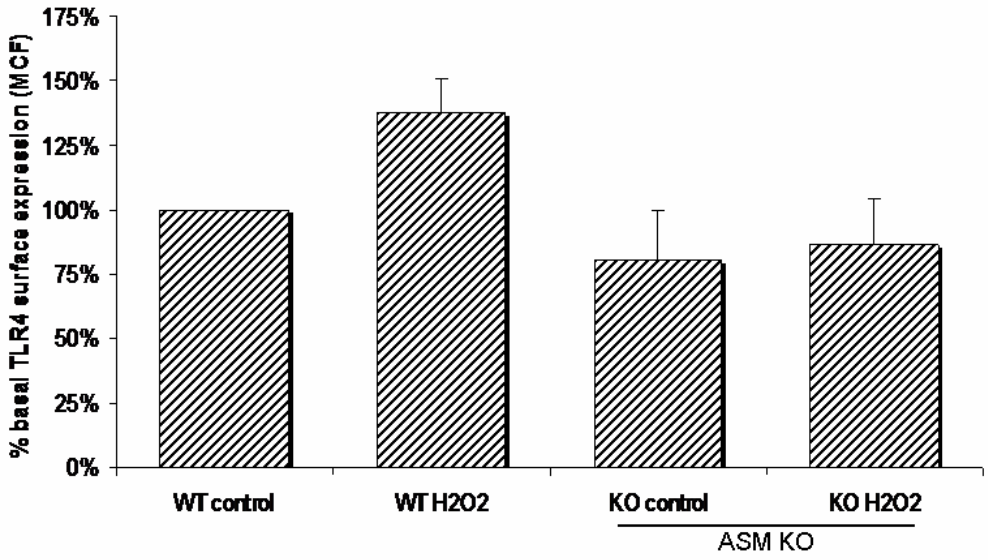

% basal TLR4 surface expression (MCF)
175%
150%
125%
100%
75%
50%
25%
0%
WT control
WT H2O2
KO control
KO H2O2
ASM KO

Figure 4. C2-induced TLR4 translocation in macrophages. A) Serum-starved RAW 264.7 macrophages were treated with C2-ceramide (200uM) for 0-30mins *in vitro*. Immunofluorescence staining for TLR4 expression in permeabilized cells was performed and representative images are shown. **B)** TLR4 surface expression was also assessed by flow cytometry on non-permeabilized cells and is reported as mean channel fluorescence (MCF) normalized to control. **C)** Alveolar macrophages (AMs) from ASM wild type (WT) and ASM knockout (KO) mice were recovered by bronchoalveolar lavage and treated with C2-ceramide (200uM) for 0-60mins *in vitro*. TLR4 surface expression was assessed by flow cytometry on non-permeabilized cells and is reported as mean channel fluorescence (MCF) normalized to WT control. Data bars represent mean +/- SEM of 3 experiments per group, and * indicate $p<0.05$ compared to respective controls.

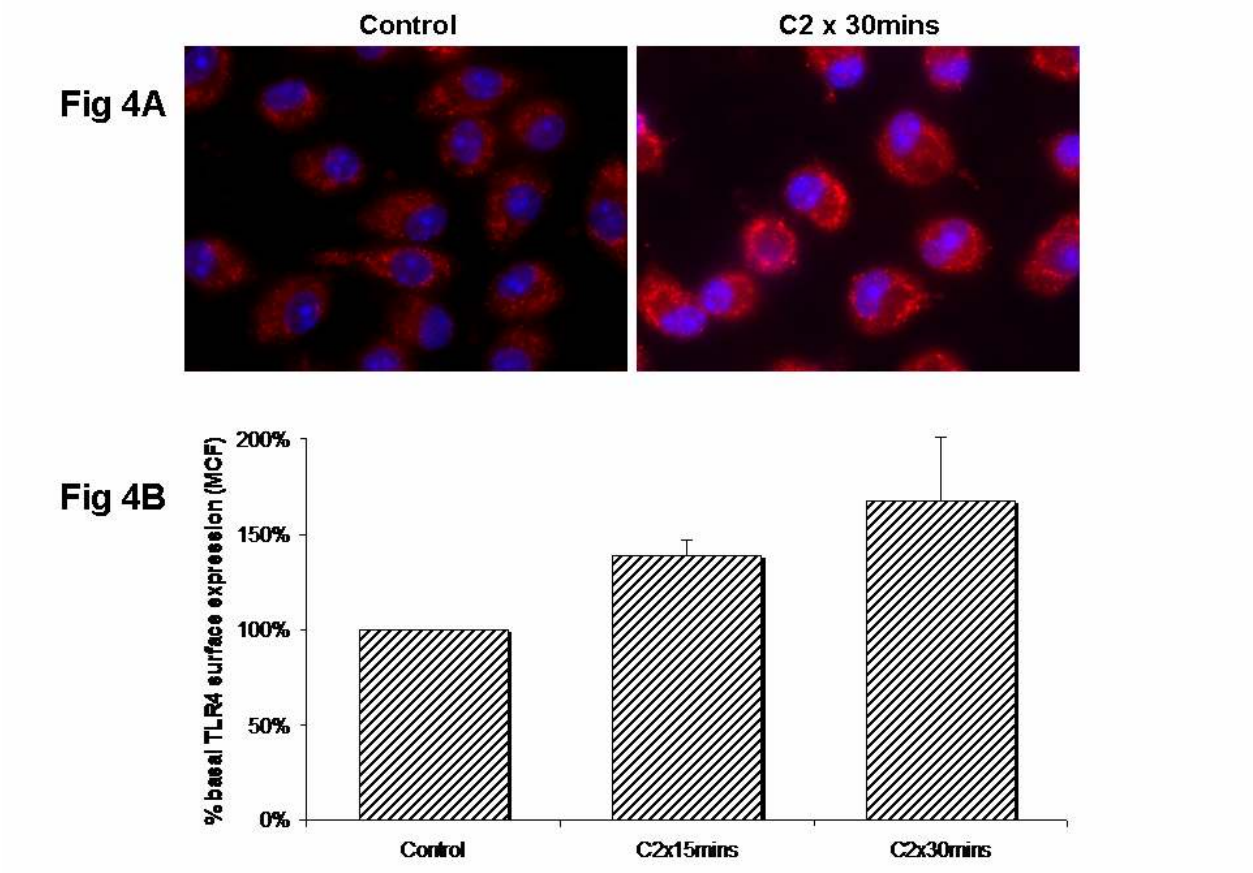


Fig 4C

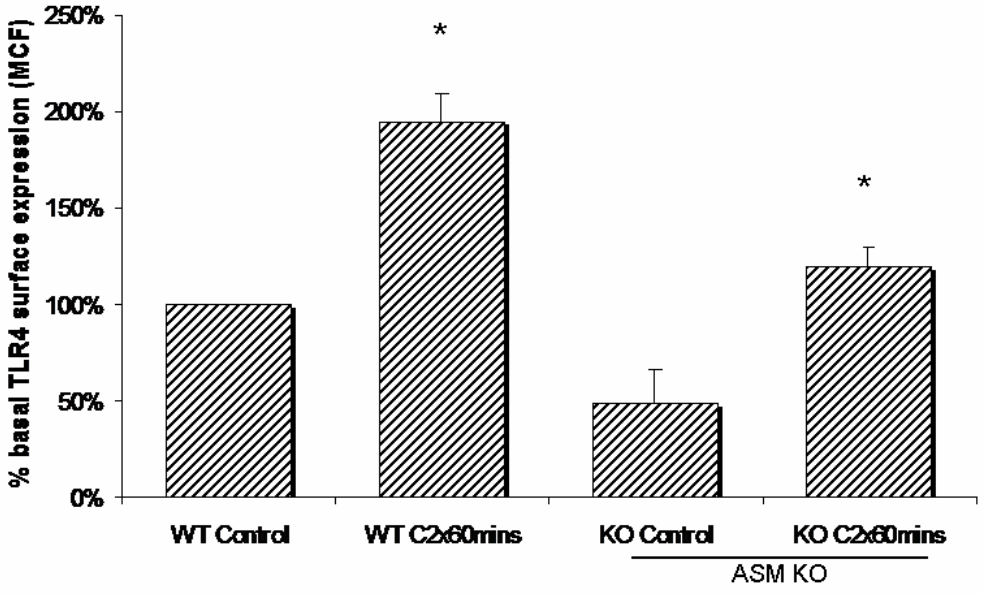
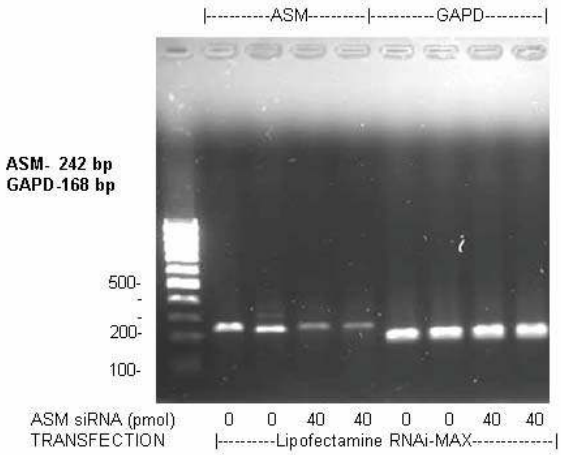

% basal TLR4 surface expression (MCF)
250%
200%
150%
100%
50%
0%
*
*
WT Control
WT C2x60mins
KO Control
KO C2x60mins
ASM KO

Figure 5. H_2O_2-induced activation of Src kinases in RAW cells is ceramide dependent. **A)** ASM knockdown was achieved *in vitro* by transfecting RAW 264.7 macrophages with ASM siRNA (40pmol) for 24hrs using Lipofectamine RNAi-MAX. To verify knockdown efficiency, ASM mRNA in RAW cells was measured in duplicate by PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPD). ASM siRNA transfection revealed a 70% reduction in ASM mRNA after 24hrs compared to control. A scrambled negative siRNA was also tested and did not cause any reduction in ASM mRNA (data not shown). **B)** Using this system of molecular ASM inhibition, RAW cells were stimulated with H_2O_2 (300uM) for 0-15mins. Western Blot analysis was performed using a phospho-Src family antibody (Y416). A representative blot is shown. Densitometry was calculated as a ratio of pSrc over Akt loading control, and normalized to the neg siRNA control (n=2). **C)** RAW cells were treated directly with C2-ceramide (100uM) for 0-15mins. Western Blot analysis was performed using a phospho-Src family antibody (Tyr 416). Representative blots from 4 experiments are shown.

Fig 5A


|----------ASM---------|----------GAPD---------|
ASM- 242 bp
GAPD-168 bp
500-
200-
100-
ASM siRNA (pmol) 0 0 40 40 0 0 40 40
TRANSFECTION |----------Lipofectamine RNAi-MAX----------|

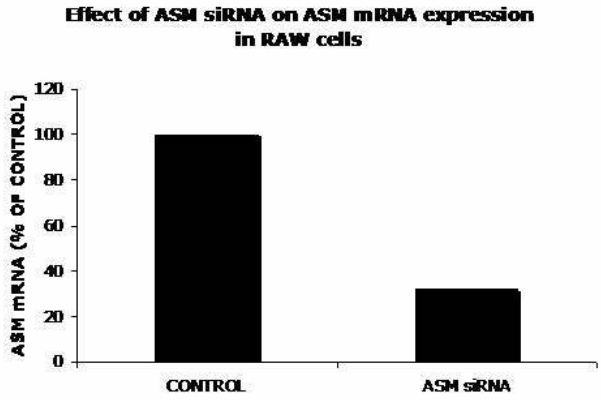
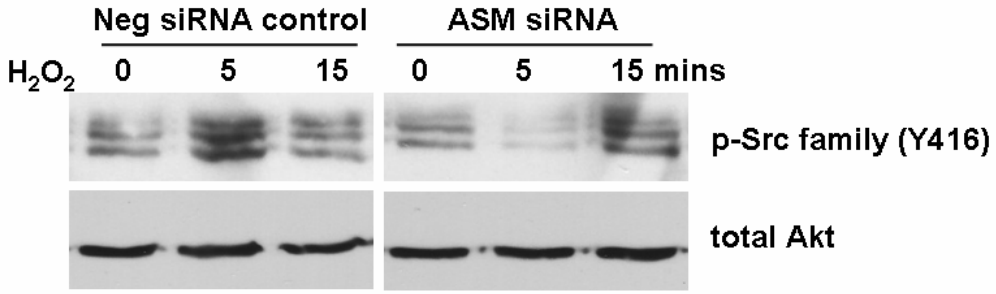

Effect of ASM siRNA on ASM mRNA expression in RAW cells
ASM mRNA (% OF CONTROL)
120
100
80
60
40
20
0
CONTROL
ASM siRNA

Fig 5B


Neg siRNA control
ASM siRNA
H2O2 0 5 15 0 5 15 mins
p-Src family (Y416)
total Akt

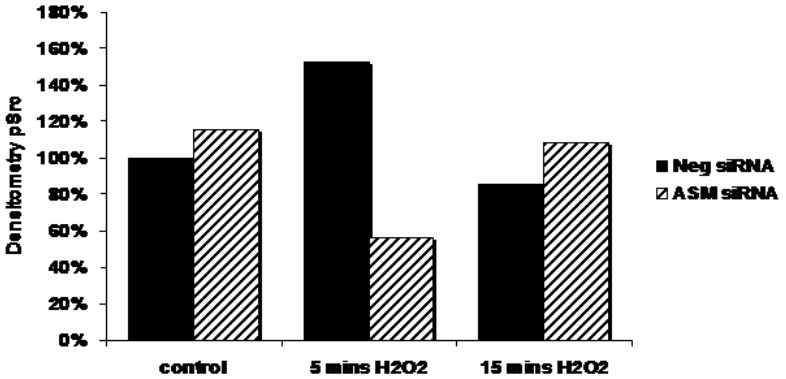

Densitometry pSrc
180%
160%
140%
120%
100%
80%
60%
40%
20%
0%
control
5 mins H2O2
15 mins H2O2
Neg siRNA
ASM siRNA

Fig 5C

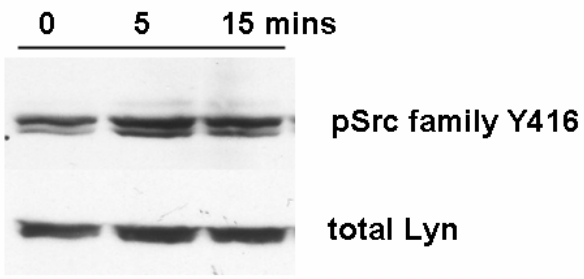


Figure 6. C2-induced TLR4 translocation in macrophages is Src dependent. TLR4 surface expression was assessed by flow cytometry on non-permeabilized cells and is reported as mean channel fluorescence (MCF) normalized to control. **A)** Serum-starved RAW 264.7 macrophages were treated with C2-ceramide (200uM) for 0-30mins +/- PP2 (10uM) *in vitro*. As a negative control, the inactive analogue of C2-ceramide, dihydroceramide (200uM), was used in parallel and did not cause any change in TLR4 surface expression (data not shown). **B)** Alveolar macrophages (AMs) from C57BL6 wild type (WT) and triple Src knockout (KO) mice ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) were recovered by bronchoalveolar lavage and treated with C2-ceramide (200uM) for 0-30mins *in vitro*. Data bars represent mean +/- SEM of 3 experiments per group.

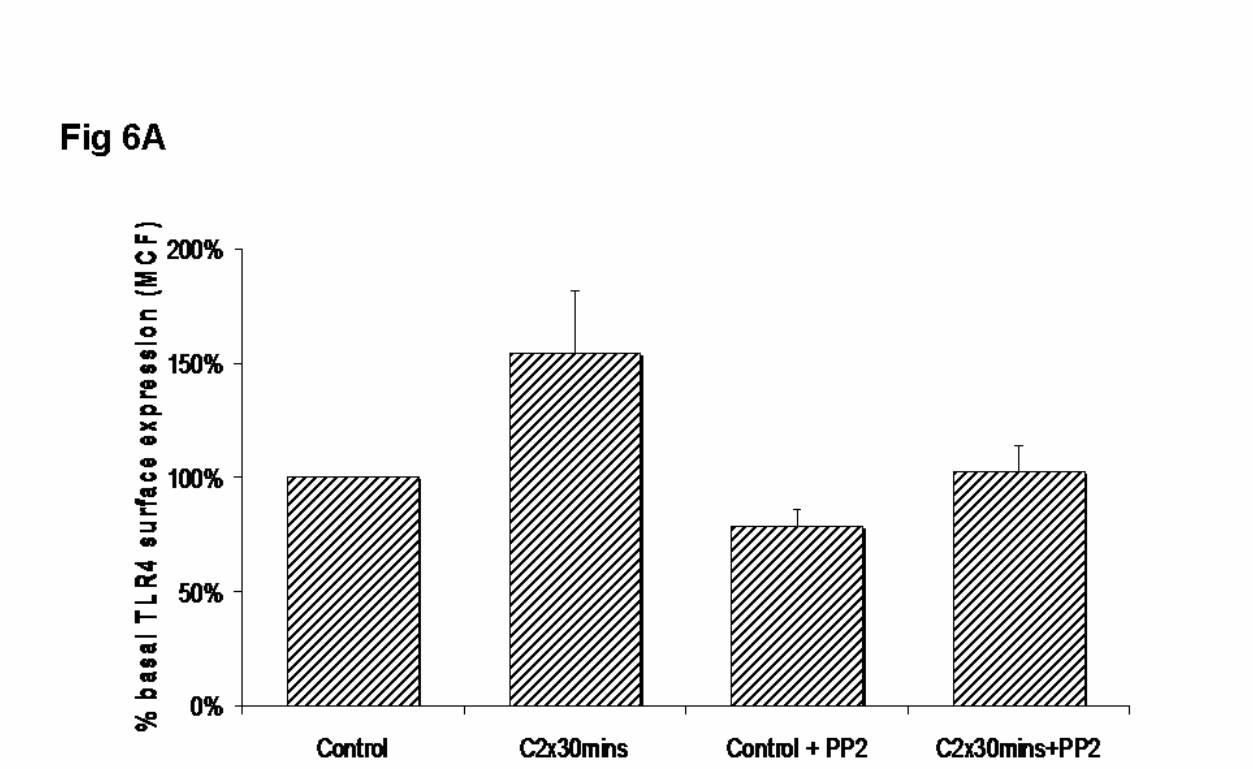


Fig 6B

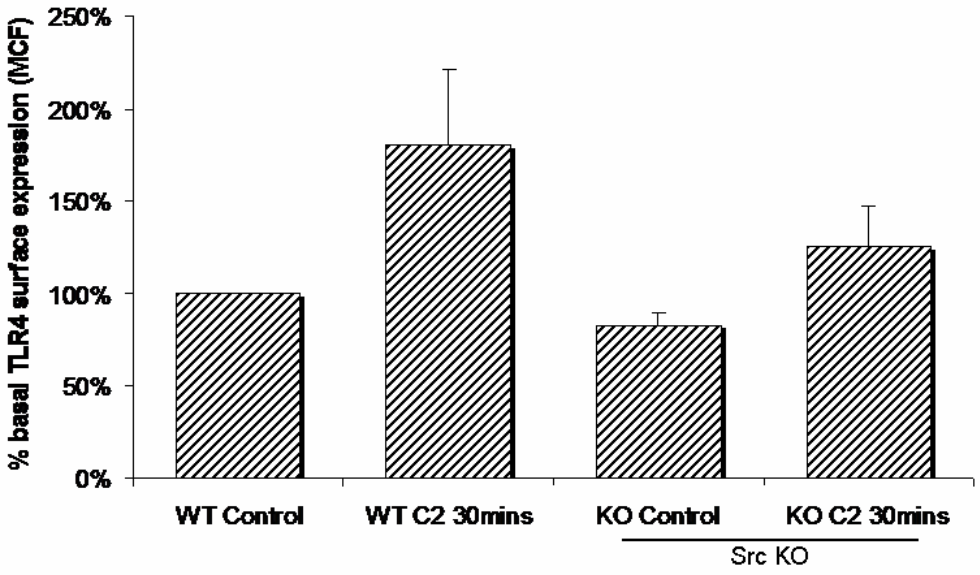

% basal TLR4 surface expression (MCF)
250%
200%
150%
100%
50%
0%
WT Control
WT C2 30mins
KO Control
KO C2 30mins
Src KO

Figure 7. S/R causes ceramide generation in lung alveolar macrophages. C57BL6 mice were subjected to hemorrhagic shock for 60mins followed by resuscitation (S/R). Their AMs were then recovered by BAL and analyzed for ceramide generation (as in figure 2) compared to sham operated animals.

