Stromal Interacting Molecule 1 Signaling
and Lung Vascular Barrier Function

BY
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B.Sc. University of Calcutta, 2006

THESIS
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I would like to dedicate this thesis to my loving brother, Debapriyo Deb Roy, who lost his battle against sepsis at the tender age of 21.
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I am lucky to have several people in my life who have helped me, motivated me and inspired me through these life-altering six years.

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<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
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<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ATIII</td>
<td>AntithrombinIII</td>
</tr>
<tr>
<td>ATF2</td>
<td>Activating transcription factor 2</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CC</td>
<td>Coiled-coil</td>
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<td>CD14</td>
<td>Cluster of differentiation 14</td>
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<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<td>CRAC</td>
<td>Calcium release-activated calcium channel protein 1</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<td>EBA</td>
<td>Evans blue dye</td>
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<td>ECs</td>
<td>Endothelial cells</td>
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<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>ERK1</td>
<td>Extracellular signal-regulated kinases</td>
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<td>ERM</td>
<td>Ezrin-Radixin-Moesin</td>
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<tr>
<td>GPCRs</td>
<td>G protein-coupled receptors</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>HLMVECs</td>
<td>Human lung microvascular endothelial cells</td>
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<tr>
<td>ID</td>
<td>Inhibitory domain</td>
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<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
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<td>IkB</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
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<td>IL6</td>
<td>Interleukin-6</td>
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<td>IP₃</td>
<td>Inositol 1,4,5-trisphosphate</td>
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<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>LBP</td>
<td>LPS-binding protein</td>
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<td>Lung endothelial cells</td>
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<td>Lipopolysaccharide</td>
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<td>MAPK3K</td>
<td>Mitogen-activated protein kinase kinase kinase</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MCP-1</td>
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<td>MEF2</td>
<td>Monocyte-specific enhancer factor 2</td>
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<td>Myosin light chain</td>
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<td>Mouse lung endothelial cells</td>
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<td>MODS</td>
<td>Multiple organ dysfunction syndrome</td>
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<td>MSK-1</td>
<td>Mitogen- and stress-activated protein kinase 1</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAR</td>
<td>Protease-activated receptors</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidyl inositol 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4, 5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PKCδ</td>
<td>Protein kinase C, delta</td>
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<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<td>QRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>ROCs</td>
<td>Receptor-operated calcium channels</td>
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<tr>
<td>RPTK</td>
<td>Receptor protein tyrosine kinase</td>
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<td>SARM</td>
<td>Sterile α and HEAT-Armadillo motifs-containing protein</td>
</tr>
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<td>Scrambled-small interfering RNA</td>
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<td>Small interfering RNA</td>
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<tr>
<td>SOAR</td>
<td>STIM1-Orai activating region</td>
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<td>SOCE</td>
<td>Store-operated Ca²⁺ entry</td>
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<tr>
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<td>Store-operated Ca²⁺ channels</td>
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<tr>
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<td>Stromal Interacting Molecule 1</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor β (TGFβ)-activated kinase 1</td>
</tr>
<tr>
<td>TER</td>
<td>Trans-endothelial monolayer resistance</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TG</td>
<td>Thapsigargin</td>
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<tr>
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<td>Thrombin</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor, alpha</td>
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<tr>
<td>Trp</td>
<td>Transient Receptor Potential</td>
</tr>
<tr>
<td>TRP-C,M,V,P,ML</td>
<td>Transient receptor potential canonical, melastatin, Vanilloid, polycystin, mucolipin</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
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SUMMARY

The lung endothelium is a cell monolayer situated strategically in between the blood and the airspace. The endothelium acts as a semi-barrier regulating the exchange of fluids, nutrients and gas between circulation and the underlying tissue. Endothelial barrier dysfunction results in uncontrolled fluid extravasation and lung edema formation, the hallmarks of sepsis. Sepsis is a systemic inflammatory response to bacterial infection. The bacterial cell wall component lipopolysaccharide (LPS) binds to specific receptors on the endothelium activating a complex downstream signaling cascade that leads to an increase in endothelial permeability and the onset of inflammation. However, the exact mechanism that triggers endothelial barrier dysfunction is still unclear.

Previous studies from our laboratory have shown that calcium (Ca$^{2+}$) overload in the endothelial cells leads to endothelial hyper-permeability. We have also shown that inflammatory mediators such tumor necrosis factor-α (TNF-α) can induce increased store-operated Ca$^{2+}$ entry (SOCE) in endothelial cells (ECs), which leads to endothelial barrier dysfunction. It is known that proinflammatory mediators generated and released during sepsis, such as thrombin and oxidants can activate Ca$^{2+}$ entry through store-operated Ca$^{2+}$ channels (SOCs) in ECs. Previously, we reported that transient receptor potential canonical (TRPC) 1 and 4 channels function as SOCs in ECs. Importantly, studies have elucidated the mechanism of the endoplasmic reticulum (ER) localized Ca$^{2+}$ sensor protein, stromal interacting molecule 1 (STIM1), in activating SOCE through TRPC and Orai1 channels. Agonist induced ER-store Ca$^{2+}$ depletion causes clustering of STIM1 at the ER/plasma membrane interface, which in turn
binds to and activates SOCs in the plasma membrane. We hypothesized in the present study that increased STIM1 expression during sepsis signals abnormally high intracellular Ca\textsuperscript{2+} in ECs, resulting in loss of endothelial barrier function and a rise in vascular permeability.

This hypothesis predicts that toll-like receptor 4 (TLR4) signaling in ECs activates STIM1 transcription and thereby contributes to lung vascular hyper-permeability. We began our studies by showing that LPS regulates STIM1 expression transcriptionally using the pharmacological transcriptional inhibitor Actinomycin D which blocked LPS-induced STIM1 transcript formation. In addition, we observe increased expression of the SOC components, TRPC1, TRPC4 and Orai1 upon LPS (a.k.a. endotoxin) challenge in ECs. The increased expression of STIM1 and SOC components was associated with augmented protease activated receptor -1 (PAR-1)-mediated SOCE. SOCE was measured in single live cells by utilizing the calcium-sensitive dye, FURA-2AM. Further the increase in SOCE resulted in elevated vascular permeability as measured by both \textit{in vitro} and \textit{in vivo} approaches.

Next, we wanted to delineate the signaling mechanism by which LPS induces STIM1 expression. Previous studies have shown that LPS ligates TLR4 to induce the expression of cytokines, chemokines, adhesion molecules, apoptotic factors and several other mediators through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 mitogen activated protein kinase (MAPK) activation. Thus, we wanted to test if NF-κB and p38 MAPK activation were required for LPS-induced STIM1 transcription. We used siRNAs and
SUMMARY (continued)

pharmacological inhibitors to specifically inhibit the NF-κB and p38 MAPK signaling pathways. We observed that inhibition of either NF-κB or p38 MAPK prevented LPS-induced STIM1 mRNA and protein expression in human lung microvascular ECs (HLMVECs). We further dissected the MAPK signaling pathway and showed that only the p38α isoform regulates LPS-induced STIM1 expression. We also analyzed the STIM1 5’-regulatory region and found consensus binding sites for the transcription factors NF-κB and activated protein 1 (AP1), both of which have been shown to regulate proinflammatory genes. To determine if these transcription factors indeed bind to the STIM1 promoter, we performed chromatin immunoprecipitation assay (ChIP) and electrophoretic mobility shift assay (EMSA). We observe that p65/RelA (NF-κB component) and c-Fos and c-Jun (AP1 components) bind to the STIM1 promoter in a time-dependent manner in response to endotoxin stimulation. To further confirm the involvement of these factors in regulating LPS-induced STIM1 expression, we knockdown the NF-κB components, p65/RelA and p50 and the AP1 components, c-Fos and c-Jun. We observed that silencing of either of these genes leads to inhibition of LPS-induced STIM1 transcription and LPS-induced potentiation of PAR-1-mediated SOCE.

P38α signaling has been shown to regulate the transcription factor AP1. Since we observed that suppression of p38α expression in HLMVECs prevented LPS-induced STIM1 expression, we wanted to investigate the possibility that LPS-induced p38α activation augments STIM1 expression via the c-Fos transcription factor. To test this hypothesis we used p38α-siRNA to specifically knockdown p38α; in p38α-depleted cells, we showed inhibition of c-Fos
activation and subsequently of LPS-induced STIM1 expression. Thus these results suggest that the co-operative signaling of NF-κB and p38α regulates LPS-induced STIM1 expression.

In the last part of the thesis, we have addressed the pathophysiological relevance of endothelial-STIM1 by utilizing endothelial-restricted STIM1 (STIM1\textsuperscript{EC-/-}) mice. Here we show that deletion of STIM1 in ECs blocks PAR-1-mediated SOCE in lung microvessels. In addition, by utilizing \textit{in vivo} and \textit{ex vivo} approaches, we measured PAR-1-induced lung vascular leak in LPS primed STIM1\textsuperscript{EC-/-} mice. We observed that STIM1\textsuperscript{EC-/-} mice were completely protected from endothelial barrier dysfunction and pulmonary edema formation associated with sepsis. These results comprehensively suggest a critical role of endothelial-STIM1 in regulating lung microvascular leak in sepsis-associated acute lung injury.
1. LITERATURE REVIEW

1.1 Endothelial Cells

1.1.1 Endothelial structure and function

The endothelium is a selectively permeable monolayer of endothelial cells lining the blood vessels (1). Endothelial cells (ECs) are essentially modified squamous epithelial cells and their function is of paramount importance in vascular biology. The endothelial cells arise from the hemangioblast along with the hematopoietic cells. Further endothelial cells also transdifferentiate into mesenchymal cells and intimal smooth muscle cells. They express different markers depending on the location and generate different responses to the same stimulus. Interestingly, endothelial cells also demonstrate polarity by expressing a myriad of surface membrane components. The endothelial cells lining the lymphatic vessels are called the lymphatic endothelial cells and the ones lining the blood vessels are called the vascular endothelial cells.

The vascular endothelium is strategically placed in between the blood and the tissue and is always exposed to blood on one side. It is responsible for maintaining the integrity of the blood vessels, blood fluidity and blood flow (1,2). The vascular ECs express inhibitors of the coagulation pathway including tissue factor pathway inhibitor (TFPIs), heparin sulphate proteoglycans, and thrombomodulin which help to maintain the fluidity of blood. Further the vascular ECs produce mediators such as nitric oxide (NO) which regulates the tone of the surrounding smooth muscle cells thus maintaining the blood flow (3).
Capillaries are made up of endothelial cells and are categorized in three groups: Continuous capillaries (create a continuous barrier excluding proteins by forming tight and adherens junctions with the adjacent endothelial cells), fenestrated capillaries (endothelial cells with discontinuous junctions forming pores, often permitting the blood to contact the underlying cells), sinusoid capillaries (they are a variant of the fenestrated capillaries with larger openings allowing red and white blood cells to pass through) (3). The continuous vascular ECs are found in the lung, central nervous system and skeletal muscles while the discontinued capillaries are found in the liver, spleen, pancreas and kidney. Vascular ECs are multifunctional. They traffic essential nutrients, plasma proteins, macromolecules and cells such as leukocyte, monocyte, lymphocyte, in a specific and highly regulated manner. Ultimately the endothelial cells regulate immune responses, inflammation and coagulation (3). Thus endothelial dysfunction gives rise to varied complications resulting in complex pathological states.

### 1.1.2 Endothelial dysfunction and inflammation

Inflammation is a protective mechanism by an organism against harmful stimuli such as pathogens. At the site of infection macrophages phagocytose bacteria and release different kinds of proinflammatory mediators such as cytokines (eg. TNF-α and interleukins) and chemokines which activates the ECs. There are two phases for the activation of ECs, type I (immediate response) and type II (slower response) (3). Both types of activation phases trigger the fundamental signs of inflammation: rubor (increased blood flow resulting in red color), calor (warmth due to inflammation of the tissues), tumor (swelling due to endothelial contraction leading to the leakage of the interstitial fluid) and dolor (pain due to the release of mediators by
the activated leukocytes recruited in the damaged tissues) (1,3). Previous studies indicate that central to the process of inflammation and sepsis lies endothelial dysfunction as noted by an increase in permeability or loss of barrier function (4).

1.2 Sepsis

1.2.1 Pathogenesis

Sepsis, a systemic inflammatory response syndrome, is a severe and debilitating clinical condition (5). It is to-date the most common cause of death among hospitalized patients in the non-coronary intensive care unit (4). Sepsis is usually manifested by two or more of these criteria, changes in body temperature, heart rate, respiratory rate and the white blood cell count (5). Depending on its severity, sepsis can lead to multi-organ failure. Sepsis leads to inappropriate regulation of a multitude of cell types, proinflammatory mediators and procoagulation factors. Sepsis is mostly induced by gram-negative bacterial infection (62% gram-negative infection, 47% gram-positive infections and 19% fungal infection) (6). The respiratory and the cardiovascular systems are usually affected the most and the classical manifestations of sepsis are acute respiratory distress syndrome (ARDS) and myocardial dysfunction (6).

Independent of the type of infection triggering the onset of sepsis (gram positive or gram negative bacteria), the mortality rates have been high (4). Interestingly, therapies involving the administration of antibodies directed against the endotoxin have largely failed during clinical trials indicating that host response plays a vital role in determining the severity of
sepsis. Monocytes, macrophages and endothelial cells recognize pathogens through the pattern recognition receptors (such as toll-like receptors or TLRs) for triggering a host response or its first line of defense (a.k.a. innate immunity). The host response upon infection yields soluble mediators responsible for enhancing the effects of the inflammatory and the coagulation cascades. Ultimately during the host’s attempt to eliminate pathogens, excessive inflammatory response and/or inadequate anti-inflammatory response can inflict collateral damage on normal tissues resulting in focal distribution of the disease state (4).

1.2.2 **Acute lung injury (ALI)/Acute respiratory distress syndrome (ARDS)**

The common causes of acute lung injury (ALI) includes sepsis, pneumonia, pancreatitis, trauma and aspiration (7). It is manifested by lung inflammation and neutrophil infiltration (7). ALI/ARDS have a reported mortality rate of approximately 25%, the majority of which are attributed to sepsis or multiple organ failure (8). During ALI/ARDS protein rich fluid extravasates into alveoli due to collapse of the alveolar-capillary barrier. Macrophages in the alveolus secrete cytokines such as interleukins and TNF-α, which further stimulates chemotaxis and activates the neutrophils. Neutrophils in turn release oxidants, proteases, leukotrienes, and other proinflammatory molecules propagating inflammation. Additionally during ALI, surfactant protein production is compromised and both alveolar-capillary and epithelial-cell injury is observed (7,9).
1.3 Lipopolysaccharide

1.3.1 Structure and function

Lipopolysaccharides (LPS) are large molecules composed of lipid A, a core polysaccharide and an O side chain. The lipid A portion is conserved amongst gram negative bacterial membrane constituent and is mainly responsible for triggering biological toxicity (10). LPS binds to the LPS-binding protein (LBP), present in serum, and LBP rapidly catalyzes the transfer of LPS to the CD14 receptor (membrane bound or soluble). GPI-linked CD14 lacks trans-membrane and intracellular domains, and the CD14-LPS complex with LPS elicits trans-membrane signaling via additional membrane receptors (11,12).

TLRs recognize antigens unique to bacteria, fungi and viruses; for example lipopolysaccharide is recognized by TLR4, flagellin by TLR5, lipopeptides by TLR2 and CpG DNA by TLR9. Depending upon the cell type and the stimuli, combinations of a diverse range of receptors are activated in the cluster thus giving rise to broad ligand specificity (13). TLR4 upon activation by LPS, undergoes oligomerisation and recruits adaptor proteins such as TIR (Toll-interleukin-1 receptor), MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain containing adaptor protein), TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule), and SARM (sterile α and HEAT-Armadillo motifs-containing protein) (14). LPS has been shown to activate multiple signaling pathways including mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and ERK1, ultimately activating proinflammatory transcription factors, most importantly,
NF-κB [Fig. 1]. Specifically, LPS has been shown to mediate NF-κB activation through TAK1 [Transforming growth factor β (TGFβ)-activated kinase 1] during inflammation (15). NF-κB, in endothelial cells functions as the major player in eliciting the effects of LPS by promoting the transcription of proinflammatory genes such cytokines such as interleukins and TNF-α. Depending upon the severity of bacterial infection there can be uncontrollable induction of inflammation leading to cardiovascular collapse, hemodynamic instability and ultimately cause fatal sepsis syndrome (13).