Fig 7

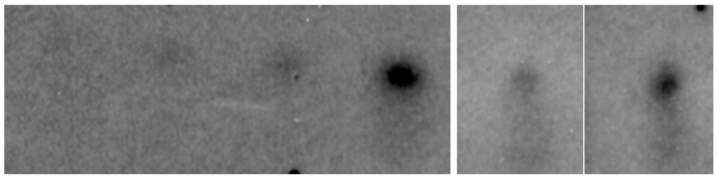


Figure 8. S/R-induced TLR4 translocation is inhibited by D609 *in vivo*. C57BL/6 mice were subjected to hemorrhagic shock for 60mins followed by resuscitation (S/R) +/- D609 (40mg/kg) *in vivo*. Alveolar macrophages were then recovered by bronchoalveolar lavage. **A)** Immunofluorescence staining for TLR4 expression in permeabilized cells was performed and representative images are shown. **B)** TLR4 surface expression was assessed by flow cytometry on non-permeabilized cells and is reported as mean channel fluorescence (MCF) normalized to control. Data bars represent mean +/- SEM of 3 experiments per group.

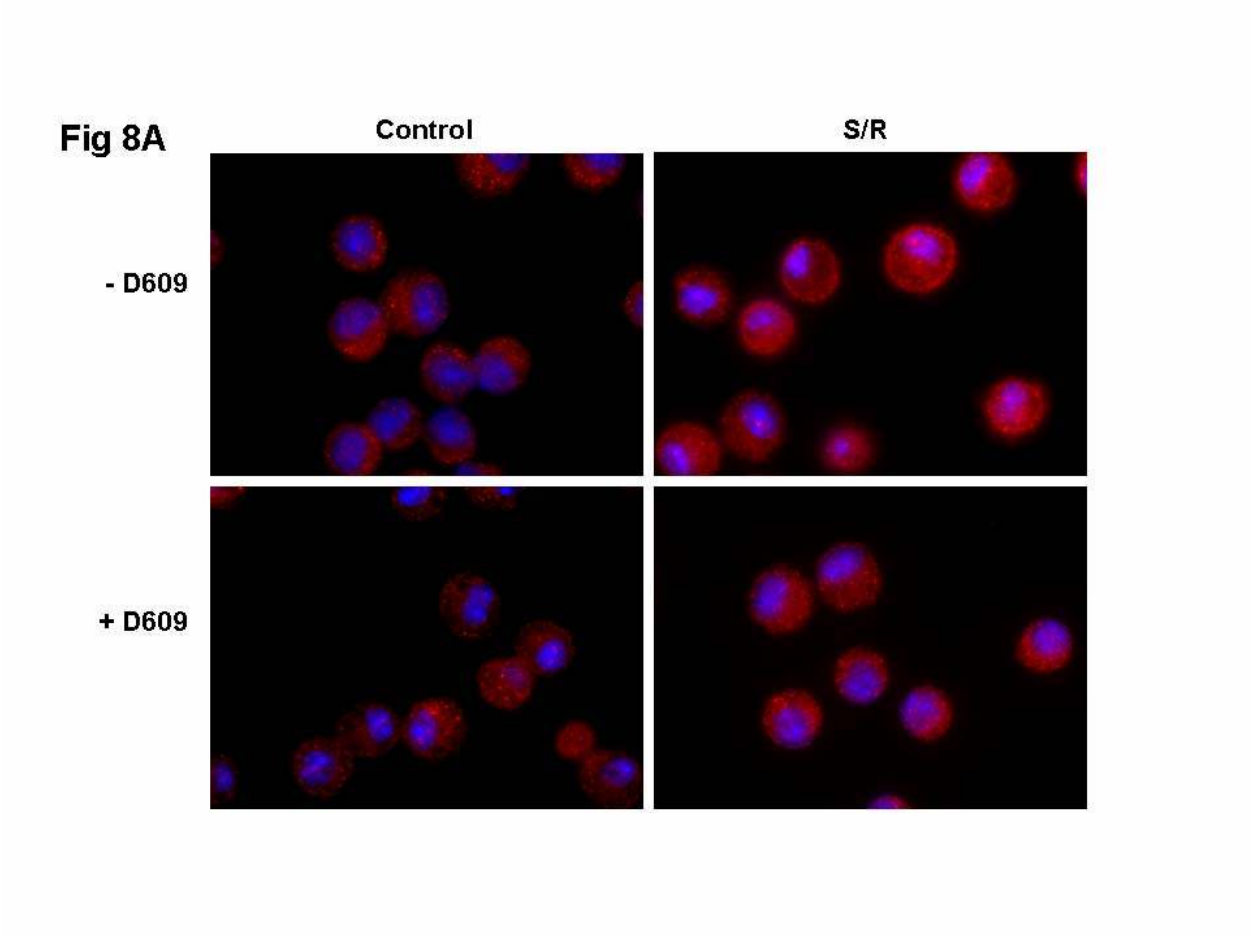


Fig 8B

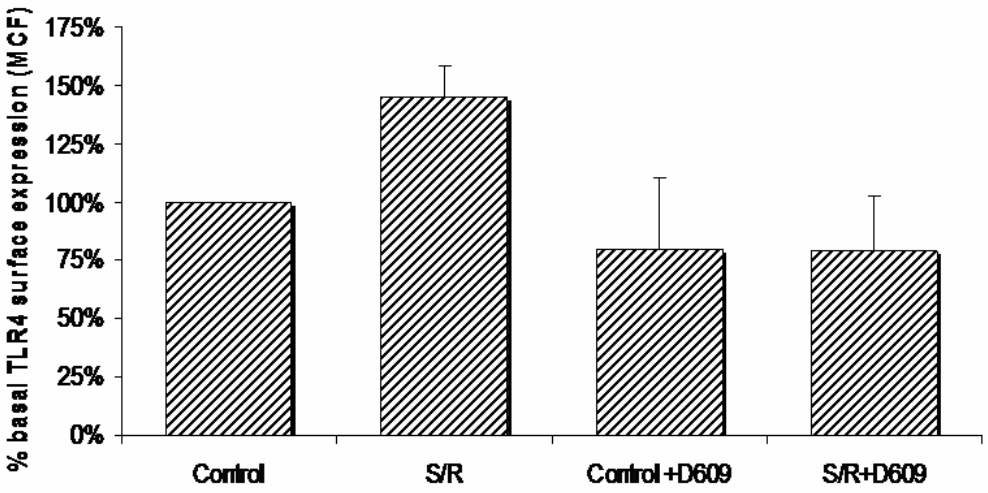

% basal TLR4 surface expression (MCF)
175%
150%
125%
100%
75%
50%
25%
0%
Control
S/R
Control+D609
S/R+D609

Figure 9. S/R-induced TLR4 translocation is inhibited in ASM KO mice *in vivo*. ASM WT and ASM KO mice were subjected to hemorrhagic shock for 60mins followed by resuscitation (S/R). Alveolar macrophages were then recovered by bronchoalveolar lavage. **A)** Immuno-fluorescence staining for TLR4 expression in permeabilized cells was performed and representative images are shown. **B)** TLR4 surface expression was assessed by flow cytometry on non-permeabilized cells and is reported as mean channel fluorescence (MCF) normalized to control. Data bars represent mean +/- SEM of 3 experiments per group.

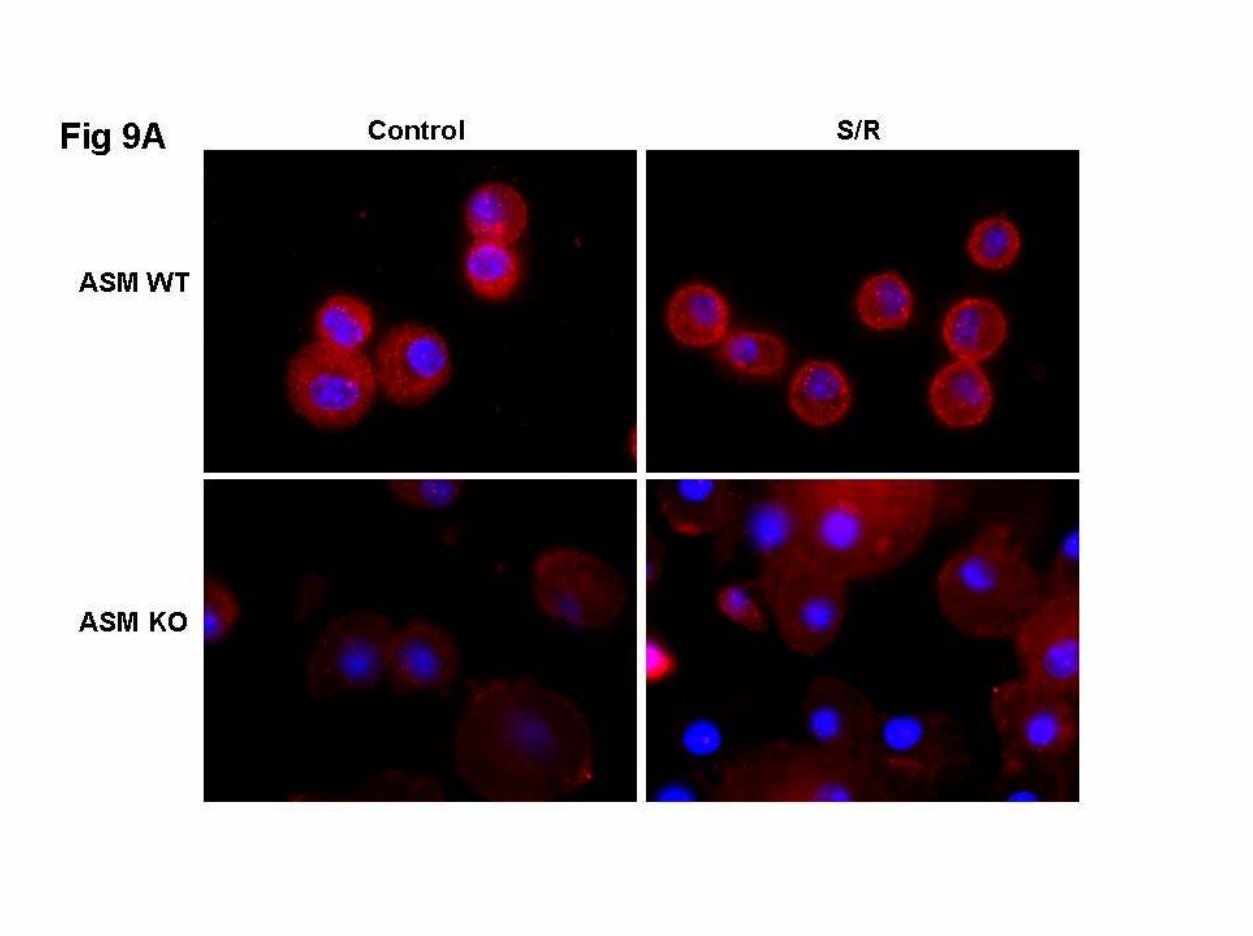


Fig 9B

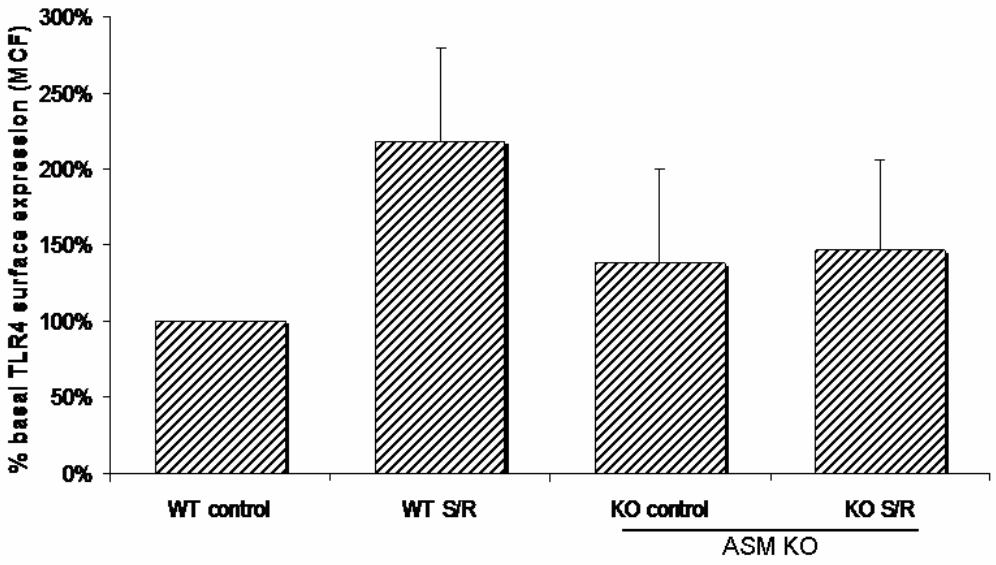

% basal TLR4 surface expression (MCF)
300%
250%
200%
150%
100%
50%
0%
WT control
WT S/R
KO control
KO S/R
ASM KO

Figure 10. S/R-induced TLR4 translocation is inhibited in Src KO mice *in vivo*. WT and triple Src knockout (KO) mice ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) were subjected to hemorrhagic shock for 60mins followed by resuscitation (S/R). Alveolar macrophages were then recovered by bronchoalveolar lavage. **A)** Immuno-fluorescence staining for TLR4 expression in permeabilized cells was performed and representative images are shown. **B)** TLR4 surface expression was assessed by flow cytometry on non-permeabilized cells and is reported as mean channel fluorescence (MCF) normalized to control. Data bars represent mean +/- SEM of 4 experiments per group and * indicates $p<0.05$ compared to control and KO S/R.

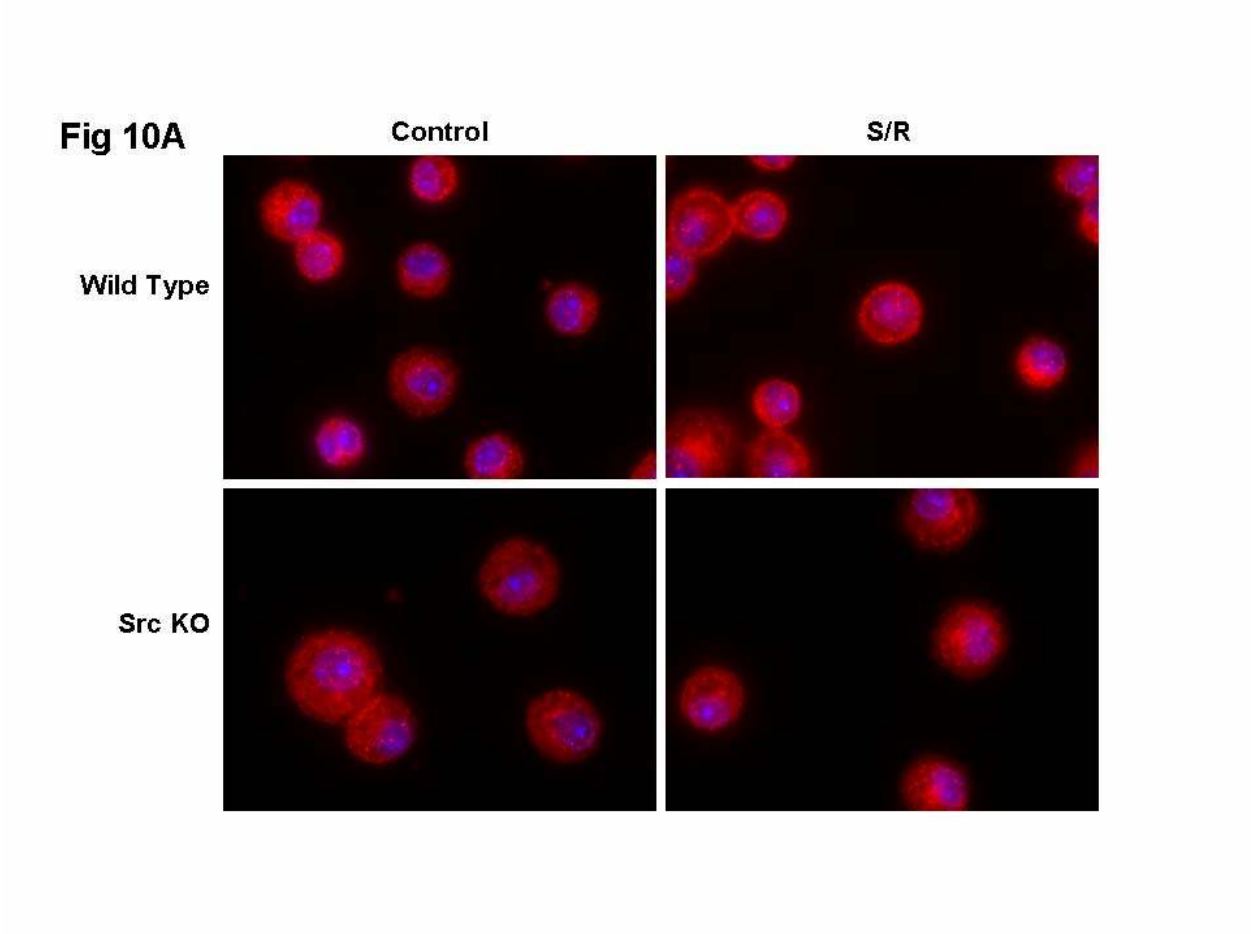
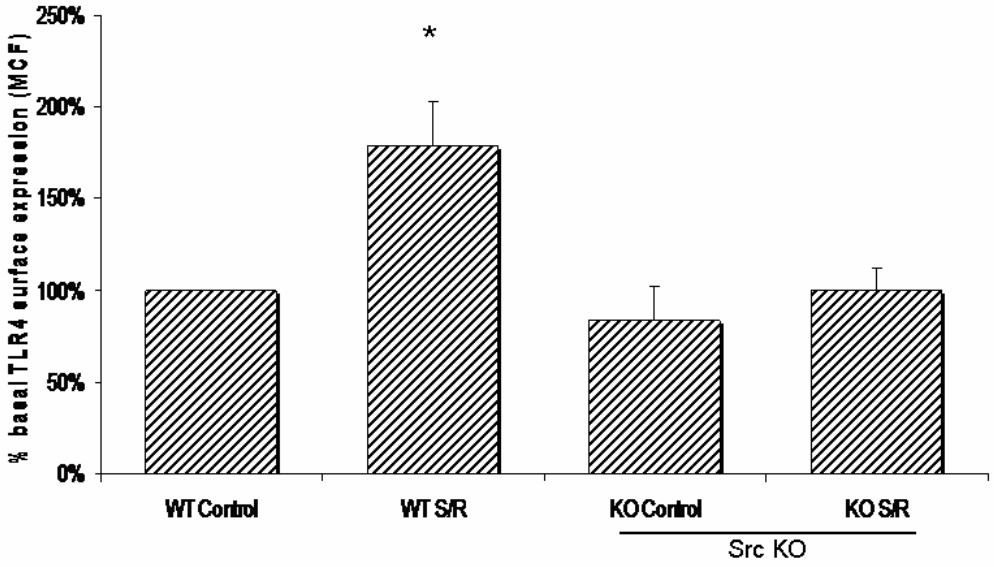


Fig 10B


% basal TLR4 surface expression (MCF)
250%
200%
150%
100%
50%
0%
*
WT Control
WT S/R
KO Control
KO S/R
Src KO

CHAPTER 3

Oxidant priming of macrophages for increased LPS responsiveness is mediated by ceramide in a src-dependent manner.

Manuscript not yet submitted for publication

ABSTRACT

Oxidative stress generated during shock/resuscitation contributes to tissue injury by augmenting cellular responsiveness to subsequent inflammatory stimuli, such as lipo-polysaccharide (LPS). Previous work by our group has shown that priming of macrophages by oxidative stress augments the pro-inflammatory response by causing earlier and greater NF-κB nuclear translocation following LPS exposure. Oxidant priming was also shown to cause re-programming of subsequent LPS signaling to a Src and phosphatidylinositol 3-kinase (PI3K) dependent pathway. The mechanism by which oxidative stress accomplishes these priming effects remains poorly elucidated. The lipid ceramide has emerged as a prominent molecule in inflammatory signaling pathways and a structural component of membrane lipid rafts. We hypothesized that oxidative stress might exert its priming effects on LPS signaling through the action of ceramide. In the present studies, oxidant re-programming of LPS signaling to a Src-dependent pathway was confirmed using macrophages from triple Src knockout mice ($hck^{-/-}fgr^{-/-}lyn^{-/-}$). Further studies *in vitro* using RAW 264.7 cells revealed that H_2O_2 causes activation of the PI3K/Akt pathway in a Src-dependent fashion. Using pharmacologic inhibitors of sphingolipid degradation (D609 and desipramine), a molecular siRNA knockdown strategy to inhibit acid sphingomyelinase (ASM), and macrophages from ASM knockout mice, we found that ceramide generation via ASM is a critical upstream regulator of H_2O_2-induced activation of Src and PI3K/Akt. Interestingly, ceramide inhibition by D609 translated into near complete inhibition of LPS-induced NF-κB translocation following oxidant priming. Together, these findings demonstrate that ceramide is a central upstream mediator of oxidant priming in macrophages,

and highlight a hierarchy of signaling molecules triggered by oxidative stress that might represent novel targets for therapy in critical illness and organ injury.

INTRODUCTION

Civilian trauma continues to be a significant health care problem in North American society [3] [4]. Studies have shown that the global ischemia/reperfusion resulting from resuscitated hemorrhagic shock can prime the innate immune system for an exaggerated response to a subsequent otherwise innocuous inflammatory stimulus, resulting in multiple organ failure or death [8, 9]. The synergistic effect of sequential stimuli has been coined the "two-hit model" for the development of organ injury in trauma patients [16, 17]. Several groups, including our own, have modeled this two-hit phenomenon *in vivo* and *in vitro*. Using a rodent model of LPS-induced lung injury, we previously demonstrated that antecedent shock/resuscitation (S/R) leads to augmented LPS-induced lung injury by way of heightened NF-κB nuclear translocation and increased transcription of pro-inflammatory genes in alveolar macrophages (AMs). Further studies revealed that oxidative stress generated during ischemia/reperfusion is responsible for the priming phenomenon, as various antioxidant strategies such as the use of N-acetylcysteine (NAC) or stylbazulenyl nitrone (STAZN) administered during the resuscitation phase were markedly effective in inhibiting the augmented lung injury caused by sequential S/R and LPS [21, 25, 26]. The mechanisms underlying the ability of oxidative stress to prime macrophages for increased LPS responsiveness remain the focus of much investigation. Our group has recently identified two significant alterations to LPS signaling under oxidative stress conditions in macrophages: 1) the rapid recruitment of the LPS receptor Toll-like receptor 4 (TLR4) to membrane lipid rafts [76], and 2) the reprogramming of LPS signaling to a Src-dependent pathway involving phosphatidylinositol 3-kinase (PI3K) [28].

The Src family of tyrosine kinases is involved in many signal transduction pathways and is known to be activated by oxidative stress [82]. However, it has been shown using hck$^{-/-}$fgr$^{-/-}$lyn$^{-/-}$ triple knockout mice that Src kinases are not obligatory for LPS-induced signal transduction in macrophages to induce NF-κB nuclear translocation and generation of TNFα, interleukin IL-1, and IL-6 [28, 90, 91]. While the mechanism by which oxidative stress redirects LPS signaling to a Src-dependent pathway remains unknown, several lines of evidence suggest that there may be a relationship between oxidant-induced Src activation and generation of the lipid ceramide.

The sphingolipid ceramide is a powerful second messenger molecule in a variety of inflammatory signaling pathways including oxidative stress, and an important structural component of membrane signaling platforms known as lipid rafts [114, 115, 118, 125]. Ceramide can be generated by *de novo* synthesis from serine and palmitoyl CoA via ceramide synthase, or by rapid degradation from complex sphingolipids that are converted to sphingomyelin by phosphatidylcholine-phospholipase C (PC-PLC), and subsequently hydrolyzed to ceramide via one of five sphingomyelinases. The latter mechanism of ceramide generation is favored upon stress stimuli such as heat, UV light, or oxidative stress, and the acid sphingomyelinase (ASM) in particular has emerged as the mediator of ceramide generation in various models of ischemia-reperfusion injury [117, 152] and lung injury [116]. Furthermore, a study in colonic smooth muscle cells demonstrated that ceramide can induce PI3K activation via tyrosine phosphorylation of a Src kinase, pp60src [165], and another study in RAW cells showed that C2-ceramide causes phosphorylation of Hck, a member of the Src kinase family [166]. Taken together, we hypothesized that oxidative stress might re-program LPS signaling to a Src-dependent pathway through the action of ceramide.

In the present studies, using pharmacologic, molecular and genetic strategies of Src, ceramide and PI3K inhibition, oxidative stress was shown to redirect LPS-induced NF-κB nuclear translocation in macrophages to a ceramide-dependent pathway. Furthermore, oxidant-induced ceramide generation was mediated predominantly through ASM, and was found to be a critical upstream activator of Src kinases and the PI3K/Akt pathway. Taken together, this study identifies ceramide as a potential molecular switch for LPS signaling following oxidative stress, and highlights a hierarchy of signaling molecules triggered by oxidant priming that might represent novel targets for therapy in critical illness and organ injury.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide from E. coli O111:B4 was purchased from Sigma Aldrich Co. (Oakville, ON). C2-ceramide was from Avanti Lipids Inc. (Alabaster, AL). Sphingosine-1-phosphate was from Calbiochem, Inc. (San Diego, CA). For pharmacological studies the following inhibitors were used: D609 and wortmannin (Biomol International Plymouth Meeting, PA), desipramine and SKI (Sigma), DMS and PP2 (Calbiochem).

Cell culture and activation

The RAW 264.7 murine macrophage cell line was purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Burlington, ON) with 10% endotoxin-free fetal calf serum (FCS, Hyclone Lab, Logan, UT) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2. RAW cells were starved in serum-free conditions for at least 2 hr prior to treatments. Experiments with H_2O_2 (100-300 μM) were performed in Hank's Balanced Salt Solution (HBSS, Gibco), those with LPS (0.1 μg/mL) were performed in DMEM with 2% fetal calf serum, while those with C2-ceramide (100-200 μM) were performed in serum-free DMEM. Where pharmacologic inhibitors were used, cells were pre-incubated with the inhibitor for 15 min prior to treatment.

Immunofluorescence microscopy

Cells plated on glass coverslips were fixed with 4% paraformaldehyde for 30 min, then permeabilized in phosphate buffered saline (PBS, Gibco) solution containing 100 mM glycine and 0.2% Triton X-100 for 20 min. Coverslips were blocked with 5% bovine serum albumin in PBS for 1 hr, incubated with anti-p65 subunit antibody (Santa Cruz

Biotechnology, Santa Cruz, CA) for 1 hr followed by a Cy3-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were stained using DAPI (Molecular Probes Eugene, OR) or Hoescht. Samples were analyzed by an Olympus IX81 microscope (60x or 100x objectives, Melville, NY) coupled to an Evolution QEi Monochrome camera controlled by the QED InVivo Imaging software (Media Cybernetics, Silver Spring, MD). Images were processed by the ImagePro Plus 3DS 5.1 software (Media Cybernetics).

Western blotting

Proteins were detected using Western blotting as described previously [28]. Briefly, following treatment cells were lysed in Triton-based lysis buffer (Cell Signaling Technologies, Danvers, MA) and equal amount of protein was subjected to SDS-PAGE. Membranes were incubated with phospho-Akt (S473), phospho-Tyr p85 PI3K binding motif or phospho-Src family (Y416) antibodies (Cell Signaling). To verify equal loading, membranes were re-probed with Akt or Lyn antibodies (Cell Signaling). Bands were visualized by Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech).

siRNA transfections

Sparsely plated RAW cells were transfected with 40 pmols of ASM siRNA construct (sc-41651, Santa Cruz) or a negative control siRNA (Ambion, Austin, TX) with Lipofectamine RNAiMax (Invitrogen Carlsbad, CA). ASM knockdown was verified 24 hr later by RT-PCR (BioRad iQ5 iCycler, BioRad Laboratories, Hercules, CA), using the following primers: sense 5'-ACAGTCTCGCCAAGATCAGC and anti-sense 5'-AGAAGCCCCCAATTCTTAGG. Data were normalized to glycerol aldehyde phosphate dehydrogenase (GAPDH) expression in the same samples using the following primers: sense

5’-TCACCACCATGGAGAAGGC and anti-sense 5’-GCTAAGCAGTTGGTGGTGCA. Experiments on RAW cells using this ASM knockdown system were performed 24 hr after transfection.

Isolation of Macrophages

Animals were cared for in accordance with the guidelines set forth by the Canadian Council on Animal Care. The following mouse strains were used: C57BL/6 mice weighing 20 to 30 g (Charles River, St Constant, Quebec), triple Src KO ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) mice obtained from Dr. Clifford Lowell (University of California, San Francisco, CA) and ASM WT/KO mice obtained from Dr. Richard Kolesnick (Memorial Sloan-Kettering Cancer Center, New York, NY). Bone marrow-derived macrophages (BMMs) were obtained from precursor cells and cultured in 20% murine L-cell conditioned media. After plating of non-adherent monocyte precursors, cells were cultured for 6-8 days prior to use. For recovery of peritoneal macrophages, mice were given a 1 mL intra-peritoneal injection of 3.9% thioglycollate 4-6 days prior to recovery. Mice were then sacrificed and peritoneal macrophages recovered by injecting and withdrawing sterile aliquots of cold PBS with 1% fetal calf serum solution. Cells were recovered by centrifugation, re-suspended in DMEM with 2% fetal calf serum, counted by hemocytometer, and plated in tissue culture dishes overnight for experiments the next day.

RESULTS

H_2O_2 redirects LPS signaling in macrophages to a Src dependent pathway

We previously demonstrated that oxidative stress primes macrophages for augmented responsiveness to LPS, causing earlier and increased NF-κB translocation in RAW cells. Those *in vitro* experiments, which utilized pharmacological and molecular strategies of Src inhibition, also demonstrated that H_2O_2 reprograms LPS signaling to a Src dependent pathway [28]. To further explore the signaling cascade triggered by oxidant priming of macrophages, we first sought to recapitulate this finding using a genetic approach of Src inhibition.

Immunofluorescence staining for the p65 subunit of NF-κB was performed in bone marrow-derived macrophages (BMMs) from wild type (WT) and triple Src knock-out (KO) mice ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) after treatment with LPS (0.1ug/mL) for 30mins, with or without antecedent treatment with H_2O_2 (100uM) for 1 hour (Fig 1). In WT BMMs, there was complete nuclear translocation of p65 after 30mins of LPS, both with and without antecedent H_2O_2. However, in Src KO BMMs, p65 nuclear translocation was completely inhibited after H_2O_2 and LPS, while remaining fully preserved after LPS alone. This experiment recapitulates, using a genetic method of Src inhibition, our previous finding that H_2O_2 reprograms LPS signaling to a Src dependent pathway. Furthermore, it supports the knowledge that direct LPS signaling (in the absence of oxidant priming) is independent of Src kinases [90].

H_2O_2 activates the PI3K/Akt pathway in a src-dependent manner

Our previous studies had further demonstrated that the Src-dependent pathway of LPS signaling induced by oxidant priming also involved activation of PI3K [28]. As PI3K is

an established upstream activator of Akt, a serine/threonine kinase capable of inducing NF-κB translocation by phosphorylation of IκBα, we proceeded to further explore the role of H_2O_2 on the PI3K/Akt pathway.

Serum starved RAW 264.7 cells were treated with H_2O_2 (50-200μM) *in vitro* for 0-60mins. A time course and dose response for activation of Akt by H_2O_2 was assessed by Western blot using a p-Akt (ser 473) antibody (Fig 2A). H_2O_2, at concentrations at or greater than 100uM, was found to induce Akt activation and cause maximal Akt phosphorylation by 15mins. To determine if H_2O_2-induced Akt phosphorylation was mediated by PI3K, the experiment was repeated using Wortmannin (1uM), a PI3K inhibitor (Fig 2B). H_2O_2-induced Akt activation was completely inhibited by Wortmannin, confirming that H_2O_2-induced activation of Akt is mediated by PI3K.

The role of Src in H_2O_2-induced activation of PI3K and Akt was investigated by treating RAW 264.7 cells with H_2O_2 in the presence or absence of PP2 (10uM), a pharmacologic inhibitor of Src kinases (Figs 3A and 3B). H_2O_2-induced activation of both PI3K and Akt was determined by Western blot and was completely inhibited by PP2 at 15 and 30mins, confirming the role of Src kinases in oxidant-induced activation of the PI3K/Akt pathway.

Oxidant-induced activation of Akt is also dependent on sphingolipid catabolism

To explore the hypothesis that a lipid metabolite such as ceramide might mediate the priming events triggered by H_2O_2, we measured serine phosphorylation of Akt, an established surrogate marker for activation of the PI3K/Akt pathway [107], in the presence of various pharmacological, molecular and genetic strategies to inhibit different aspects of the sphingolipid degradation pathway.

First, H_2O_2-induced activation of Akt in serum starved RAW 264.7 cells was assessed in the presence or absence of D609, a pharmacologic inhibitor of phosphatidylcholine-phospholipase C (PC-PLC), the most upstream enzyme in the sphingolipid degradation pathway (Fig 4A). H_2O_2-induced activation of Akt at 15 and 30 mins as assessed by Western blot was completely inhibited by D609, suggesting that a downstream product of PC-PLC is necessary to mediate H_2O_2-induced activation of Akt. To further explore this finding, the same experiment was performed in the presence or absence of desipramine, a pharmacologic inhibitor of acid sphingomyelinase (ASM) [164], which more specifically converts sphingomyelin to ceramide (Fig 4B). Here again, while the modest activation of Akt shown after 5 mins of H_2O_2 was unaffected, the strong activation of Akt by 15mins of H_2O_2 was completely inhibited by desipramine, suggesting that a product of ASM, possibly ceramide, is necessary to mediate H_2O_2-induced activation of Akt.