1.3.2 Downstream signaling pathways of TLR4

a) Nuclear factor-κB (NF-κB)

NF-κB plays a central role in mediating inducible transcription in both the innate and adaptive immune system (16). It regulates the generation of several cytokines, chemokines and adhesion molecules in response to TLR4 activation in the vascular endothelial cells and tissues. This leads to the recruitment and activation of effector cells, such as the neutrophils, macrophages and other leukocytes. It is activated by diverse stimuli and is involved in regulating a host of immunoregulatory mediators thus modulating a broad range of cellular responses. These attributes have made NF-κB a key player in numerous pathological conditions including inflammation, acute respiratory distress syndrome (ARDS), sepsis, multiple organ dysfunction syndrome (MODS), apoptosis and cancer (16-18).
Following ligation of the TLR4 receptor with LPS, binding proteins such as TRAF6 and Myd88 (not shown) are recruited to the receptor. Consequently TAK1 is activated which now activates the IKK complex inducing phosphorylation and translocation of the transcription factor NF-κB into the nucleus (15). TAK1 stimulates MAP3Ks activating p38 MAPK, ERK (not shown) and JNK (not shown) pathways (19). AP1 is activated downstream of p38 MAPK signaling pathway which now drives the transcription of target genes.

Figure 1: Schematic representation of the potential cascade of events downstream of TLR4 activation.
The mammalian NF-κB family consists of five proteins, all of which share a conserved amino-terminal region called the Rel-homology domain (RHD) (16-18), dimerization domains and also the nuclear localization signal (NLS). NF-κB, present in the cytoplasm, usually exist as homo- or hetero- dimers of these five structurally related proteins; p65 (a.k.a. RelA), c-Rel, RelB, p50/p105 (a.k.a. NF-κB1) and p52/p100 (a.k.a. NF-κB2). The first three proteins, p65, c-Rel and RelB are produced transcriptionally active; however, NF-κB1 and NF-κB2 are produced as longer precursor molecules, 105 and 100 kDa respectively, which are further processed to smaller transcriptionally active form (16-18).

Eight IκB family members regulate NF-κB by sequestering them, in an inactive form within the cytoplasm (16-18). Among the eight members, IκBα, IκBβ, IκBε, IκBδ, BCL-3, IκBNS, p100 and p105, the first three are the most important ones (16). The IκB family contain multiple copies of a 30-33 amino acid sequence called the ankyrin repeat domain, which are conserved and are used to regulate NF-κB by masking its NLS domain, preventing nuclear translocation and DNA binding (17). IκBα activation is the prototype for the rest of the IκB family of proteins (16). Following stimulation IκBα undergoes phosphorylation directing it towards proteasomal degradation. Consequently, NF-κB bound to the IκBα is released and the cytoplasmic NF-κB dimer is translocated to the nucleus where it drives transcription of numerous genes including that of IκBα itself. Thus IκBα regulates NF-κB transcriptional activity in a negative feedback fashion (16-18). IκB proteins are phosphorylated by activated IκB kinase (IKK complex). The IKK complex consists of three components, the two homologous kinases, IKKα and IKKβ and a regulatory subunit, IKKγ (also known as NEMO). Previous studies have
shown that IKKβ is necessary and sufficient to phosphorylate IκBα in the canonical NF-κB signaling pathway, although recently a significant role for IKKα was also suggested (16). IKKα plays an important role in regulating NF-κB signaling by phosphorylating non-IκB substrates such as transcriptional co-activators and co-repressors (16). It can also phosphorylate NF-κB itself, specifically the p65 subunit, thus modulating the recruitment of co-activators or co-repressors to NF-κB, which in turn regulates NF-κB mediated gene transcription (16). IKKγ is required for classical (canonical) signaling in the NF-κB pathway. The other signaling cascade activating NF-κB is the alternate (non-canonical) pathway which is mainly activated downstream of TNF receptors. This pathway relies on the NF-κB inducing kinase (NIK) along with IKKα, which induces phosphorylation dependent ubiquitination and processing of p100 thus releasing the p52 containing dimers (18). This pathway, in contrast to the canonical pathway, is dependent on p100 phosphorylation rather than IκB. Kinases such as IKKα, IKKβ, NIK, PKCδ, PKA, PKG and GSK3β can directly phosphorylate p65 and enhance NF-κB transactivation activity, depending upon the stimuli.

\[b) \textit{MAPK pathways}\]

MAPKs are evolutionarily conserved enzymes which are activated during stress and immune responses and regulate cell survival and differentiation (20). They are critical in mediating the functional effects of LPS by translating extracellular stimuli into a wide range of cellular responses (19). It is a three-tiered signaling cascade starting with the MAP3Ks [A/B/C-Raf, MEKK1/4, ASK1/2, MLK1/2/3, MEKK 2/3], then MAP2Ks [MKK1-7] and finally MAPK [ERK1/2/5, p38α/β/γ/δ, JNK1/2/3]. Depending upon the stimuli and the tissue affected,
MAPKs activate varied transcription factors and affect numerous cellular processes such as proliferation, cell division, apoptosis, inflammation, and cell differentiation. Activation of MAPKs requires either mono- or dual-phosphorylation on the Thr-X-Tyr motif by the MAP2Ks in the activation loop. Subsequently MAPKs phosphorylate substrates on serine and threonine residues and signaling is turned off by generic or dual-specificity phosphatases (19).

Published evidence shows that the p38 MAPK signaling pathway plays a major role in mediating inflammation (21,22). Since then, in-depth analysis and studies have mapped out the differential expression of the p38 MAPK isoforms in inflammatory cell lineages (21). Four different MAPK genes, MAPK11, MAPK12, MAPK13 and MAPK14, encode the four different p38 isoforms, p38α [expressed ubiquitously in most cell types], p38β [brain and endothelial cells], p38γ [skeletal muscle] and p38δ [endocrine glands and endothelial cells] respectively. Thus endothelial cells express α, β, and δ isoforms, which are activated by stress or inflammatory stimuli via the canonical signaling pathway. MKK6 kinase is an activator of p38 MAPKs, and MKK3 and occasionally MKK4 can also activate the p38 isoforms other than p38β (19,21). P38 MAPK signaling regulates two groups of proteins: transcription factors including p53, NF-κB, ATF2 (activating transcription factor 2), monocyte-specific enhancer factor 2 (MEF2), C/EBPβ MSK1 and protein kinases such as MAPK-activated kinase 2 (MK2 a.k.a. MAPK2), mitogen- and stress-activated protein kinase 1 (MSK1) and MAP kinase-interacting serine/threonine kinase 1 & 2 (MNK1, MNK2).
P38α/β/δ are abundant in the endothelial cells; however, immunokinase assays show comparatively higher activation of the p38α isoform in LPS-challenged ECs (19). Similar observations were made in macrophages. In addition, genetic evidence shows p38α to be a critical player during inflammatory responses in myeloid and epithelial cells (19). Pyridinylimidazole drugs are used to target p38α which inhibits cytokine production and inflammation (20). P38α has been shown to regulate the production of proinflammatory cytokines, such as TNF α, IL-1 and IL-6, by modulating the function of the transcription factor NF-κB or the mRNA stability of the target genes post-transcriptionally. It also facilitates the binding of NF-κB to DNA by chromatin modifications and by increasing the accessibility of NF-κB to encrypted sites (22). Strong activation of p38α is also evident when ECs are stimulated with TNF α, IL-1, IL-6 or LPS, suggesting a feedback mechanism which regulates and maintains p38 signal transduction and is consistent with its important role in inflammation (20).