Second, to complement the finding that oxidant-induced activation of Akt is dependent on sphingolipid catabolism through ASM, a molecular strategy for ASM inhibition was achieved by transfecting RAW cells with an ASM siRNA. PCR analysis after 24hrs revealed that ASM siRNA transfection caused a 70% reduction in ASM mRNA compared to a scrambled negative siRNA control (Fig 5A). Using this molecular ASM knockdown system, H_2O_2-induced Akt activation was markedly inhibited in cells transfected with ASM siRNA at 5 and 15 mins (Fig 5B), supporting the role of an ASM product in mediating H_2O_2-induced activation of Akt.

Third, a genetic model of ASM inhibition was used to recapitulate these findings. H_2O_2-induced activation of Akt was assessed *in vitro* by Western blot on peritoneal macrophages from ASM KO and WT mice (Fig 6). H_2O_2-induced activation of Akt shown

at 15 mins was completely inhibited in the ASM deficient macrophages. Taken together, these data suggest that H_2O_2-induced Akt activation in macrophages is mediated by a product of acid sphingomyelinase.

Ceramide recapitulates the effects of oxidative stress on Src and the PI3K/Akt pathway

To further investigate the role of the candidate lipid ceramide in H_2O_2-induced activation of the PI3K/Akt pathway, we investigated its ability to recapitulate PI3K and Akt activation. First, serum starved RAW 264.7 cells were treated with the ceramide analogue C2-ceramide (100μM) *in vitro* for 0-60mins and activation of Akt was assessed by Western blot using p-Akt (ser 473) antibody (Fig 7A). Ceramide recapitulated the effect of H_2O_2 on Akt with an earlier time course, causing marked Akt activation by 10mins. Second, the role of PI3K in ceramide-induced Akt activation was similarly assessed by stimulating RAW cells with C2-ceramide (100μM) *in vitro* for 0-15mins in the presence or absence of the PI3K inhibitor Wortmannin (1uM) (Fig 7B). As with H_2O_2, ceramide-induced Akt phosphorylation was PI3K-dependent. Thus in addition to being necessary for H_2O_2-induced activation of the PI3K/Akt pathway as shown above, ceramide appears sufficient to recapitulate this effect.

The role of Src kinases in ceramide-induced Akt activation was also assessed. Serum-starved RAW cells were treated with C2-ceramide (100μM) *in vitro* for 0-15mins in the presence or absence of the Src kinase inhibitor PP2 (10uM) and activation of Akt was assessed by Western blot using p-Akt (ser 473) antibody (Fig 8A). Interestingly, ceramide-induced Akt phosphorylation was also Src-dependent, and most obvious at the 5mins time point. To further explore this finding, serum-starved RAW cells were treated with C2-ceramide (100μM) *in vitro* for 0-15mins and directly assessed for Src activation using the

phospho-Y416 Src family kinase antibody (Fig 8B). Consistent with Fig 8A, ceramide induced Src activation by 5mins. Finally, to determine if H_2O_2-induced activation of Src is similarily mediated by ceramide, RAW cells transfected with ASM siRNA as previously described were treated with H_2O_2 and revealed that early (5mins) H_2O_2-induced Src kinase activation was inhibited in the ASM knockdown cells (Fib 8C), suggesting a critical early requirement for ceramide generation through ASM.

Taken together, these data suggest a hierarchy of signaling events following oxidative stress in macrophages: oxidative stress causes sphingolipid degradation through ASM which leads to ceramide generation, which in turns activates Src kinases, which activate PI3K, which activates Akt.

Oxidative stress redirects LPS signaling in macrophages to a ceramide-dependent pathway

The findings described thus far support a critical upstream role for H_2O_2-induced generation of sphingolipid metabolites such as ceramide through the ASM pathway. In order to determine whether these findings translate into a meaningful aspect of oxidant priming for augmented LPS signaling in macrophages, we examined the effect D609 on LPS-induced NF-κB nuclear translocation with or without oxidant priming.

Immunofluorescence staining for the p65 subunit of NF-κB was performed on RAW cells after treatment with LPS (0.1ug/mL) for 0, 15 or 30mins, with or without antecedent treatment with H_2O_2 for 1 hour. In the absence of D609, the two-hit model of oxidant priming was demonstrated as complete p65 nuclear translocation occurred in RAW cells after 30mins of LPS, while p65 translocation was observed earlier by 15mins with antecedent H_2O_2 priming. However, in the presence of D609 which inhibits ceramide generation from

sphingolipid degradation, LPS-induced p65 nuclear translocation after H_2O_2 priming was almost completely inhibited. Interestingly, LPS-induced p65 nuclear translocation in the absence of H_2O_2 priming was not inhibited by D609. Taken together, these data support the novel finding that oxidative stress re-programs LPS signaling in macrophages to a ceramide dependent-pathway.

Oxidant-induced activation of Akt is mediated by the ceramide metabolite S1P

Last, we sought to determine whether oxidant priming is mediated by the lipid ceramide or a metabolite of ceramide such as sphingosine-1-phosphate (S1P). S1P was a natural candidate as it is rapidly converted from ceramide through the action of sphingosine kinase and is known in the literature to have pro-survival effects, in contrast to ceramide which normally exerts pro-apoptotic ones. Using H_2O_2-induced activation of Akt as the downstream measure of oxidant priming in macrophages, we sought to isolate the mediator of Akt phosphorylation by using various inhibitors of sphingosine kinase. Serum starved RAW 264.7 cells were treated with H_2O_2 (200μM) *in vitro* for 0-15mins with or without the sphingosine kinase inhibitor SKI (30uM) and activation of Akt was assessed by Western blot using p-Akt (ser 473) antibody (Fig 10A). H_2O_2-induced activation of Akt was completely inhibited by SKI. To corroborate these findings, a similar experiment was conducted using DMS (30uM), another inhibitor of sphingosine kinase (Fig 10B). Here again, H_2O_2-induced activation of Akt was completely inhibited by DMS, suggesting that Akt activation is actually mediated by S1P rather than ceramide itself. To further corroborate these findings, RAW 264.7 cells were directly treated with S1P (15μM) *in vitro* for 0-60mins and activation of Akt was assessed by Western blot as previous (Fig 10C). S1P did indeed cause Akt

activation, peaking at 5mins and supporting the role of S1P in oxidant-induced activation of Akt.

DISCUSSION

The impact of oxidative stress generated during ischemia/reperfusion injury on cellular responsiveness has been the focus of much investigation across many fields. Whether it is global ischemia/reperfusion as in resuscitated hemorrhagic shock caused by trauma, or a focal ischemic event such as a cerebral or myocardial infarct, oxidative stress has been linked as a causative factor in the ensuing cellular pathology. To elucidate the mechanism by which oxidative stress primes macrophages for increased responsiveness to subsequent stimuli such as Gram-negative bacterial endotoxin would provide novel targets for modulating the excessive immune response that causes lung injury and multiple organ failure in the late post-trauma period. In this study, we identified the lipid ceramide as a novel upstream regulator of oxidant priming. Ceramide generation via ASM following oxidative stress was both necessary and sufficient to mediate activation of Src kinase and the PI3K/Akt pathway. Furthermore, LPS-induced NF-κB translocation following oxidant priming was found to be ceramide-dependent. Taken together, these findings support the hypothesis that oxidative stress may redirect LPS signaling by causing activation and assembly of different TLR4 receptor components, such that it is poised to respond more vigorously when subsequently exposed to LPS.

We began by providing genetic evidence to support our previous finding that oxidative stress redirects LPS signaling to a Src-dependent pathway. In our previous work, the evidence was based on *in vitro* use of the pharmacologic Src inhibitor PP2, and a molecular strategy of Src inhibition via constitutive expression of the natural Src antagonist Csk in RAW cells [28]. In the present work, bone marrow-derived macrophages from triple Src knockout $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mice were used to reaffirm the role of Src kinases in LPS

signaling following oxidative stress. Despite that there are at least 9 kinases in the Src family, peritoneal or bone marrow derived macrophages from these triple Src KO mice have been shown to have no residual Src kinase activity, suggesting that Hck, Fgr and Lyn are the only functional Src kinases in the monocyte/macrophage lineage [82, 90]. Using this method of genetic Src inhibition, we confirm that LPS-induced NF-κB translocation is independent of Src kinase, while oxidant priming redirects LPS signaling to a Src-dependent pathway.

Oxidative stress is known to generally activate Src kinases, but the mechanism by which Src kinases become essential to subsequent LPS signaling toward NF-κB translocation is unclear. Our previous work on the LPS receptor TLR4 suggested that oxidative stress causes translocation of TLR4 to membrane lipid raft domains [76]. Incidentally, several studies have described various roles for Src kinases in lipid rafts. Yasuda et al described a role for the Src kinase Fyn in orchestrating Csk-binding protein (Cbp) phosphorylation in lipid rafts and thereby recruiting Csk to the raft domain, a critical negative regulator of Src kinases [93]. Filipp et al showed that upon T-cell receptor (TCR) and CD4 co-aggregation, the Src kinase Lck is activated and translocates into lipid raft domains where it induces activation of another Src kinase, Fyn, thus triggering proximal TCR signaling [94-96]. Recruitment of Src kinases to lipid rafts following oxidative stress may therefore represent a plausible critical step in the re-programming of LPS signaling.

The requirement for lipid rafts in LPS signaling through TLR4 is not unique to the setting of oxidative stress. Indeed, TLR4 activation requires LPS interaction with the acute phase protein LPS binding protein (LBP), which facilitates interaction with CD14, a glycosylphosphatidyl-inositol (GPI)-anchored recognition receptor found on lipid rafts [168, 169]. CD14 in turns mediates interaction with MD-2 and TLR4 to trigger signal transduction

[32, 72]. However, significant changes have been reported to occur with regards to lipid raft organization following oxidative stress. The lipid ceramide, a component of lipid rafts, is known to be generated in response to various stimuli including oxidative stress, and many recent studies have shown that ceramide generation in lipid membranes by sphingomyelin hydrolysis can lead to the coalescence of lipid rafts into larger, more stable ceramide-enriched membrane platforms [121, 134-136]. Dumitru et al showed that ceramide generation through acid sphingomyelinase and the formation of ceramide-enriched membrane platforms induced by TRAIL (a TNF-related apoptosis inducing factor) were both inhibited by the antioxidant NAC, supporting the role of reactive oxidant species in mediating these events [140]. In the setting of macrophage priming, ceramide generation may therefore alter surface raft organization and thereby affect TLR4 distribution and LPS signaling.

In the present studies, we demonstrate using the sphingolipid degradation inhibitor D609 that oxidant priming in macrophages also redirects LPS-induced NF-κB translocation to a ceramide-dependent pathway. Interestingly, LPS-induced NF-κB translocation alone was not inhibited by D609, despite the fact that LPS has been shown to also induce ceramide generation [65, 170, 171]. Consistent with this finding, ceramide has previously been shown not to be an essential component of LPS-mediated actions such as ERK phosphorylation and NF-κB activation [170]. LPS-induced NF-κB translocation is normally the result of a redundant pathway that culminates in phosphorylation and ubiquitination of IκBα by a number of upstream kinases including IKKβ, TAK1, TRAF6, MAPK, atypical PKC, and Akt [30, 49, 172]. While LPS has previously been shown capable of activating the PI3K/Akt pathway via ceramide in macrophages [65], there has also been conflicting data indicating

that ceramide causes Akt downregulation [173, 174]. However, the recruitment of Src kinases to the TLR4 signaling complex by oxidative stress creates a mechanism by which subsequent LPS signaling might involve Akt as several studies have implicated important interactions between Src kinases and TLR4 signaling molecules such as TRAF6 [100, 101]. In those studies, Src and TRAF6 were shown to collaborate in mediating PI3K activation, which we previously implicated as part of the oxidant-primed LPS signaling cascade to NF-κB activation [28], and also a well known activator of Akt through the action of PDK-1 [105].

Here we demonstrate in RAW cells that oxidative stress alone causes activation of the PI3K/Akt pathway via Src kinases. While others have shown in endothelial cells that H_2O_2 can activate the pro-survival signaling pathways PI3K/Akt and ERK1/2 [175], the upstream dependence on Src is novel and highlights its role as a potential mediator of oxidant priming in macrophages. We also demonstrate that oxidant-induced ceramide generation is both necessary and sufficient to mediate PI3K/Akt activation via Src. While ceramide's ability to activate Src kinases has previously been described [165, 166], the implication that ceramide mediates oxidant-induced activation of Src is novel and highlights another upstream mediator of oxidant priming in macrophages. Using the pharmacologic inhibitors D609 and desipramine, a molecular siRNA knockdown of ASM, and ASM knockout macrophages, we further demonstrated that oxidant-induced ceramide generation occurs primarily through ASM, a finding which is supported by data on oxidant-activation of ASM [121]. In this context, however, these results highlight yet another novel target for modulation of oxidant priming.

Last, in assessing the overall feasibility of ceramide as the perpetrator of oxidant priming via activation of Src and the PI3K/Akt pathway, we pondered the possibility that the effects observed could be caused by a closely related metabolite of ceramide: sphingosine-1-phosphate (S1P). While ceramide has generally been ascribed pro-apoptotic roles, S1P has been implicated in mechanisms of cell survival, motility, proliferation and inflammation [176]. S1P has also been shown to activate the PI3K/Akt pathway in endothelial cells [177]. In our experiments, the pharmacologic, molecular and genetic strategies for ceramide inhibition would also have resulted in S1P inhibition, and exogenous addition of C2-ceramide does not preclude endogenous conversion to S1P. Using two different inhibitors of sphingosine kinase and exogenous addition of S1P, we demonstrate that H_2O_2-induced activation of Akt is mediated by S1P. While this does not preclude the effects of ceramide on Src kinase and its possible role in restructuring the TLR4 receptor complex in ceramide-enriched membrane platforms, it offers another target to consider in the hierarchy of molecules triggered by oxidant priming.

FIGURES

Figure 1. H_2O_2 redirects LPS signaling in macrophages to a Src dependent pathway. Immunofluorescence staining for the p65 subunit of NF-κB was performed in bone marrow-derived macrophages (BMMs) from wild type (WT) and triple Src knock-out (KO) mice ($hck^{-/-}fgr^{-/-}lyn^{-/-}$) after treatment with LPS (0.1ug/mL) or vehicle for 30mins, with or without antecedent treatment with H_2O_2 (100uM) for 1 hour. The p65 subunit of NF-κB was labelled with Cy3 coupled secondary antibody (red), and the nuclei are stained with DAPI (blue). The top panel shows complete nuclear translocation of p65 in WT BMMs after 30mins of LPS, both with and without antecedent H_2O_2. The bottom panel shows complete inhibition of p65 nuclear translocation in Src KO BMMs after H_2O_2 and LPS, while being fully preserved after LPS alone. There was no p65 translocation in control or H_2O_2-treated WT or Src KO BMMs (data not shown).

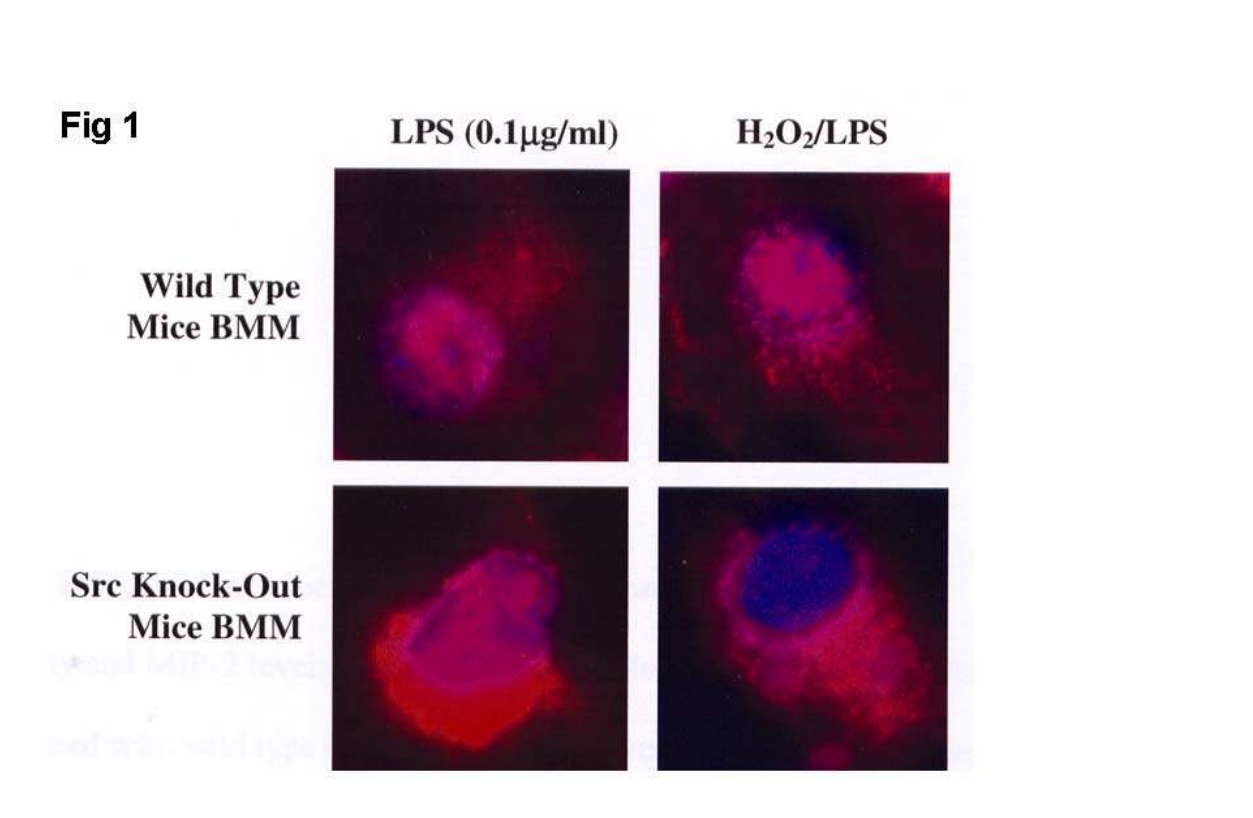


Figure 2. H_2O_2 activates Akt in macrophages in a PI3K-dependent manner.