Jun N-terminal kinase (JNK) proteins are also known as (a.k.a.) stress activated protein kinases (SAPKs), which are encoded by three different genes MAPK8 (jnk1), MAPK9 (jnk2) and MAPK10 (jnk3). The mRNAs generated are spliced alternatively to generate ten different isoforms (19,23). MKK4 and MKK7 can act synergistically to activate the JNK pathway; however, specifically during inflammation MKK7 plays a vital role in activating the latter (23). Usually activation involves a dual phosphorylation on the threonine and tyrosine residues within the threonine-proline-tyrosine (TPY) motif. The JNK proteins primarily target the members of the activator protein 1 (AP1) family of transcription factors. AP1 consists of Jun and Fos family members, mainly c-Fos and c-Jun, that form homo- or hetero- dimers, and the
JNK pathway has been shown to primarily induce c-Jun resulting in the activation of a plethora of target genes (19,23). The p38 MAPK and the JNK pathway share a number of upstream regulators and thus multiple stimuli such as LPS can activate both the pathways simultaneously. This might result in the synergistic activation of the transcription factor AP1; however, reports of antagonistic interactions between the two pathways have also appeared (19). For example, p38α inhibits the upstream activator of JNK, TAK1 and MLK3 (19). Thus crosstalk between these two pathways brings about a variety of functions depending upon the cell type and the stimulus. They play a key role in chronic inflammation which is also a potent cancer promoter (19). A number of p38 MAPK and JNK inhibitors have also been tested in clinical trials, such as, Talmapiomid (targeting p38), SB-681323 (targeting p38) and XG-102 (targeting JNK) but most of them, although showed promising results in the preclinical trial, could not progress beyond Phase I clinical trials owing to side effects such as liver toxicity (19). A new generation of drugs selectively targeting p38 MAPK is currently being developed that seem to have lesser toxicity in animal models.

1.4 Thrombin

1.4.1 Structure and Function

Thrombin is a trypsin-like serine protease which plays a key role in maintaining hemostasis (24). It is responsible for regulating various physiological and pathophysiological processes including blood coagulation, anti-coagulation, inflammation, tissue repair and artherogenesis (25). It cleaves substrates with high specificity, determined by the “classical”
active site surrounded by some critical features, loops and charged patches. Two additional recognition centers, anion-binding exosite 1 (a.k.a. fibrinogen recognition site) and anion-binding exosite 2 (a.k.a. heparin-binding site), interact with complementary sites of specific substrates, inhibitors and receptors thus implementing critical regulatory functions (25). Another loop with Na\(^+\) binding site is responsible for the allosteric regulation of thrombin (24,25). Two Na\(^+\) binding sites are present. Depending upon the occupancy of the site, conformational changes occur directing thrombin to a “slow” [Na\(^+\)-free] or a “fast” [Na\(^+\)-bound] form (25). The fast form is characterized by higher affinity and catalytic activity toward fibrinogen, favoring pro-coagulant activities. By contrast, the slow form specifically activates Protein C, shifting thrombin towards anti-coagulant activities (25). Thus allosteric regulation is critical to thrombin function. Thrombin generation activates various downstream signaling pathways resulting in calcium influx, cytoskeletal reorganization, release of soluble mediators, growth factors, metalloproteinases and enhanced expression of genes involved in inflammation, cell proliferation, leukocyte adhesion, vasomotor tone and hemostasis (26).

1.4.2 **Coagulation**

Depending upon the mode of initiation, there are two pathways by which the coagulation cascade is activated, the extrinsic (tissue factor) pathway and the intrinsic pathway (surface contact) (27). Both pathways converge at the thrombin production step, mediated by the proteolytic action of factor Xa on its substrate prothrombin. The thrombin generated in turn acts on fibrinogen, converting it into fibrin (27). Coagulation relies on a group of soluble factors synthesized in the liver, circulating in the plasma in inactive zymogen forms. They are denoted
with roman numerals and the active forms are denoted with a lower case “a” following the Roman numeral (27). These factors have been categorized accordingly: Factor I [Fibrinogen], Factor II [Prothrombin], Factor III [Tissue factor], Factor IV [Calcium], Factor V [Proaccelerin], Factor VII [Proconvertin], Factor VIII [Antihaemophilic factor], Factor IX [Christmas factor], Factor X [Stuart-Prower factor], Factor XI [Plasma thromboplastin antecedent], Factor XII [Hageman factor] and Factor XIII [Fibrin stabilizing factor] (27). Most of the factors, other than factor XIII, are serine proteases in their active forms and are related to the digestive enzyme trypsin. Other factors including tissue factor, factor V, Factor VIII and high molecular weight kininogen (HK) act as co-factors.

a) **Extrinsic pathway**

Extrinsic pathway is the main mechanism of thrombinogenesis in the blood stream which is triggered by exposure of the tissue factor on the surface of the endothelial cells and monocytes (25). Tissue factors are glycoproteins composed of extracellular, transmembrane and cytosolic domains; they are expressed on the sub-endothelium of blood vessels, and thus not exposed to circulating blood. Tissue factor requires phospholipid for activation thus ensuring coagulation complex formation at the site of injury. Factor VII, consisting of two chains, circulates in the blood as an active protease; VIIa is cleaved at Arg152, a conformation that prevents plasma activation (25,27). However when bound to tissue factors, the conformation changes into an active form which in turn activates the factors IX and X.
b) **Common Pathway**

This is the point at which both the intrinsic and the extrinsic pathway converge. Factor Xa, V and II form the “prothrombinase” complex which plays an instrumental role in generating thrombin (27). Factor Xa can cleave the prothrombin to produce thrombin. Thrombin cleaves fibrinogen to expose the centre which enables bulbous ends of other fibrinogen to tether together. Fibrins composed of β and γ chains are produced that form a meshwork surrounding the site of injury and also capturing about 40% of the thrombin present to protect it from the natural inhibitors (27). To ensure that fibrin formation is at the desired location and is not washed off by the circulating blood, two methods are usually employed. Firstly, platelets bind and stabilize the fibrin strands with their phospholipids, and a series of inhibitors constrain the platelet hemostatic plug to the site of injury (27). Following activation, a platelet-specific enzyme flipase reverses the charged phospholipids on the platelets to direct the negatively charged phosphatidylserines to face outward. On the other hand the Vitamin K dependent coagulation factors possess negatively charged glutamic acids at their N-terminal regions. Ca\(^{2+}\) ions buffer the highly negatively charged acid phospholipids and the proteins and induce conformational change on the latter stabilizing the platelet plug (27). Secondly, natural inhibitors such as, antithrombin III (ATIII), heparin cofactor II (HCII), tissue factor pathway inhibitor (TFPI), thrombomodulin and protein C in the blood restrain the platelet plug at the site of injury by various methods (27).

1.4.3 **Protease-activated receptors (PAR)**

The PARs belong to a G-protein coupled receptor super family having four
members, PAR-1, PAR-2, PAR-3 and PAR-4. Signal transduction by PARs is sustained intracellularly by heterotrimeric G-proteins which bind to the intracellular domain of the receptor (28). Following activation, G-proteins coupled to intermediates including phospholipase C (PLC), mitogen activated protein kinase (MAPK), protein kinase C (PKC), phosphatidyl inositol 3-kinase (PI3K) and AKT (26). Mammalian PAR activation occurs by a number of trypsin-like serine proteases including thrombin, prothrombin intermediates, granzyme A, Factor Xa, trypsin IV and activated protein C. Thrombin specifically activates PAR-1, PAR-3 and PAR-4 receptors which are coupled to different G-protein such as, $G_a$ (types: $G_{z11}$, $G_0$, $G_{a12}$, $G_{a13}$ and $G_i$), $G_\beta$ and $G_\gamma$, thus depending upon the stimulus and the tissue, various intracellular signaling cascades get activated (25). PAR-2 does not get activated by thrombin but does by the coagulation proteases VIIa and Xa (29). Reports show that human umbilical vein endothelial cells express predominantly PAR-1 receptors with a lesser extent of PAR-2, PAR-3 and PAR-4 (26). Genetic studies performed with single knockout (PAR-1/PAR-2/PAR-4) or double knockouts (PAR-1/PAR-2; PAR-2/PAR-4) confirm that the main thrombin receptor on the microvascular endothelial cells is PAR-1 (29). A direct relation between PAR-1 expression and $Ca^{2+}$ concentration was established in the human microvascular endothelial cells as well by utilizing PAR-1 knockout mice (30,31).

Thrombin binds to a hirudin-like domain on the extracellular sequence of PAR-1 and cleaves PAR-1 between Arg41 and Ser42. Consequently the new N-terminus is exposed (26,28). The tethered ligand (SFLLRN) now interacts with extracellular loop 2 (amino acids 248 to 268) of the receptor, resulting in its activation [Fig. 2]. Agonist bound PAR-1 binds to $G_{z11}$
Figure 2: Thrombin-mediated PAR-1 cleavage and activation. Thrombin binds to a hirudin-like domain on the extracellular sequence of PAR-1 and cleaves PAR-1 between Arg41 and Ser42. The new exposed N-terminus is the tethered ligand (SFLLRN) which now interacts with the extracellular loop 2 (PAR-AP) of the receptor. This leads to the activation of the PAR-1 receptor. Reproduced with permission from the American College of Chest Physicians, Lawrence F. Brass “Thrombin and Platelet Activation”, 2003 (32).
phosphatidylinositol-4, 5-bisphosphate (PIP2) to yield the second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 induces downstream signaling ultimately mobilizing Ca^{2+} into the cytosol from the endoplasmic reticulum (ER) (28). On the other hand, DAG activates PKCα, which can phosphorylate VE-cadherin junctional proteins leading to the disassembly of VE-cadherin junctions. Also, PKCα can activate MLC generating contractile forces and further enhancing endothelial retraction by activating Rho-kinase (33).

1.4.4 Coagulation-Inflammation

Inflammation and coagulation are two major signaling cascades that exhibit cross-talk with each other triggering the onset of sepsis (4,34). During inflammation, lipopolysaccharide induces expression of tissue factor on the surface of endothelial cells, monocytes and macrophages. This leads to the activation of the extrinsic pathway of the coagulation cascade, thrombin generation and consequently fibrin formation. Thrombin generation by the extrinsic pathways further activate the intrinsic pathway amplifying blood coagulation. In addition, thrombin, a potent activator of the PAR-1 receptor, induces PAR-1-mediated downstream signaling in the cells including endothelial cells. In addition, it has been shown in our laboratory that the major proinflammatory transcription factor, NF-κB is activated downstream of thrombin-mediated PAR-1 activation (35). Along with the activation of the coagulation pathway there is usually a concomitant activation of the anti-coagulation pathway. During sepsis, there is an inhibition of the natural coagulation inhibitors including reduction in the levels of the circulating protein C, ATIII and decreased thrombomodulin expression. Sepsis is also associated with impaired fibrinolysis which usually facilitates engulfment and disposal of
the pathogens (4). Further thrombin enhances expression of P-selectin, E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) which dictates changes in the cell shape, cell permeability, proliferation and leukocyte adhesion. Additional inflammation is also triggered by thrombin’s ability to increase expression of PAR-1, secretion of von Willebrand factor (vWF), matrix metalloproteinsae and stimulate release of soluble mediators including platelet activating factor (PAF), IL8, monocyte chemo-attractant protein 1 (MCP-1) and growth factors (4). The positive feedback loop mechanism between inflammation and coagulation, amplifies individual responses triggering a potentially explosive host response during sepsis (4). Previous publications from our laboratory have shown a direct relation between enhanced PAR-1-mediated store-operated \( \text{Ca}^{2+} \) influx and increased endothelial permeability using animal model as well as in vitro cell cultures (31,36,37).

1.5 Calcium Signaling

1.5.1 Types & Function

Calcium is an essential second messenger in nearly all eukaryotic cells. The importance of extracellular \( \text{Ca}^{2+} \) influx within the cell in maintaining contraction of isolated hearts was first recognized by Ringer (38). The concentration of \( \text{Ca}^{2+} \) in the cell is strictly maintained at \( \sim 100 \text{ nM} \) and alteration of this concentration triggers a plethora of cellular responses such as muscle contraction, mitochondrial metabolism, neurotransmitter release, cell growth, cell proliferation, and gene expression (33,39). Indiscriminate release of \( \text{Ca}^{2+} \) can lead to major pathophysiological conditions including cell death, necrosis, inflammation and sepsis (39).
Others and we have shown a direct link between increased calcium overload and endothelial hyper-permeability (33,37,40,41). Calcium within the cell enters from the extracellular space by three mechanisms: Voltage-dependent calcium channels (VDCCs), Receptor-operated calcium channels (ROCs), and store-operated channels (SOCs) (33,42). The VDCCs are found in the membranes of excitable cells including muscle, neurons, and glial cells. They are activated upon depolarization of the membrane potential and thus called “voltage-dependent” (41). Calcium measurements revealed that there were periodic rises of calcium concentration in the cells originating from a fixed point and propagated with waves. This phenomenon was termed calcium oscillations (43). In non-excitable cells such as endothelial cells, several studies have shown the major calcium influx pathway is the store-operated Ca\(^{2+}\) entry (SOCE) (44,45).

### 1.5.2 Store-operated Ca\(^{2+}\) entry

In 1986, Putney proposed the concept of capacitative Ca\(^{2+}\) entry where he suggested that agonist-stimulated generation of second messengers could deplete the ER-stored Ca\(^{2+}\) (46). This brief elevation of Ca\(^{2+}\) in the cell could evoke sustained increase in Ca\(^{2+}\) influx within the cell from the extracellular medium in non-excitable cells including mast cells, endothelial cells, thymocytes, and T cells (47). In 1993, Hoth and Penner went on to describe Ca\(^{2+}\) release activated Ca\(^{2+}\) current (CRAC current) in mast cells, which is a highly Ca\(^{2+}\) selective, non-voltage gated, inward rectifying current (48). SOCE involve two distinct phases, activation of the EC membrane-localized receptors including receptor protein tyrosine kinase (RPTK), GPCRs (eg. PAR-1) induces the transient release of the stored calcium from the endoplasmic reticulum, followed by the sustained influx of calcium from the extracellular
medium to inside the cell. Upon activation of the ECs, activated phospholipase C (PLC) produces a second messenger, diacylglycerol (DAG) which is known to activate the receptor-operated calcium channels (41) [Fig. 3]. Studies performed by us and others have established a critical link between the SOC and endothelial permeability associated with inflammation (41).

An inflammatory mediator such as thrombin, histamine, bradykinin and oxidants stimulate endothelial cells eliciting downstream signaling which results in the generation of IP$_3$ by PLC. IP$_3$ binds and activates the IP$_3$ receptor (IP$_3$R) on the ER membrane (41). There are three distinct IP$_3$Rs (IP$_3$RI, IP$_3$RII, IP$_3$RIII) approximately 300 kDa each and originating from the ryanodine channel family. They have cell-specific expression with varying sensitivity to IP$_3$. IP$_3$RII is the most sensitive and IP$_3$RIII being the least (41). IP$_3$R exist in tetrameric structures and are regulated by both concentration of its ligand IP$_3$ as well as the intracellular concentration of Ca$^{2+}$. All the isoforms have consensus phosphorylation site for kinases including, AMP-dependent kinase II (PKA), Ca$^{2+}$/calmodulin-dependent kinase II (CamKII), PKA and PKC (41). Activation of the IP$_3$R induces release of the stored Ca$^{2+}$ in the ER. This activates an ER-membrane localized protein, Stromal Interacting Molecule 1 (STIM1), which oligomerises and interacts directly with the cell-membrane localized channels eliciting store-operated calcium entry (SOCE) (49). This allows Ca$^{2+}$ entry into the cells and maintains a sustained increase in [Ca$^{2+}$]$_i$ (intracellular calcium concentration).
Figure 3: Schematic representation of the mechanism of ER-store Ca^{2+} release and Ca^{2+} entry in endothelial cells. Activation of Receptor tyrosine kinases and G-protein coupled receptors can release the stored Ca^{2+} from ER by activating PLC and generating IP_3. IP_3 binds to IP_3R on the ER membrane inducing stored Ca^{2+} release (33).
1.5.3 Store operated Ca\(^{2+}\) channel [SOC]

\(\text{a) Transient receptor potential (Trp) canonical channel}\)

The name “Transient Receptor Potential” (Trp) was thus coined (50). In 1989, Montell and Rubin first cloned sequenced and characterized the Drosophila Trp gene, and suggested it encodes a protein which is a receptor/channel (51). However, previous studies had electrophysiological recordings indicating Trp functions normally under dim light, suggesting it was not a light-sensitive channel (51). Further experiments with Limulus photoreceptors showed that G-proteins mediates light excitation and light-sensitive channels can be activated in the dark by the exogenous application of inositol-1,4,5-trisphosphate (InsP\(_3\)) (52,53). Further screening of genes identified two genes encoding light sensitive G-protein subunits, G\(_q\)\(\alpha\) and G\(_q\)\(\beta\) (54,55). Mutation of the G\(_q\)\(\alpha\) subunit highly reduced sensitivity to light in the presence of unchanged levels of G\(_q\)\(\beta\), PLC or rhodopsin (56). This suggests G\(_q\)\(\alpha\) subunit to be the critical and essential mediator of stimulations by light (56).