A) Serum starved RAW 264.7 cells were treated with H_2O_2 (50-200μM) *in vitro* for 0-60mins. A time course and dose response for activation of Akt was assessed by Western blot using a p-Akt (ser 473) antibody. Maximal H_2O_2-induced Akt phosphorylation occurred at 15mins. **B)** Similarily, serum starved RAW 264.7 cells were treated with H_2O_2 (200μM) *in vitro* for 0-15mins, with and without the PI3K inhibitor Wortmannin (1uM). H_2O_2-induced activation of Akt was completely inhibited by Wortmannin at all time points. Representative blots are shown, n>3.

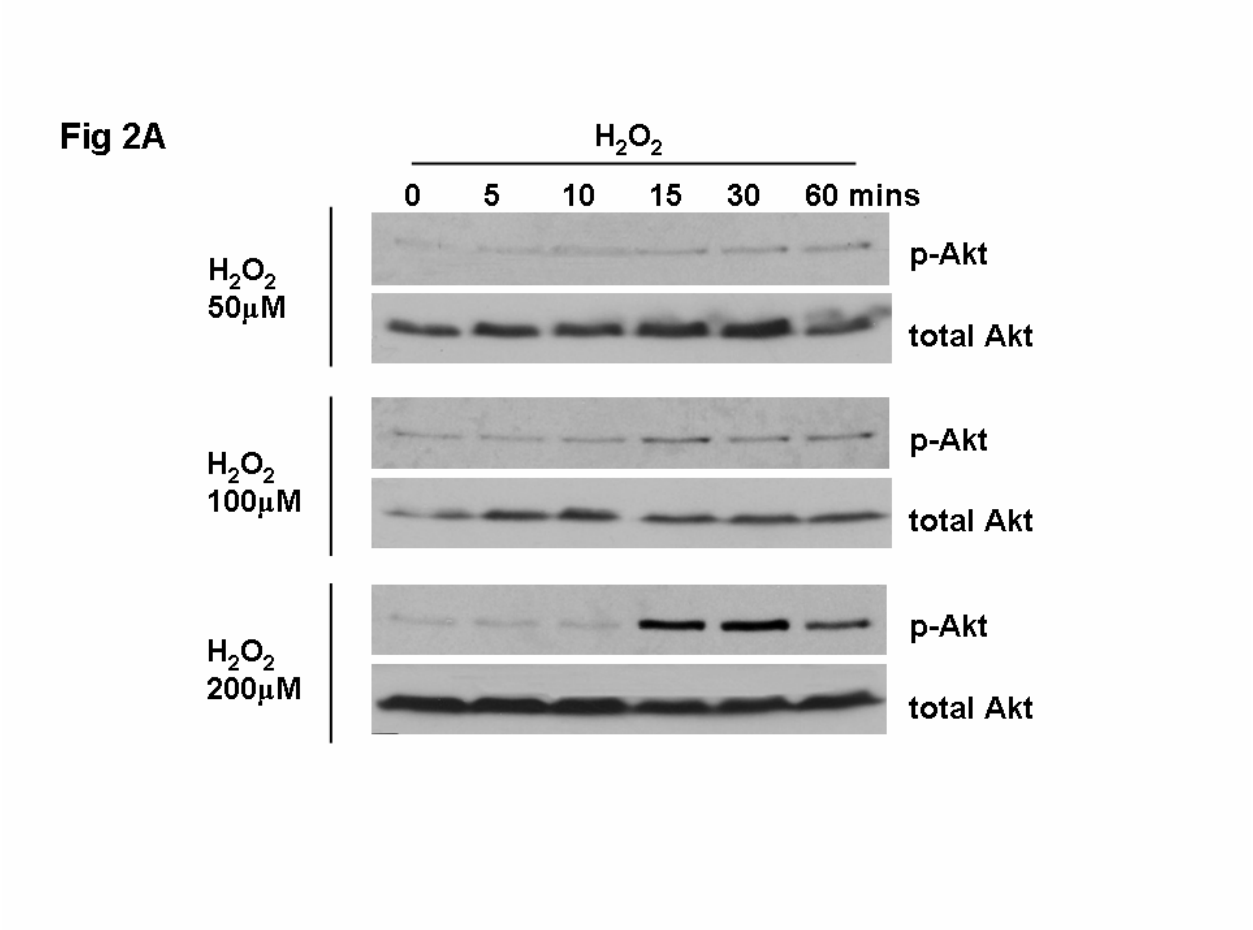


Fig 2B

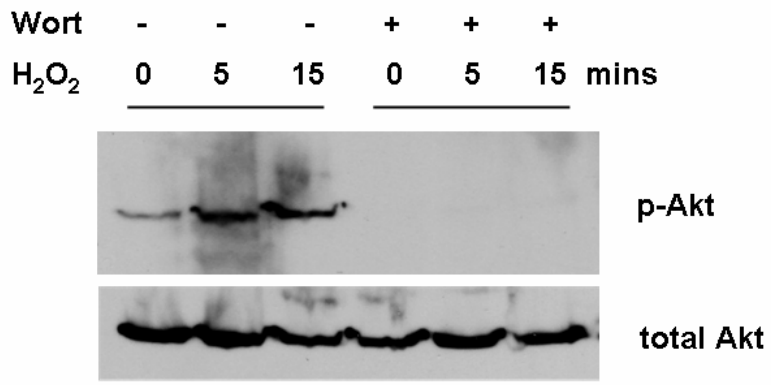


Figure 3. H_2O_2 activates PI3K and Akt in macrophages in a src-dependent manner.

A) Serum starved RAW 264.7 cells were treated with H_2O_2 (200μM) *in vitro* for 0-30mins, with and without the Src inhibitor PP2 (10uM). Activation of PI3K was assessed by Western blot using the phosphor-(Tyr) p85 PI3K binding motif antibody and was completely inhibited by PP2 at 15 and 30mins. **B)** Similarily, H_2O_2-induced Akt activation was completely inhibited by PP2 at 15 and 30mins. Representative blots are shown, n>3.

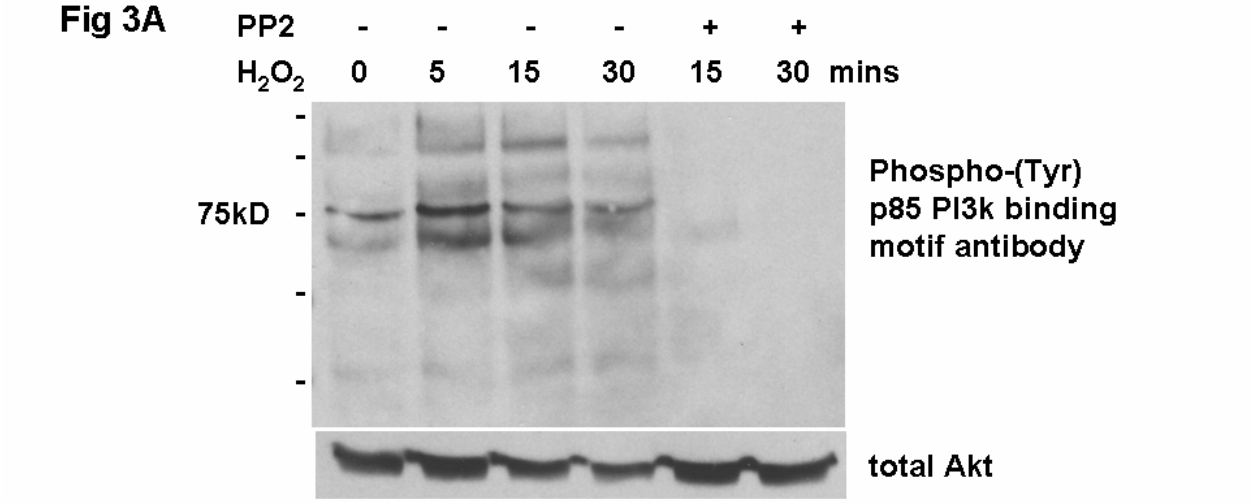


Fig 3B

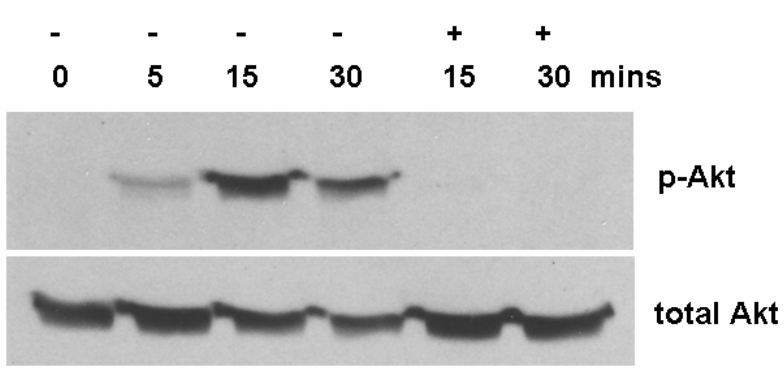


Figure 4. H_2O_2-induced activation of Akt is dependent on sphingolipid catabolism.

Serum starved RAW 264.7 cells were treated with H_2O_2 (200μM) *in vitro* for 0-30mins, and Akt activation was assessed by Western blot using p-Akt (ser 473) antibody. **A)** The role of sphingolipid degradation in H_2O_2-induced activation of Akt was assessed using D609 (100uM), an inhibitor of PC-PLC. **B)** The role of ASM in H_2O_2-induced activation of Akt was assessed using desipramine (5uM). Representative blots are shown, n>3.

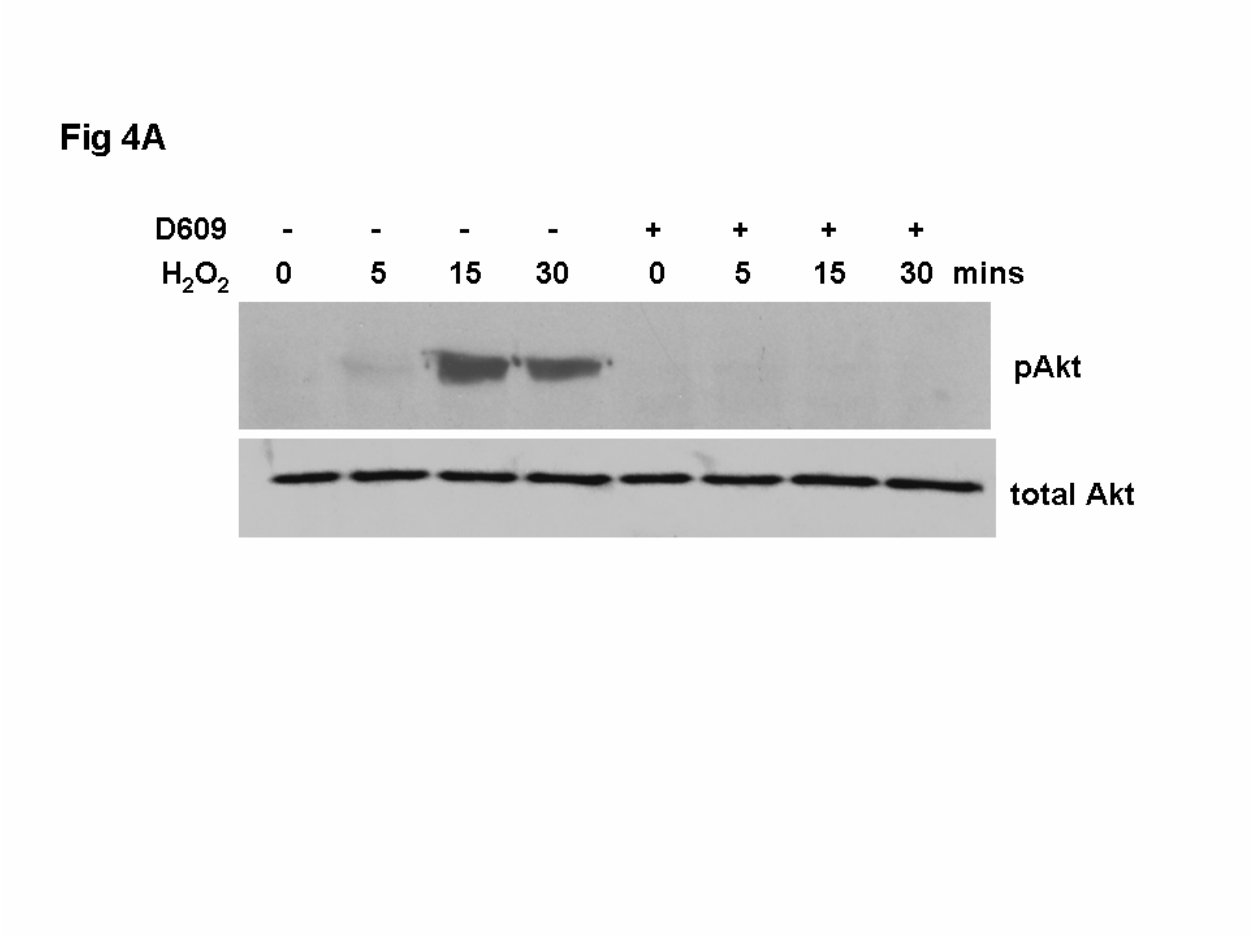


Fig 4B

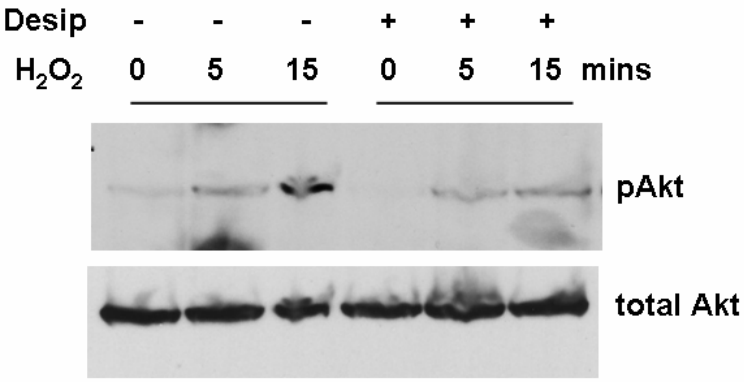


Figure 5. H_2O_2-induced activation of Akt in macrophages is inhibited by ASM siRNA.

A) ASM knockdown was achieved *in vitro* by transfecting RAW 264.7 macrophages with ASM siRNA (40pmol) for 24hrs using Lipofectamine RNAi-MAX. To verify knockdown efficiency, ASM mRNA in RAW cells was measured in duplicate by PCR and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPD). ASM siRNA transfection revealed a 70% reduction in ASM mRNA after 24hrs compared to control. A scrambled negative siRNA was also tested and did not cause any reduction in ASM mRNA (data not shown). **B)** Using this system of molecular ASM inhibition, RAW cells were stimulated with H_2O_2 (300uM) for 0-15mins. Western Blot analysis was performed using a p-Akt (ser 473) antibody. A representative blot is shown. Densitometry was calculated as a ratio of pAkt over Akt loading control, and normalized to the neg siRNA control (n=4).

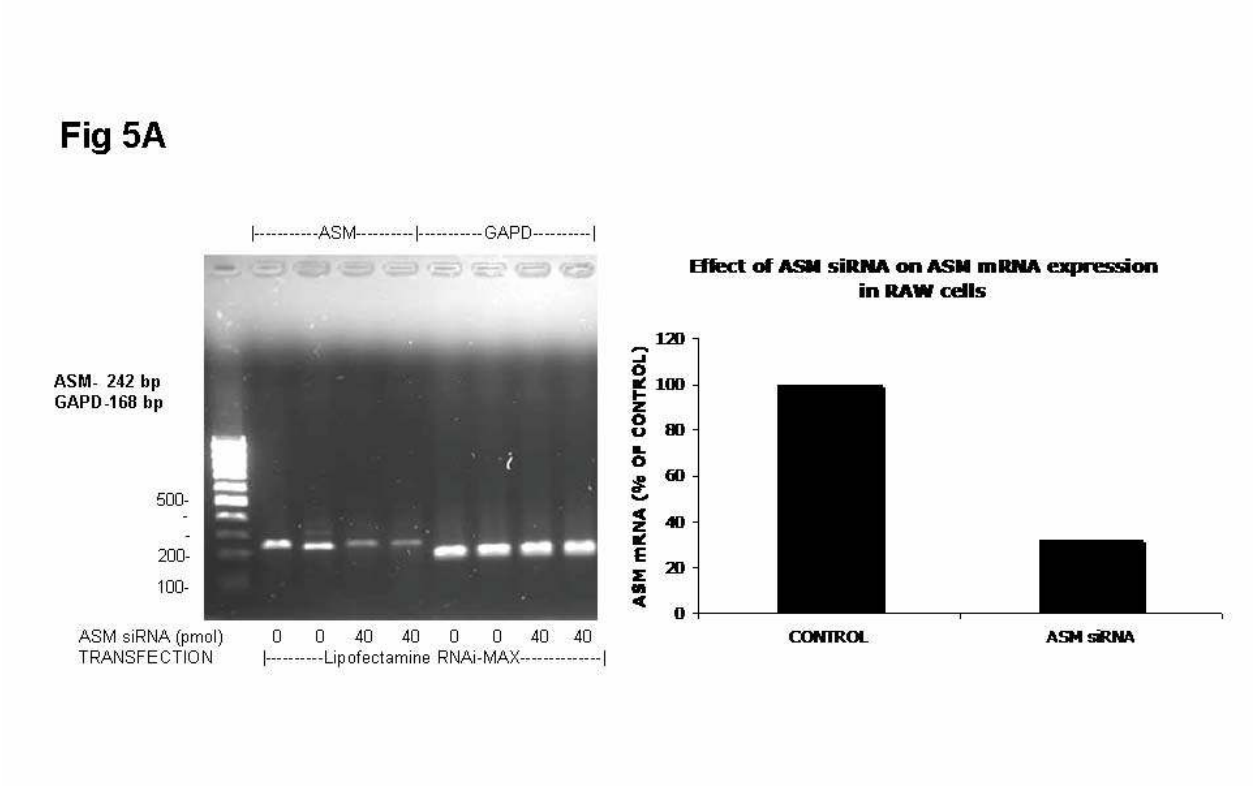


Fig 5B

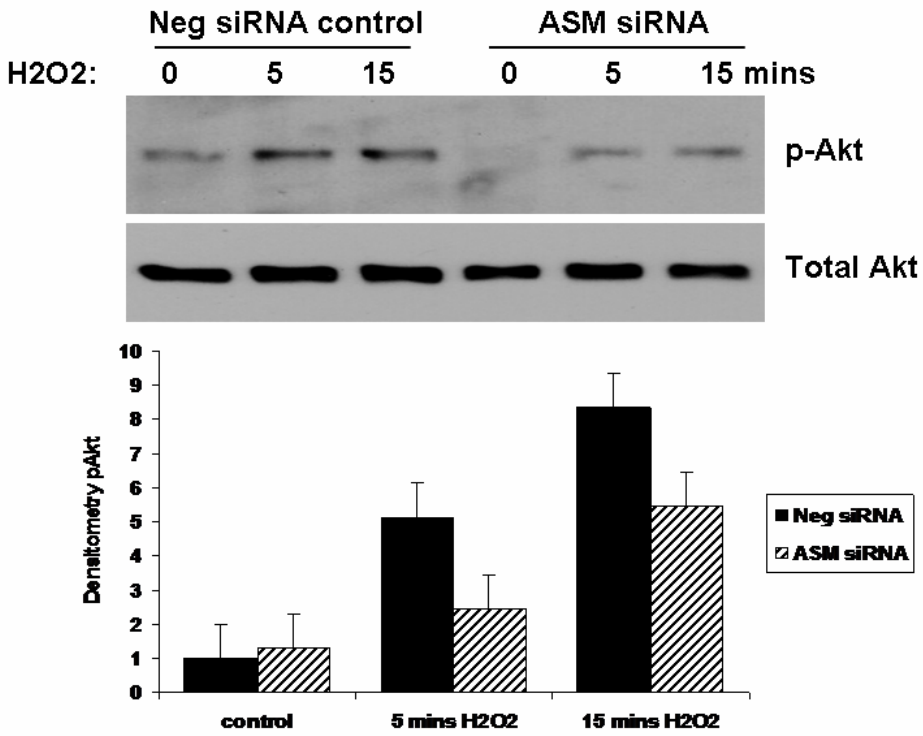

Neg siRNA control
ASM siRNA
H2O2:
0
5
15
0
5
15 mins
p-Akt
Total Akt
Densitometry pAkt
10
9
8
7
6
5
4
3
2
1
0
control
5 mins H2O2
15 mins H2O2
Neg siRNA
ASM siRNA

Figure 6. H_2O_2-induced activation of Akt is inhibited in macrophages from ASM KO mice. Peritoneal macrophages from WT and ASM KO mice were treated with H_2O_2 (100μM) *in vitro* for 0-30mins, and Akt activation was assessed by Western blot using p-Akt (ser 473) antibody. A representative blot is shown, n=4.