Trp family of receptors comprises of more than twenty related cation channels with diverse modes of activation and cation selectivities (57). Trp genes encode a superfamily of proteins with significant sequence homology and structural similarities. They are grouped in 7 subfamilies: TRPC (classical or canonical), TRPM (melastatin), TRPV (vanilloid), TRPA (ankyrin), TRPP (polycystin), TRPML (mucolipin) and TRPN (no mechano-receptor potential C) (58). The mammalian proteins that are most related to the Drosophila TRP are TRPV, TRPM, TRPC, the latter having the highest sequence homology. TRPP and TRPML are distantly related.
to TRP. TRPC homologues have been shown to play a critical role in regulating endothelial permeability (33,40,41). They are nonselective, monovalent cation channels also allowing the entry of Ca\(^{2+}\). They consist of 7 isoforms: TRPC1 to TRPC7 (33). Members of the TRPC protein subfamily contain 700-1000 amino acids and 6 transmembrane helices (33,44,59). The TRPCs are divided in four subfamilies ~ a) TRPC4 and TRPC5 (high Ca\(^{2+}\) selectivity as assessed sensitivity to La\(^{3+}\), 1µM) b) TRPC1 which is also closely related to TRPC4 and TRPC5 (less Ca\(^{2+}\) selectivity as assessed sensitivity to La\(^{3+}\), 40 to 100 µM), c) TRPC3, TRPC6, TRPC7 and lastly d) TRPC2, it is present in mice sperm and vomeronasal organ while its function is unknown in humans (41). Similar to Trp activation in Drosophila, where PLC activity is required for its activation, TRPC1, TRPC4 and TRPC5 gets activated by IP\(_3\) generation (catalyzed by PLC) and functions as Store operated Ca\(^{2+}\) channels (SOCs) while TRPC3, TRPC6 and TRPC7 gets activated by DAG (catalyzed by PLC) and function as Receptor operated Ca\(^{2+}\) channels (ROCs).

TRPCs are six-transmembrane helices protein with both the N- and C-terminus facing towards the cytoplasm (41)[Fig. 4]. The N-terminus consists of 2-5 highly conserved ankyrin repeats which interact with the cytoskeleton. The C-terminus consists of a proline-rich motif and a calmodulin binding domain. All the TRPCs also contain a highly conserved 25 amino acid sequence called the TRP domain and a cation permeable pore located between transmembrane helices 5 and 6 (33). TRPCs function by forming homo- or hetero-tetramers such as TRPC1/1, TRPC4/5 or TRPC3/6/7.
Figure 4: Structure of TRPC. The N-terminus of TRPC contains three ankyrin repeats (yellow) facing the cytosol. The extracellular domain, between transmembrane helix 5 and 6 contains the calcium binding pore (indicated with an arrow). The C-terminus contains highly conserved proline rich motif (P). Reprinted from Vascular Pharmacology, 39, Tiruppathi C, Minshall RD, Paria BC, Vogel SM, Malik AB. “Role of Ca^{2+} signaling in the regulation of endothelial permeability”, 2002, 173-185, with permission from Elsevier (41).
The most commonly expressed TRPCs in endothelial cells isolated from vascular beds of humans are TRPC1, TRPC3, TRPC4 and TRPC6. We have shown the knockdown of the dominantly expressed isoform in human vascular endothelial cells, TRPC1 and the dominantly expressed isoform in mouse lung endothelial cells, TRPC4, abolished thrombin-induced Ca\(^{2+}\) entry in the respective cell types (35,60). We further showed that knockdown of these isoforms also impaired thrombin-induced increase in permeability (37,61). It has also been published from our laboratory that TNF-α and thrombin enhance the expression of TRPC1 via NF-κB signaling in human endothelial cells (36,40). Interestingly, enhanced TRPC1 expression augmented thrombin-induced Ca\(^{2+}\) influx and permeability (40). These unique findings suggest that Ca\(^{2+}\) entry through TRPC channels in endothelial cells may contribute to the hyper-permeability associated with sepsis.

b) Orai

Orai1 was discovered in 2006 as a calcium selective channel in the T cells, a mutation in which lead to severe combined immune deficiency (SCID) (62). Hence Orai was named after the Greek keepers of Heaven’s gate. Recently Drosophila Orai1 structure was crystallized (63) and as previous studies utilizing biochemical and functional approaches had suggested, it is a four-transmembrane segment protein with both its N- and C-terminals located in the cell cytoplasm (62,64) [Fig. 5]. Orai has three isoforms Orai1, Orai2 and Orai3. Numerous studies dedicated to dissect the components of native SOCE have shown the exclusive function of Orai1 in mediating Ca\(^{2+}\) influx regulated by store-depletion through STIM1, despite the presence of the other Orai isoforms in abundance (65). Orai1 is about 30 kDa in molecular
Figure 5: Structure of Orai1. Orai1 consists of four transmembrane subunits, TM1, TM2, TM3 and TM4. TM1 lines the channel pore and contains the glutamate-106 residue (not shown) providing the channel with high Ca\textsuperscript{2+} selectivity. The C-terminus, facing towards the cytosol, contains a coiled-coil (CC) domain that acts as the binding site for STIM1 (66).
weight although this might considerably increase due to glycosylation. Results from single amino acid mutations, glutamate to alanine at positions 106 in the first transmembrane segment of human Orai1 and glutamate to glutamine at 190 in the third transmembrane domain affected the selectivity of the channel. These two glutamates form a Glu-ring selectivity filter and are also conserved in all the Orai isoforms as well as amongst different species. This indicates Orai’s function as a pore-forming subunit of the CRAC channel. Beyond the pore-forming subunit, the cavity is lined by hydrophobic side chains from valine, phenylalanine and leucine (Val\textsuperscript{102}, Phe\textsuperscript{99} and Leu\textsuperscript{95} in human Orai1). These are highly conserved and mutation of these residues leads to alteration of the pore selectivity as well as conductance. The crystal structure of Drosophila Orai1 indicates the presence of transmembrane helices (M1, M2, M3 and M4) with cytoplasmic extensions of two of the helices (M1 and M4). Evidence suggests that M4 extension (amino acids 263 to 301 in human Orai1) are required to “tether” the SOAR domain of STIM1, facilitating their interaction. Also the M1-extension is essential for activation of Orai1 by STIM1. Stoichiometric studies suggest Orai1 functions as a hexamer which requires a STIM dimer to bind to each subunit for activation (2:1 ratio) (67). Overexpression of either STIM1 or Orai1 alone did not alter Calcium influx however, overexpressing both STIM1 and Orai1 lead to a significant increase in Calcium influx.

1.5.4 **Stromal Interacting Molecule proteins (STIMs)**

Various theories were suggested to explain the phenomenon of stored calcium release from the ERs leading to the opening of the channels on the plasma membrane. In 2005, two independent research groups Roos et. al. (68) and Liou et. al. (69) identified STIM proteins
to be the key player in the ER-plasma membrane junctional coupling model, proposed by Putney (70,71) and Berridge (72) more than a decade ago (33). Roos et al. performed RNA interference (RNAi) screens in Drosophila melanogaster S2 cells and identified a single STIM protein (68). On the other hand, Liou et al. monitored calcium signaling in HeLa cells identifying a pair of STIM proteins (69). These proteins were described as a tumor-suppressor gene product (73,74) located on the surface of stromal cells. Its original function was deduced to be a mediator of interactions between Stromal cells and haematopoietic cells (75,76). Hence the acronym, STIM.

STIM has two isoforms, STIM1 and STIM2 (76). They are structurally homologous except in the amino- and the carboxy-terminals. STIM1 and STIM2 are located in the ER-membrane while some evidences suggest the presence of STIM1 in the plasma membrane to some extent. They are ubiquitously expressed in different cell types of vertebrates. Although in most tissues the level of STIM1 is much higher than STIM2, brain and dendritic cells express the latter predominantly (76). STIM proteins interact with SOC [TRPC family] channels and ICRAC [Orai] present on the plasma membrane inducing Ca\(^{2+}\) influx (76).