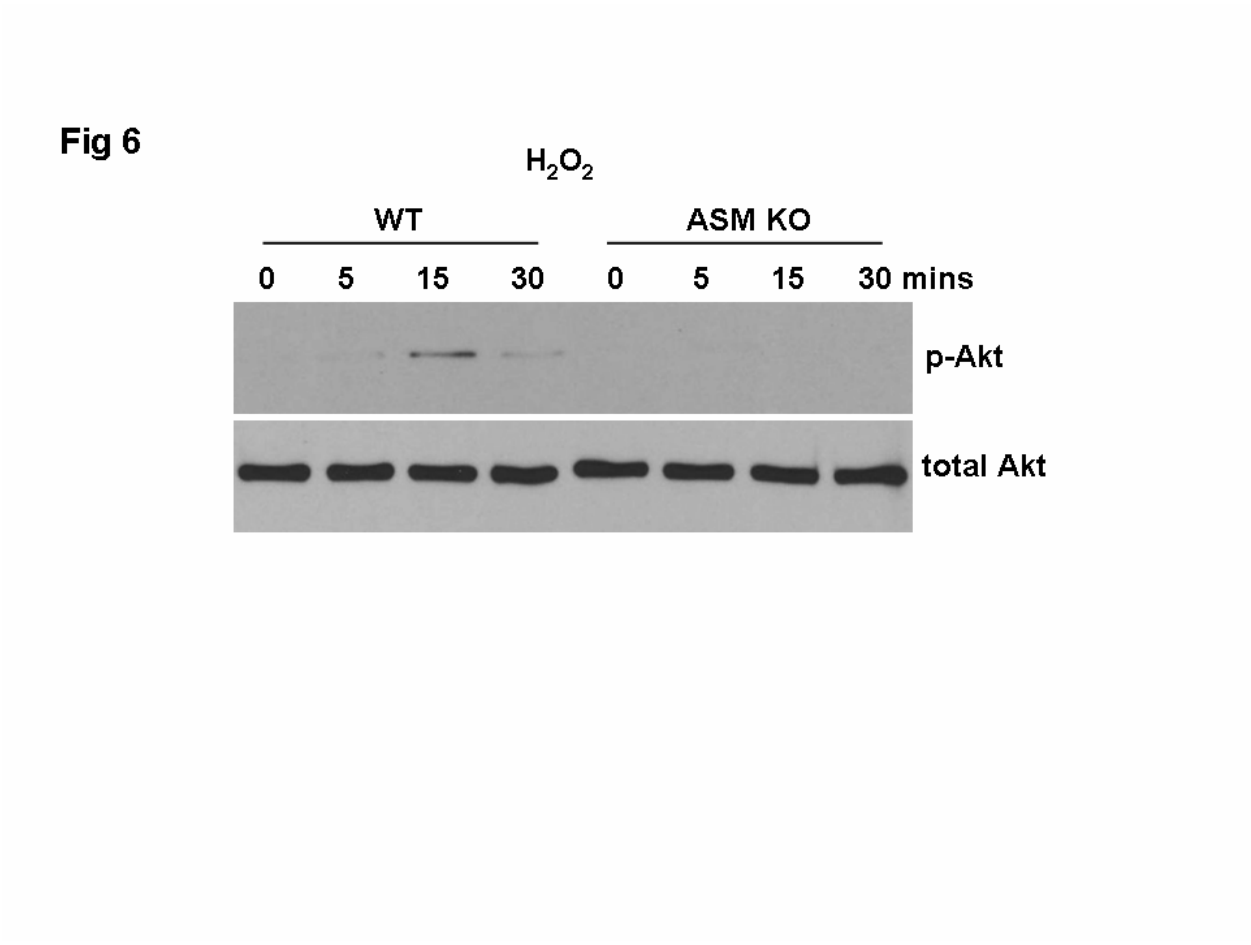


Figure 7. Ceramide induces activation of the PI3K/Akt pathway.

A) Serum starved RAW 264.7 cells were treated with the ceramide analogue C2-ceramide (100μM) *in vitro* for 0-60mins. C2-induced activation of Akt was assessed by Western blot using p-Akt (ser 473) antibody and revealed marked Akt activation by 10mins. **B)** The role of PI3K in ceramide-induced Akt activation was similarily assessed by treating RAW cells with C2-ceramide (100μM) *in vitro* for 0-15mins in the presence or absence of the PI3K inhibitor Wortmannin (1uM). C2-induced activation of Akt was completely inhibited by Wortmannin at all time points. Representative blots are shown, n=3.

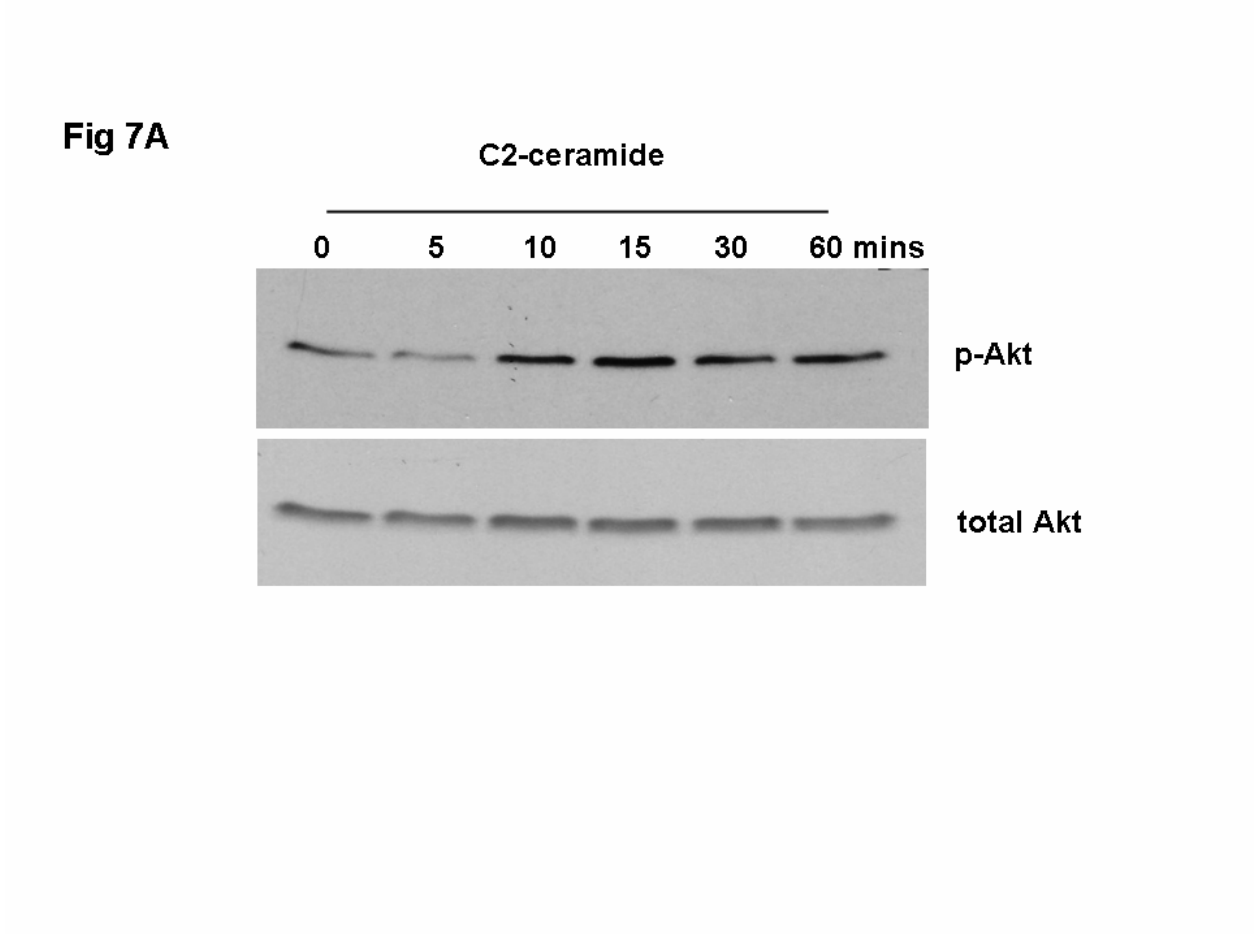


Fig 7B

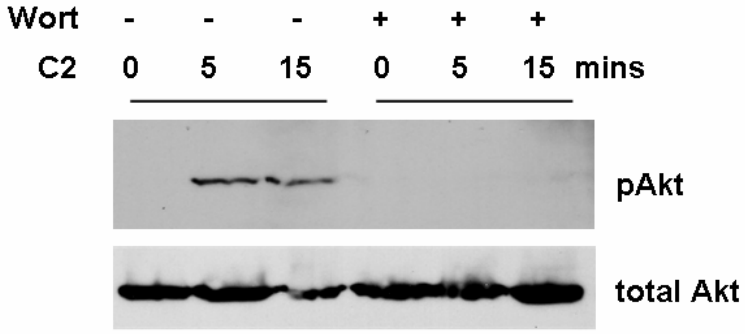


Figure 8. Ceramide-induced activation of Akt is also Src-dependent.

A) Serum-starved RAW cells were treated with the ceramide analogue C2-ceramide (100μM) *in vitro* for 0-15mins in the presence or absence of the Src kinase inhibitor PP2 (10uM), and activation of Akt was assessed by Western blot using p-Akt (ser 473) antibody. C2-induced activation of Akt was inhibited by PP2 at 5mins. A representative blot is shown, n=3. **B)** RAW cells were treated directly with C2-ceramide (100uM) for 0-15mins and Src activation was assessed by Western Blot using a phospho-Src family antibody (Tyr 416). Ceramide caused Src activation by 5mins. A representative blot is shown, n=3. **C)** RAW 264.7 macrophages transfected with ASM siRNA as in figure 5 were treated with H_2O_2 (300uM) for 0-15mins and Src activation was assessed by Western blot using a phospho-Src family antibody (Tyr 416). H_2O_2-induced early Src activation (5mins) was significantly inhibited in the ASM knockdown cells. A representative blot is shown. Densitometry was calculated as a ratio of pAkt over Akt loading control, and normalized to the neg siRNA control (n=2).

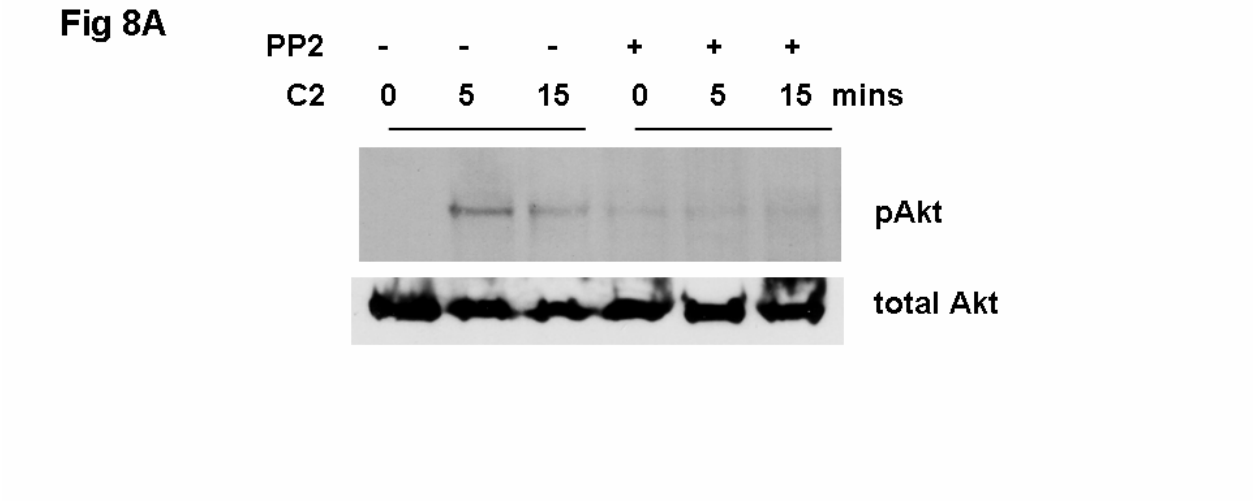


Fig 8B

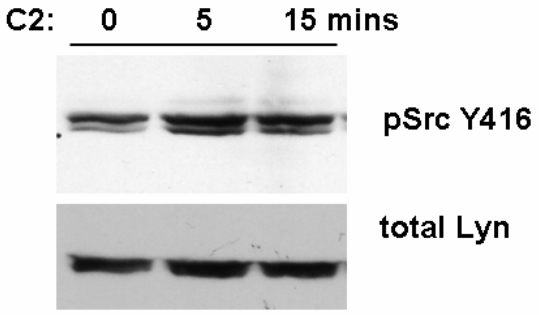

C2: 0 5 15 mins
pSrc Y416
total Lyn

Fig 8C

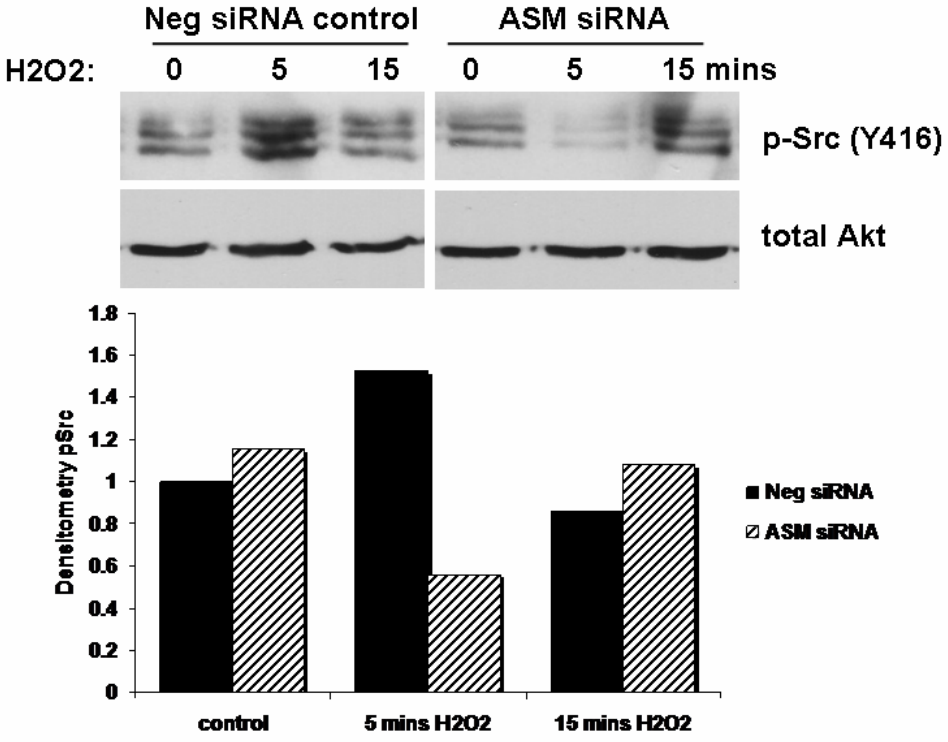

Neg siRNA control
ASM siRNA
H2O2: 0 5 15 0 5 15 mins
p-Src (Y416)
total Akt
Densitometry pSrc
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
control
5 mins H2O2
15 mins H2O2
Neg siRNA
ASM siRNA

Figure 9. H_2O_2 redirects LPS signaling in macrophages to a ceramide dependent pathway.

Immunofluorescence staining for the p65 subunit of NF-κB was performed on RAW cells after treatment with LPS (0.1ug/mL) or vehicle for 0, 15 or 30mins, with or without antecedent treatment with H_2O_2 (300uM) for 1 hour. The p65 subunit of NF-κB was labelled with Cy2 coupled secondary antibody (tainted red), and the nuclei were stained with Hoechst (tainted blue). As shown in the top panel, there is complete nuclear translocation of p65 in RAW cells after 30mins of LPS treatment, and it occurs earlier at 15mins with antecedent oxidative stress (H_2O_2). However, as shown in the bottom panel, D609 completely inhibits p65 nuclear translocation after H_2O_2 and LPS, while it remains unaffected after LPS alone. There was no p65 translocation in control or H_2O_2-treated RAW cells (not shown).