\(\textbf{a) STIM1}\)

STIM1 is a single pass trans-membrane protein with an amino-terminal Ca\(^{2+}\) binding motif in the ER lumen. Studies were performed deleting specific regions to identify the functions of each domain (77,78). The luminal domain or the N-terminus contains clusters of short \(\alpha\)-helices comprising of two EF-hand domains [a Ca\(^{2+}\)-binding canonical EF-hand (cEF) domain and a non-Ca\(^{2+}\)-binding hidden EF-hand (hEF) domain] and a sterile \(\alpha\)-motif (SAM) domain with Asn-linked glycosylation sites [Fig. 6]. The EF-hand domain senses small
changes of Ca$^{2+}$ concentration in the ER-lumen. Following store Ca$^{2+}$ depletion, Ca$^{2+}$ dissociates from the EF-hand, unfolding and destabilizing the N-terminal domain. As a result STIM1 is activated, triggering oligomerisation through the SAM domain, stabilizing the exposed hydrophobic residues (78). The carboxy-terminus (C-terminus) faces towards the cytosol containing the Ezrin-Radixin-Moesin (ERM) domain. It is comprised of the coiled-coil (CC) regions, STIM1-Orai activating region (SOAR), inhibitory domain (ID) followed by the serine/proline (S/P) rich domain, a microtubule interacting domain (TRIP) and a lysine (K) rich domain (78) [Fig. 6]. The CC region consists of CC1, CC2 and CC3 domains and the CC1 domain is again divided into three $\alpha$-helices termed $\alpha$1, $\alpha$2 and $\alpha$3. The $\alpha$3 helix has acidic sequences, EEELE that inhibits SOAR function. The CC2 (363-389) and CC3 (399-423) region extends into the SOAR domain.

The CC regions mediate the STIM1-STIM1 interactions during the resting state as well as during activation. The SOAR domain is the central core for STIM1 activation and is essential for activating Orai1. SOAR contains a polybasic active site, KIKKKR, which acts as a critical sequence for the interaction between STIM1 and Orai1 (78). SOAR consists of four $\alpha$ helices called S$\alpha$1, S$\alpha$2, S$\alpha$3 and S$\alpha$4. Downstream of SOAR another inhibitory domain (ID) is present which inactivates Orai1. Next, the K-domain is required for the activation of SOCs like the TRPC channels. It is conserved in all vertebrates. It is speculated that the positive charges of lysine interact with the anionic phospholipids in the membrane or proteins to facilitate channel activation.
Figure 6: Domain structure of STIM1. STIM1 protein consists of Ca\textsuperscript{2+} binding EF-hand domain at the N-terminus facing the ER-lumen. The EF-hand is followed by the SAM (sterile-\(\alpha\)-motif) and the TM (transmembrane) domain. The cytosolic side contains the ERM (ezrin-radixin moesin domain) domain, which consists of C-C (coiled coil) domain and the SOAR (STIM1-Orai activation) region. It is next followed by the S/P (serine-proline rich domain) and the K (lysine) rich-domain \cite{73, 74}.
The K-domain also provides a major driving force for junctional assembly during redistribution of STIM1 at close proximities to the plasma membrane (78). The ERM and the K domains are necessary and sufficient to bind and activate the TRPCs [Fig. 7]. Except, TRPC7, all TRPCs interact with SOAR domain of STIM1 (4, 5, 25). Evidences show that specific electrostatic interactions occur between the basic residues at the C-terminus (K684 and K685) of STIM1 and the acidic residues in the C-terminus of TRPC1 proteins (D639 and D640) (77-80). TRPC4 C-terminus also contains acidic amino acid residues to interact with STIM1 [Fig. 7]. Further co-immuno-precipitation studies and immuno-staining studies confirmed the biochemical association of TRPC-STIM1 during SOCE (81). After stored Ca\(^{2+}\) depletion, STIM1 proteins oligomerise and accumulate in the ER/plasma membrane interface, forming discrete punctae, which in turn interact with the SOCs (Orai1, TRPCs) activating them (49, 78). Puncta formation is reversible and microtubules facilitate its formation. Refilling and depleting the stores dissolves and restores punctae, respectively, at the same loci as before, indicating that puncta formation is not a random process. The sites are predetermined by structural or scaffolding molecules acting as place markers (43).

**b) STIM2**

STIM2 is the second homologue of STIM1 belonging to the same STIM family. It shares 66% homology with STIM1 at the N-terminus while largely diverges at the C-terminus (82). STIM2 domain structure is conserved in vertebrates with 92% homology between the mouse and the human proteins. While STIM1 and STIM2 both are involved in maintaining homeostasis, STIM2 function is mainly attributed to refilling of the ER-store, suggesting STIM2
Figure 7: STIM1-TRPC interaction. STIM1 undergoes electrostatic interaction with the C-terminus of TRPC, activating it and opening the channel inducing Ca\(^{2+}\) influx into the cell (79).
mainly has a “housekeeping” function. Oh-Hora et al. proposed redundant functions for STIM1 and STIM2, deficiency of both leading to the development of autoimmunity in mice (83). However, other reports show complete inhibition of SOC in STIM1 knockout mice comparable to conditions where there was no extracellular Ca\textsuperscript{2+} available or complete inhibition of the stored Ca\textsuperscript{2+} release (83). Expression of STIM1 is also higher in certain cell types compared to STIM2. STIM2, in contrast to STIM1 remains partially active constitutively and becomes fully activated upon moderate decrease of Ca\textsuperscript{2+} within the ER. This can be attributed to the differential affinities of the isoforms to Ca\textsuperscript{2+}. STIM1 have a lot higher affinity compared to STIM2 (83).

c) **Physiological functions of STIM proteins**

Since the discovery of the STIMs, there have been a number of studies in different cells and tissues to unravel their functions (83-85). Initially, STIMs were identified as glycosylated phosphoproteins localized at the cell surface (75). Subsequent studies showed it to be predominantly located on the ER-membrane (76). In 2005, studies identified its critical role in SOCE (49,68,69). Initially the importance of SOCE was majorly dedicated to the refilling of the depleted Ca\textsuperscript{2+} store, ER (83). However studies utilizing whole-genome knockout mice and cell-specific knockout mice mapped STIM functions during physiological and pathophysiological conditions (83). Evidences suggest a definite importance of SOCE for T-cell function, where familial deletion of I\textsubscript{crac} resulted in severe immunodeficiencies (83). Double knockouts of STIM1 and STIM2 in mice have shown that both the isoforms are required for the development and functions of regulatory T-cells and deficiency in both lead to the development of autoimmunity in mice.
Previously from our laboratory it has been shown that \( \text{Ca}^{2+} \) overload leads to an increase in endothelial permeability (40). Thus we wanted to test if LPS induced the expression of STIM1 and SOCs to augment SOCE leading to hyper-permeability of the microvascular endothelial cells, a hallmark of sepsis.
2. OBJECTIVES

- To determine whether up-regulation of STIM1 during sepsis results in Ca\(^{2+}\) overload in endothelial cells and thereby signals lung microvascular leak.

- If so, what is the transcriptional mechanism underlying the up-regulation of STIM1 expression during sepsis.

- To determine if endothelial cell-STIM1 is critical in mediating lung microvascular leak associated with sepsis.
Figure 8: Proposed model. During sepsis, LPS binding to TLR4 on the endothelial cell membrane and activates NF-κB and p38 signaling pathways to induce STIM1 transcription. LPS also triggers TLR4 on monocytes and endothelial cells to produce TF and thereby stimulating the production of thrombin via factor VIIa (4). Proposed hypothesis is that sepsis-mediated STIM1 expression in endothelial cells through activating transcription factors NF-κB and AP1 and thrombin generation contributes to the observed leaky vessel syndrome by increasing SOCE in endothelial cells of lung microvessels.
3. MATERIALS AND METHODS