Fig 9

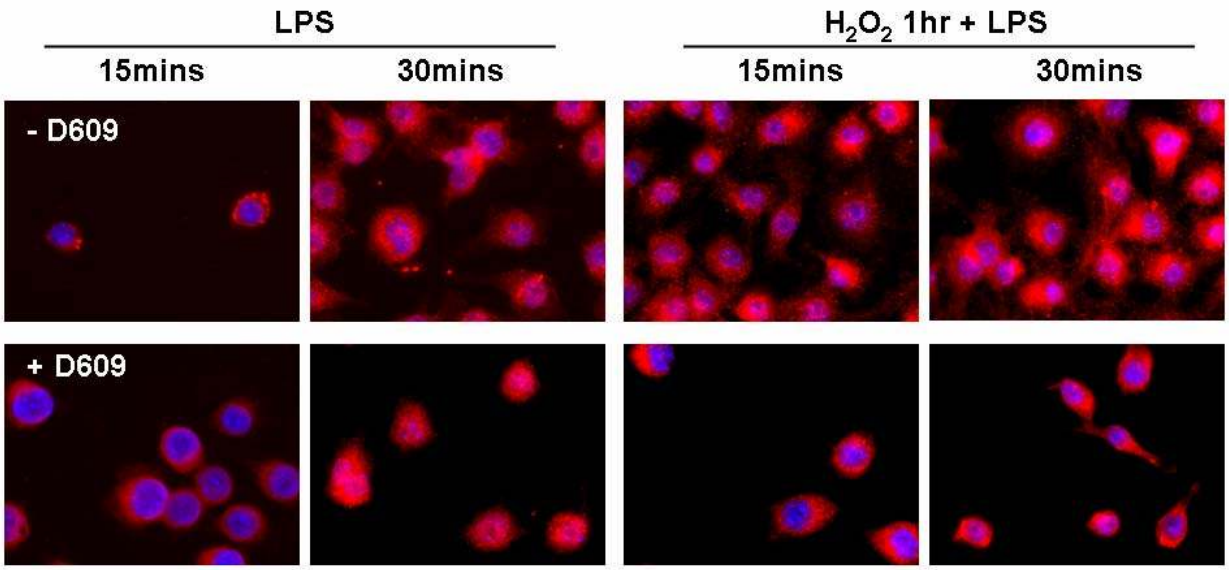


Figure 10. H_2O_2-induced activation of Akt in macrophages is mediated by S1P. Serum starved RAW 264.7 cells were treated with H_2O_2 (200μM) *in vitro* for 0-15mins with or without: **A)** the sphingosine kinase inhibitor SKI (30uM), and **B)** another sphingosine kinase inhibitor DMS (30uM). Activation of Akt was assessed by Western blot using p-Akt (ser 473) antibody. **C)** RAW 264.7 cells were treated with S1P (15μM) *in vitro* for 0-60mins. Activation of Akt was assessed by Western blot using p-Akt (ser 473) antibody. Representative blots are shown, n=3.

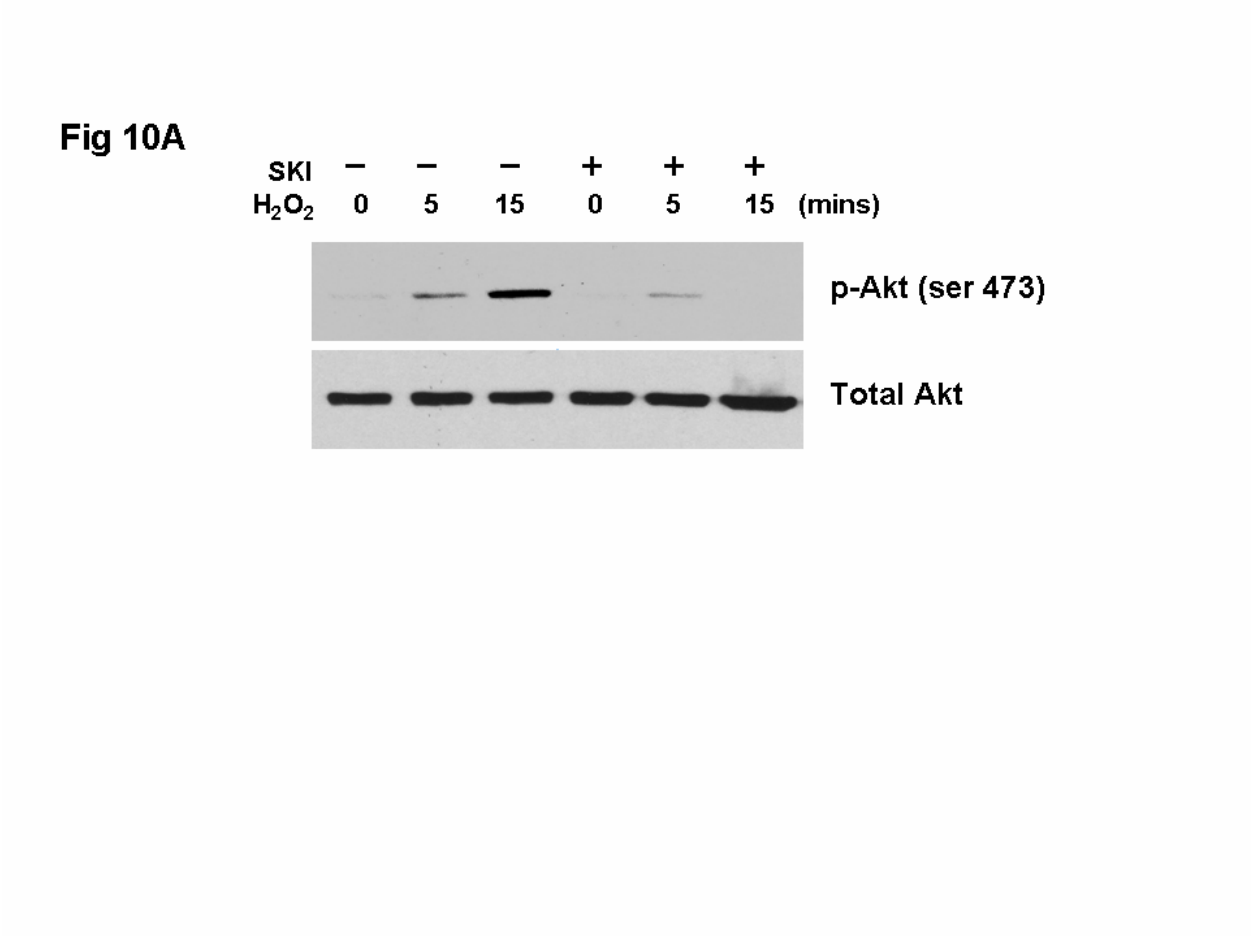


Fig 10B

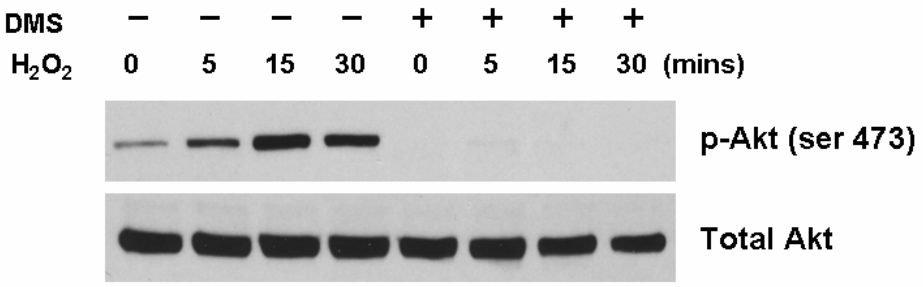
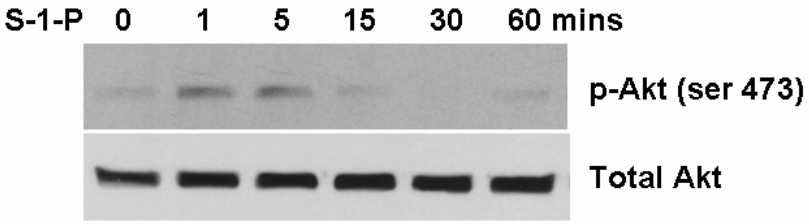


Fig 10C



DISCUSSION

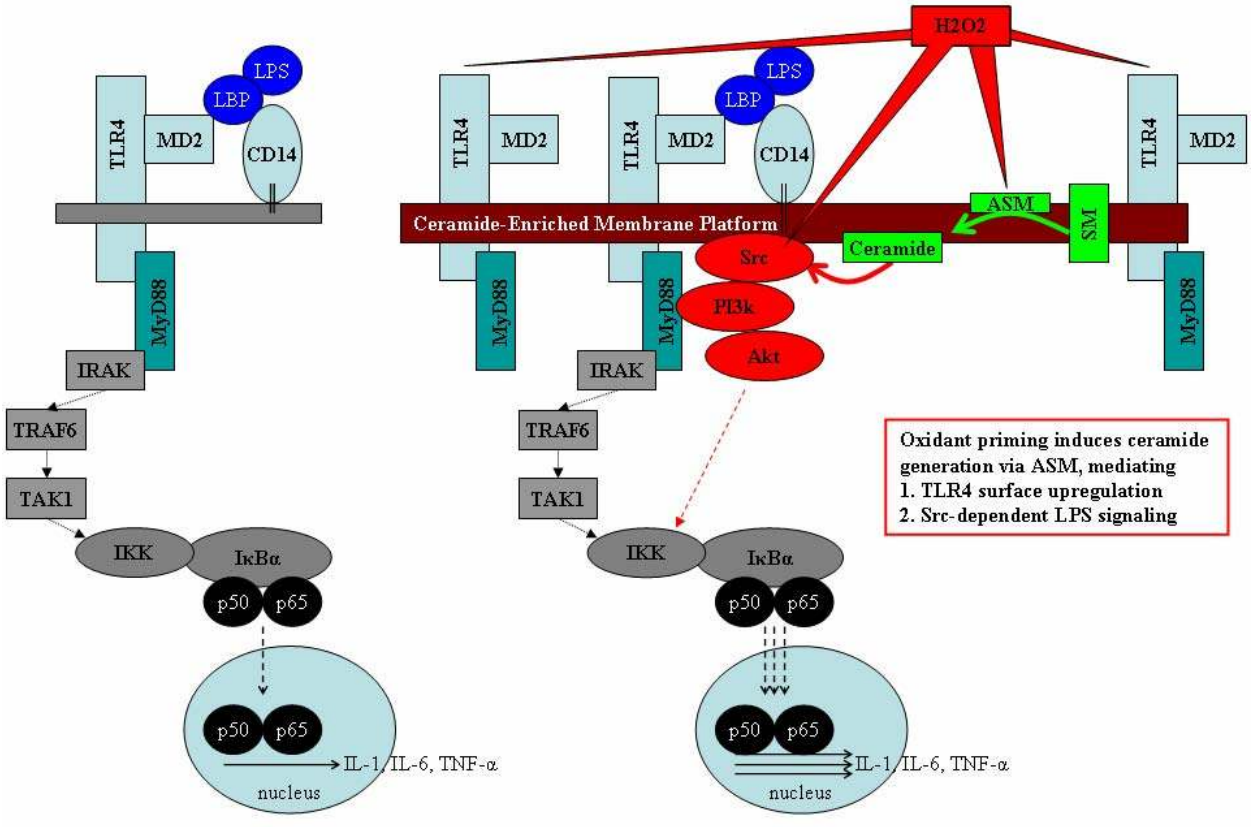
SUMMARY

The burden of multiple organ failure and death as late complications of trauma remains significant and devastating. Better strategies must be devised to modulate the immune response and abrogate the augmented cellular responsiveness we observe following hemorrhagic shock and resuscitation (S/R).

In Chapter 1, we demonstrate that the antioxidant STAZN, a novel second-generation azulenyl nitrone free radical scavenger, is effective in significantly reducing lung injury in a two-hit model of hemorrhagic S/R and LPS. It achieves this protection by reducing the circulating oxidant load, and by abrogating nuclear translocation of NF-κB and the elaboration of the pro-inflammatory cytokine TNF-α. This finding underscores the importance of understanding the mechanisms by which oxidative stress "primes" alveolar macrophages for an exaggerated response to a delayed inflammatory stimulus such as LPS.

In Chapters 2 and 3, we define a hierarchy of signaling events that underscore two mechanisms of oxidant priming for increased LPS responsiveness. Summarized in **Figure 1**, oxidative stress causes a rapid rise in ceramide generation through the acid sphingomyelinase enzyme. Ceramide then mediates early oxidant-induced Src activation. Together, these events cause: 1) upregulation of membrane TLR4 in macrophages, and 2) activation of the PI3K/Akt pathway. Ultimately, we observe that oxidant stress reprograms LPS-induced NF-κB translocation to a ceramide-dependent pathway. The hierarchy of signaling molecules and interactions described herein represent novel targets for modulating oxidative stress in the treatment of critical illness and organ injury.

Figure 1: Oxidant mediated priming of macrophages for LPS signaling



OXIDATIVE STRESS MEDIATES CELLULAR PRIMING FOLLOWING S/R

The observation in rodent models that antioxidant supplements such as N-acetylcysteine (NAC) administered during the resuscitation phase were markedly effective in inhibiting macrophage priming and resultant lung injury provided support for the role of oxidants in this process [21]. Similarly, in man, the use of an antioxidant "cocktail" in trauma patients was shown to reduce alveolar cytokines, to impact positively on ventilation parameters and to lessen the incidence of multiple organ failure [143].

The Role of STAZN

In our studies, we provide further evidence in support of oxidative stress being the central mediator of cellular priming following S/R. Indeed, the azulenyl nitrone compound STAZN was shown to prevent oxidative stress as demonstrated by reduced 8-isoprostanes in the plasma. As a result, STAZN was shown to prevent lung inflammation in LPS-induced lung injury with or without antecedent shock/resuscitation. Since STAZN had only previously been shown to confer neuro-protection in models of focal ischemic stroke and traumatic brain injury, this is the first reported evidence of its efficacy in a model of lung injury. The finding that STAZN could protect against LPS-induced lung injury without prior S/R was unexpected. Previous *in vitro* investigations have implicated intracellular induction of oxidative stress as a key mechanism leading to nuclear translocation of the transcription factor NF-κB and downstream induction of pro-inflammatory genes [148, 149]. In this regard, we suspect that STAZN was highly effective in protecting against lung neutrophil infiltration when LPS was given intra-tracheally by virtue of its high lipid solubility, in contrast to other antioxidants such as NAC. The finding that STAZN reduced whole lung NF-κB translocation and TNF-α levels following LPS treatment is consistent with its ability to exert antioxidant properties in the lung. It also suggests that the effect is, at least in part, mediated by inhibition of pro-inflammatory signaling pathways. Further studies on this and other related compounds should be pursued in animal and human models as a therapeutic organ protective strategy following ischemia/reperfusion injury.

MECHANISMS OF CELLULAR PRIMING BY OXIDATIVE STRESS

Our subsequent studies on the molecular events underlying oxidant priming in macrophages led to a common upstream role for the lipid ceramide. A vast body of literature has evolved over the last decade on the role of ceramide and related sphingolipids in membrane function and cellular signaling. In our studies, we demonstrate that oxidative stress leads to a rapid doubling of ceramide in macrophages through the action of acid sphingomyelinase, which then mediates TLR4 upregulation on the cell surface and activation of the PI3K/Akt pathway. Interestingly, both effects were shown to occur in a Src-dependent manner. Thus, oxidant priming appears to be a ceramide-mediated process of altered activation and assembly of TLR4 receptor components, causing it to signal more vigorously when subsequently exposed to LPS. The next few paragraphs will further discuss those findings.

Oxidative stress causes ceramide generation

First, oxidative stress *in vitro* was shown to cause rapid doubling of ceramide in macrophages. Ceramide generation in alveolar macrophages *in vivo* was also demonstrated after shock/resuscitation. This finding forms the basis of the proposed oxidant priming mechanism in macrophages. Data from the literature support the generation of ceramide following oxidative stress. A previous report in lung epithelial cells found a 150% increase in ceramide generation after treatment with H_2O_2 (100uM) by 5 to 10 mins [135]. We further demonstrated that this early peak was the result of sphingolipid degradation as two independent inhibitors of the degradation pathway, D609 and desipramine, inhibited oxidant-induced ceramide generation. The use of desipramine specifically suggested that oxidant-

induced ceramide generation was occurring primarily through ASM as desipramine is specific for this enzyme [164].

Ceramide mediates oxidant-induced TLR4 translocation

Ceramide was then shown to be necessary and sufficient to mediate oxidant-induced TLR4 surface upregulation. We know from our previous report that TLR4 upregulation occurs in membrane lipid rafts [76]. Significant changes have been reported to occur with regards to lipid raft organization following oxidative stress, including membrane recruitment and clustering of the LPS receptor complex [27, 75, 157]. Many recent studies have shown that ceramide generation in lipid membranes by sphingomyelin hydrolysis can lead to the coalescence of lipid rafts into larger, more stable ceramide-enriched membrane platforms [121, 134-136]. Dumitru et al showed that ceramide generation through acid sphingomyelinase and the formation of ceramide-enriched membrane platforms induced by TRAIL (a TNF-related apoptosis inducing factor) were both inhibited by the antioxidant NAC, supporting the role of reactive oxidant species in mediating these events [140]. In a similar manner, oxidative stress has also been shown to have a critical role in ligand-mediated TLR4 trafficking [158, 159]. Nakahira et al demonstrated that LPS mediated TLR4 assembly in lipid rafts was mediated by NADPH oxidase-dependent ROS generation [77]. Thus, in the setting of macrophage priming by oxidative stress, ceramide generation may alter surface raft organization and thereby affect TLR4 distribution and LPS signaling.

Src mediates oxidant-induced TLR4 translocation

Src was also shown to mediate oxidant-induced TLR4 surface upregulation. This finding complements our earlier description in macrophages that oxidative stress redirects LPS signaling to a Src-dependent pathway [28]. But while oxidative stress is known to

activate Src kinases, the exact mechanism by which Src kinases can mediate oxidant-mediated TLR4 surface upregulation remains undefined. Several studies, particularily in T cells, have described various roles for Src kinases in lipid rafts. Yasuda et al describe a role for the Src kinase Fyn in orchestrating Csk-binding protein (Cbp) phosphorylation in lipid rafts and thereby recruiting Csk to the raft domain [93]. Recruitment of Src kinases to lipid rafts by oxidative stress may therefore represent a critical step in organizing TLR4 upregulation at the cell surface.

Alternatively, there is also an established role for Src kinases in mediating receptor traffic and vesicular transport. Sandilands et al demonstrated that Src kinase modulates the activation, transport and signaling dynamics of fibroblast growth factor receptors upon ligand binding [98]. They demonstrated very rapid Src activation (within 5 mins of ligand stimulation), and an essential role as part of the Src kinase/RhoB-dependent shuttle of receptor containing endosome delivery to the plasma membrane. Src inhibition by pharmacologic and genetic strategies was shown not only to impair receptor exocytosis, but also to decrease downstream activation of Akt. In a separate earlier study, Mocsai et al had demonstrated a role for the Src kinases Fgr and Hck in mediating adhesion-dependent degranulation in neutrophils [99]. Src may therefore mediate oxidant-induced TLR4 translocation to the cell surface by facilitating receptor transport.

Ceramide mediates oxidant-induced Src activation

Ceramide was also shown to cause Src activation. H_2O_2-induced early activation of Src by 5 mins, and was significantly inhibited in RAW cells transfected with ASM siRNA, suggesting a role for ceramide in mediating Src activation. C2-ceramide added directly to RAW cells also caused Src activation by 5 mins. These finding are consistent with a study in

colonic smooth muscle cells where ceramide-induced PI3K activation was found to be mediated by tyrosine phosphorylation of pp60src [165], and another study in RAW cells where C2-ceramide was found to cause Hck phosphorylation [166]. However, the involvement of ASM in mediating ceramide generation and downstream Src activation is novel. The exact mechanism of the interaction between Src and ceramide remains unclear, although proximity between ceramide and Src kinases in lipid rafts following oxidative stress is a plausible mechanism by which they may interact.

When the emerging hierarchy between ceramide and Src was further tested, C2-induced TLR4 upregulation was assessed by flow cytometry with Src-inhibition via PP2 and in triple Src KO AMs. Both strategies revealed partial inhibition of C2-induced TLR4 translocation, suggesting that Src kinases are not the only mechanism underlying TLR4 recruitment to lipid rafts. Indeed, some studies have demonstrated that ceramide can also play a role in regulating endocytic and exocytic vesicular processes through associations with Rab proteins [167]. Nevertheless, C2-induced TLR4 translocation was reduced by about half under Src inhibitory conditions and served to reaffirm the apparent hierarchy involved in oxidant-induced TLR4 translocation.

Oxidative stress induce Src and PI3K/Akt activation via ceramide generation

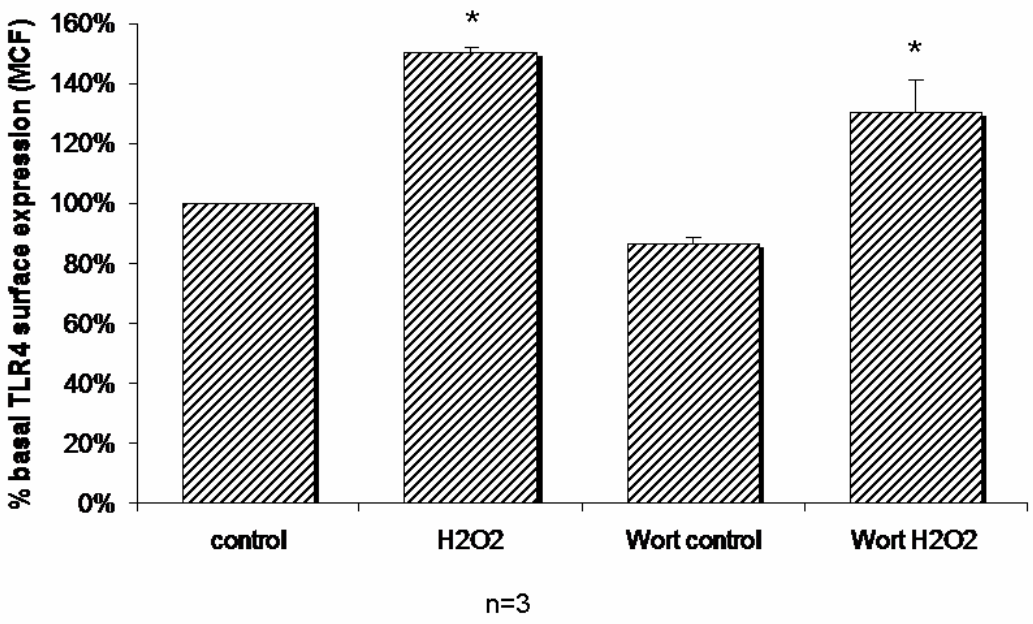
Oxidative stress was shown to cause activation of the PI3K/Akt pathway via Src kinases in RAW cells. While others have shown in endothelial cells that H_2O_2 can activate the pro-survival signaling pathways PI3K/Akt and ERK1/2 [175], the upstream dependence on Src is novel and highlights its role as a potential mediator of oxidant priming in macrophages. We also demonstrate that oxidant-induced ceramide generation via ASM is both necessary and sufficient to mediate PI3K/Akt activation via Src. While ceramide's

ability to activate Src kinases has been discussed, and Src is a known potential activator of PI3K through tyrosine phosphrylation of p85, the finding that oxidative stress triggers this signaling pathway in RAW cells is novel. The upstream dependence on ceramide generation through ASM is also novel. Consistent with our previous finding that oxidative stress redirects LPS signaling to a Src and PI3K dependent pathway, it appears that oxidative stress imparts its priming effects by causing recruitment of Src and PI3K into the TLR4 receptor complex such that subsequent signaling is enhanced by, and dependent on, those molecules. Relevant to these findings, some studies have implicated important interactions between Src kinases and TLR4 signaling molecules such as TRAF6 in the setting of oxidative stress [100, 101]. In those studies, Src and TRAF6 were shown to collaborate in mediating PI3K activation. While non-primed LPS signaling has been shown in some studies to signal through the PI3K/Akt pathway, it is not an obligatory component of the traditional cascade to NF-κB activation. Our findings suggest that oxidative stress activates this pathway such that it adopts an obligatory position in the TLR4 receptor complex, and thereby enhances the strength of subsequent signaling. Another potential mechanism which may contribute to activation of PI3K in the setting of oxidative stress is concomitant inhibition of its negative regulator, PTEN. Evidence now shows that oxidative stress can inhibit the phosphatase PTEN, resulting in PI3K activation [178].

Given the finding that oxidative stress activates PI3K via ceramide and Src, and given that PI3K is known to have a role in receptor trafficking, we further hypothesized that PI3K might also contribute to oxidant-induced TLR4 translocation. However, a recent experiment (**Figure 2**) where RAW cells were treated with H_2O_2 with or without the PI3K inhibitor wortmannin, demonstrates no disruption in oxidant-induced TLR4 translocation by Flow

Cytometry. This finding effectively identifies PI3K as the diverging point between the mechanism of oxidant-induced TLR4 translocation and the oxidant-induced signaling cascade.

Figure 2: H2O2-induced TLR4 translocation in macrophages is not PI3k-dependent



Oxidative stress redirects LPS signaling to a ceramide dependent pathway

Last, we demonstrate in an immunofluorescence experiment that prior oxidant exposure redirects subsequent LPS-induced NF-κB translocation in RAW cells to a ceramide-dependent pathway. Interestingly, LPS-induced NF-κB translocation alone was not inhibited by D609, despite the fact that LPS has been shown to also induce ceramide generation [65, 170, 171]. This is consistent with previous findings as, in a manner similar to

PI3K and Akt, ceramide has also previously been shown not to be an essential component of LPS-mediated actions such as ERK phosphorylation and NF-κB activation [170]. LPS-induced NF-κB translocation is normally the result of a redundant pathway that culminates in phosphorylation and ubiquitination of IκBα by a number of upstream kinases including IKKβ, TAK1, TRAF6, MAPK, atypical PKC, and Akt [30, 49, 172]. While LPS has previously been shown capable of activating the PI3K/Akt pathway via ceramide in macrophages [65], there has also been conflicting data about the ability of ceramide to cause Akt downregulation [173, 174]. The novelty of this finding is that it identifies an obligatory upstream target, ceramide, in the pathway of LPS signaling following oxidative stress.

FUTURE DIRECTIONS

Ultimately, it is my hope that the findings described herein will advance our understanding of cellular priming and may eventually lead to novel therapeutic strategies aimed at modulating the immune response in the setting of critical illness and multiple organ failure following hemorrhagic shock and resuscitation (S/R).

In Chapter 1, we demonstrate for the first time that the antioxidant STAZN, a novel second-generation azulenyl nitrone free radical scavenger, is effective in significantly reducing lung injury in a two-hit model of hemorrhagic S/R and LPS. Further studies on this and other related compounds should be pursued in animal and human models as a therapeutic organ protective strategy following ischemia/reperfusion injury.

In Chapters 2 and 3, we define a hierarchy of signaling events that underscore two mechanisms of oxidant priming for increased LPS responsiveness. Oxidative stress causes a rapid rise in ceramide generation through acid sphingomyelinase. Ceramide then mediates early oxidant-induced Src activation. Together, these events cause: 1) upregulation of surface TLR4 in macrophages, and 2) activation of the PI3K/Akt pathway. Ultimately, we observe that oxidant stress reprograms LPS-induced NF-κB translocation to a ceramide-dependent pathway. The hierarchy of signaling molecules and interactions described herein represent novel targets for modulating oxidative stress in the treatment of critical illness and organ injury.

Of particular interest is the upstream nature of ceramide and src activation in the proposed mechanisms of oxidant-mediated macrophage priming. There are certainly numerous fascinating and very relevant studies that could be performed to further elucidate the exact mechanisms of interactions between the proposed molecules, and whether they are regulated by other as of yet uninvolved molecules.

However, testing our findings *in vivo* on the rodent two-hit lung injury model of hemorrhagic S/R and LPS would provide tangible information about the systemic relevance of these novel upstream targets of cellular priming. To that end, we performed some preliminary experiments:

Ceramide inhibition *in vivo*

Figure 1: Using the pharmacologic inhibitor of sphingolipid degradation D609 *in vivo*, preliminary results suggest a marked reduction in lung neutrophil sequestration and myeloperoxidase activity following S/R and LPS by i.p. administration of D609 (40mg/kg) during the resuscitation phase.

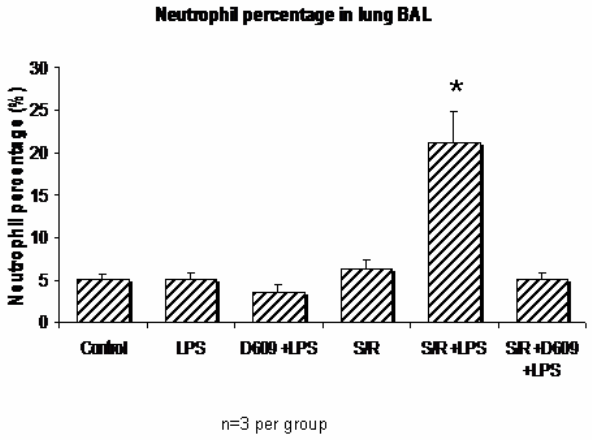
Figure 2: Using WT and ASM KO mice, *in vivo* elaboration of pro-inflammatory cytokines TNF-α and KC by alveolar macrophages after S/R and LPS was measured by standard ELISA technique following bronchoalveolar lavage. Preliminary results suggest that ASM deficiency confers significant reduction in the elaboration of pro-inflammatory cytokines after S/R and LPS.

Src inhibition *in vivo*

Figure 3: Using WT and triple Src KO mice, preliminary results suggest a marked reduction in lung neutrophil sequestration following S/R and LPS in the Src KO mice.

Figure 4: Similarily, using WT and triple Src KO mice, preliminary results suggest that Src deficiency confers significant reduction in the elaboration of pro-inflammatory cytokines after S/R and LPS. Interestingly, this effect is not apparent following LPS alone. This is consistent with S/R causing LPS reprogramming to a Src-dependent pathway.

Figure 1: D609 *in vivo* prevents lung neutrophil sequestration following S/R and LPS in mouse two-hit lung injury model



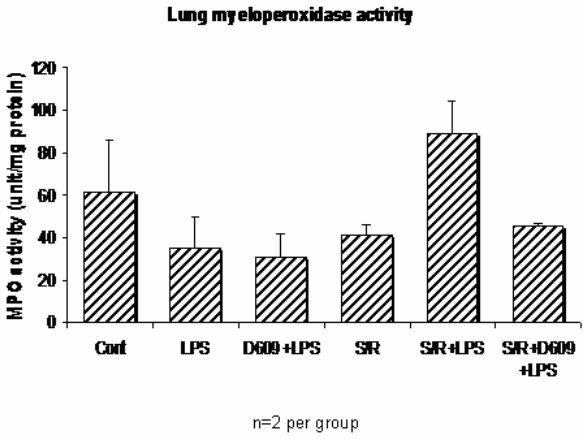


Figure 2: ASM KO genotype inhibits elaboration of TNFa and KC following S/R and LPS in mouse two-hit lung injury model

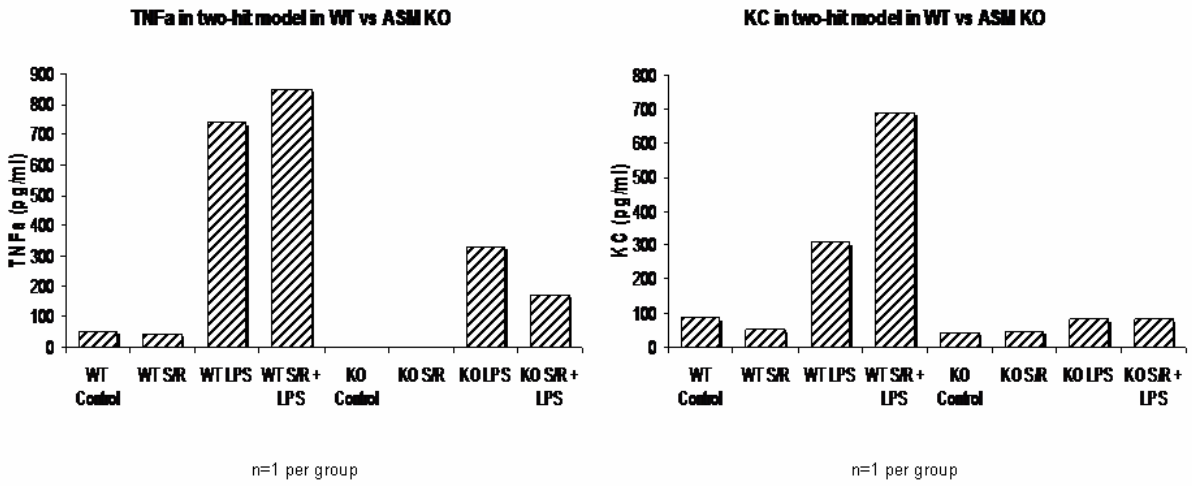


Figure 3: Src KO genotype protects against lung neutrophil sequestration following S/R and LPS in mouse two-hit lung injury model

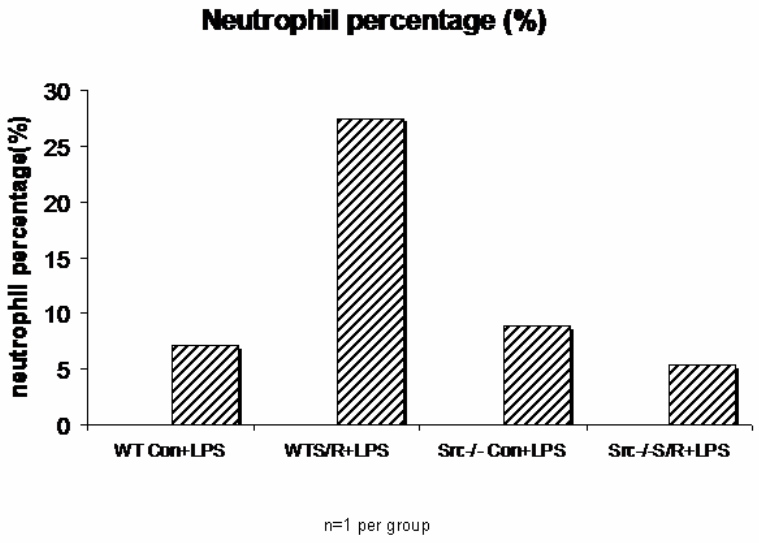
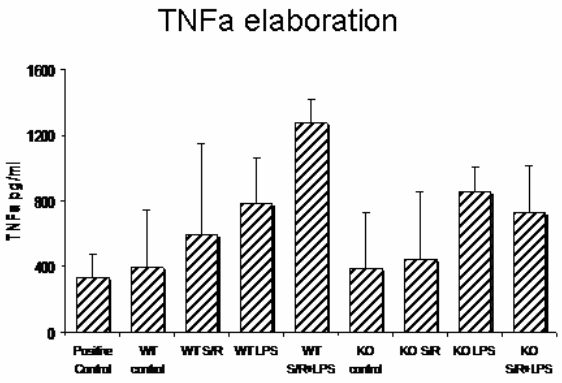
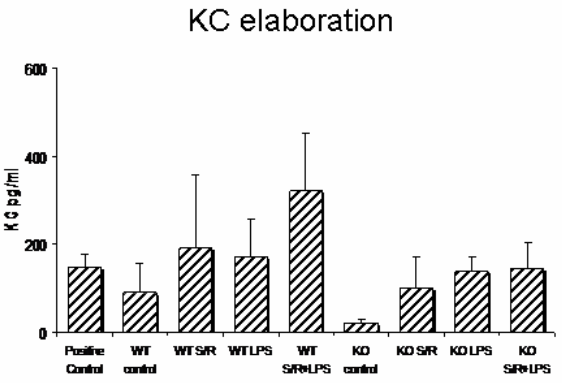


Figure 4: Src KO genotype inhibits elaboration of TNFa and KC following S/R and LPS in mouse two-hit lung injury model





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