3.1 Materials

Endothelial growth medium-2 (EGM-2) were from Lonza Walkersville, Inc. (Walkersville, MD). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Hanks’ balanced salt solution (HBSS), L-glutamine, trypsin, trizol reagent, and Taq DNA polymerase, and Fura-2AM were from Invitrogen (Carlsbad, CA). Human \(\alpha\)-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). LPS (ultrapure *E. coli* 0111:B4) was obtained from InvivoGen (San Diego, CA). Actinomycin D, thapsigargin, SB203580, and 6-amino-4-(4-phenoxyphenylamino)quinzoline (NF-\(\kappa\)B inhibitor), PD 98059, SP600125 were from Calbiochem (La Jolla, CA). qPCR primers were custom synthesized by IDT (Coralville, IA). Human \((h)\)-specific siRNA to target STIM1, p65/RelA, p38\(\alpha\), p38\(\beta\), c-Fos, and scrambled siRNA
(Sc-siRNA) were obtained from Dharmacon (Lafayette, CO). Anti-STIM1 mAb was purchased from BD transduction Laboratories. Anti-STIM1 pAb was from Proteintech Group (Chicago, IL). Antibodies against c-Jun, phos-ERK1/2, total ERK1/2, Phos-JNK, total JNK were obtained from Cell signaling (Danvers, MA). Antibodies specifically reacting with TRPC1, Orai1, c-Fos, and p50 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). si-RNA transfection reagent and Human (h)-specific siRNA to target p38γ, p50 and c-Jun were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). SuperFect transfection reagent was obtained from QIAGEN (Valencia, CA). Anti-p65/RelA pAb was purchased from Millipore Corp. (Billerica, MA). Anti-β-actin mAb was from Sigma (St. Louis, MO). Phospho (Thr-325)-c-Fos pAb was from Abcam (Cambridge, MA). Anti-TRPC4 pAb was purchased from Everest Biotech Ltd (Ramona, CA). PAR-1-activating peptide (TFLLRNPNKD-NH₂) peptide was custom synthesized by GenScript (Piscataway, NJ). Fast SYBR Green Master mix was purchased from Applied Biosystems (Grand Island, NY).

3.2 Animals

C57BL/6 mice were obtained from Charles River laboratories. STIM1^{fl/fl} mice were a gift from Dr. Oh-hara (Tokyo). B6.Cg-Tg(Tek-cre)1Ywa/J and B6.Cg-Tg(Cdh5-Cre)7Mlia/J mice were obtained from Jackson Laboratories.

STIM1^{fl/fl} mice were backcrossed with C57BL/6 mice (Wild-type/WT) to produce a STIM1^{fl/fl} mice colony. After establishing a stable colony, STIM1^{fl/fl} mice was crossed with B6.Cg-Tg(Tek-cre)1Ywa/J mice for two generations. B6.Cg-Tg(Tek-cre)1Ywa/J (Tie2Cre⁺) mice have the endothelial-specific receptor tyrosine kinase (Tek or Tie2) promoter drive the
expression of Cre. The second generation offsprings generated endothelial-restricted STIM1 (STIM1^{EC-/-}) mice. In the present study the loss of STIM1 in the STIM1^{EC-/-} mice was confirmed by qPCR, measuring transcript formation of STIM1, immuno-staining and confocal imaging of lung sections prepared from lungs isolated from STIM1^{EC-/-} mice, confirming the depletion of STIM1 expression specifically in the endothelial cells. In addition, western blot analysis was performed in lung tissue homogenates to determine significant decrease in STIM1 protein expression in STIM1^{EC-/-} mice compared with STIM1^{fl/fl} mice. Freshly isolated endothelial cells from these mice were used to further confirm the successful deletion of the gene by measuring protein expression. STIM1^{EC-/-} mice was also generated by crossing STIM1^{fl/fl} mice with B6.Cg-Tg(Cdh5-Cre)7Mlja/J (VE-cadherin promoter driving the expression of Cre).

3.3 Primary endothelial cell culture

Primary human lung microvessel endothelial cells (HLMVECs) were obtained from Lonza Walkersville, Inc. (Walkersville, MD). They were grown in EGM-2 MV supplemented with 10% FBS were used between passages 2 and 4.

According to an approved protocol of the University of Illinois Animal care committee, mice weighing 25 to 30 g were anesthetized with 3% halothane, and heparin (50 U/mouse) was injected into the jugular vein. The abdominal cavity was opened and the pulmonary artery was cannulated. Krebs-Henseleit solution supplemented with bovine serum albumin (5 g/100 mL) was infused to remove blood. Lungs were isolated and placed inside a culture hood. Lung tissue slices from at least 3 mice were prepared, washed and suspended in HBSS. Excess HBSS was aspirated, and the tissue slices were minced and transferred to a 15-ml
sterile tube. The minced tissues were suspended in 10 mL of collagenase A (1.0 mg/mL in HBSS) and digested for 60 min at 37°C with gentle shaking. The released cells were centrifuged at 200g for 10 min. The pellet was suspended in 10 mL suspension buffer (Ca\textsuperscript{2+} - and Mg\textsuperscript{2+} -free containing 0.5 g/100 mL bovine serum albumin, 2 mmol/L EDTA, and 4.5 mg/ml D-glucose), and filtered through 200 µm mesh filter. The filtered cells were suspended in 10 mL of suspension buffer. To this cell suspension, 1.5 µg/ml anti-mouse PECAM-1 antibody (BD Pharmingen) was added and incubated at 4°C for 30 min with gentle shaking. The cell suspension was centrifuged to remove unbound antibody and washed once with suspension buffer. The washed cells were then incubated with Dynabeads M-450 (Sheep anti-rat IgG) for 30 min at 4°C. Following this, the cell suspension was attached to a magnetic column and the unbound cells were aspirated. Cell bound with magnetic beads were washed once with HBSS and digested with trypsin for 3 min at 22°C. The cells released from the magnetic beads were separated, washed, suspended in growth medium (EGM-2 supplemented with 10% FBS). The cell suspension was plated on matrigel (BD Biosciences)-coated 35 mm culture dish and allowed to grow to confluence for 10 days. Cells were then harvested from the matrigel plates with dispase (BD Bioscience) for 60 to 90 min. Cells were washed after dispase treatment once with growth medium and plated on 0.1% gelatin coated culture dish. Cells passaged between 3 and 4 times were used in experiments. Mouse lung endothelial cells (MLECs) were characterized by their cobblestone morphology, PECAM-1 (platelet/endothelial cell adhesion molecule-1) (or CD31) expression, and Dil-Ac-LDL uptake (37). To study LPS effect, ECs were incubated with 1% FBS containing medium overnight at 37°C and were then exposed to LPS in 1% FBS containing medium.
3.4 **Genotyping**

To determine the genotype of the mice, tail snips from the mice were collected in tubes and labeled appropriately. The tubes were kept in ice and a solution of 120 µl of 0.5M EDTA and 500 µl Nuclei lysis solution (Promega) were added to it. Further 17.5 µl of 20 mg/mL of Proteinase K was added to the mixture. It was incubated overnight at 55°C. Next day, 200 µl of protein precipitation solution (Promega) was added and then the mixture was vortexed at high speed for 20 seconds. Sample was then chilled for 5 min and centrifuged at 13000 g for 5 more min. The supernatant was separated and 600 µl Isopropanol was mixed by inversion. Then it was centrifuged at 13000 g for 5 min. 600 µl of Ethanol was added to it and inverted to mix. Samples were centrifuged at 13000 g for 5 min and the supernatant is separated without disturbing the pellet. The pellet is air-dried and 30-50 µl TE buffer was added to it. To mix, it was incubated at 65°C for 1 h.

3.5 **Polymerase chain reaction**

The DNA extracted from the mice tails is tested by PCR method to check the deletion of the gene. DNA was amplified using the following primer sets: STIM1 wt 5’-CGATGGTCTCAGGTCTAGTTTC, STIM1 KO 5’-AACGTCTTTGCGTTGCTGAGGC, STIM1 AS 5’-GGCTCTGCTGACCTGGAACTATAGTGC; The reaction condition include: an initial 96°C for 1 min, then 30 cycles of (96°C for 10 s, 55°C for 30 s and 72°C for 30 s), followed by 72°C for 5 min. PCR product for STIM1 fl/fl was 399 bp, STIM1 fl/+ bands were 399 and 348 bp and for STIM1 EC/– three bands, 348, 399 and 580 bp were observed. To test the presence of the Tie-2 Cre gene in the mice, this gene was amplified using primers 5’-TTC CAT GAG TGA ACG AAC CTG GTC-3’ and 5’-AGT GAT GAG GTT CGC AAG AAC CTG-3’
with the reaction condition: 94°C for 1 m 30 s, 30 cycles of (94°C for 30 s, 63°C for 1 m 15 s and 72°C for 1 m), followed by 72°C for 7 m. The product size is 400 bp. For the Ve-Cadherin Cre gene, primers used were 5’-GCG GTC TGG CAG TAA AAA CTA TC-3’, 5’-GTG AAA CAG CAT TGC TCT CAC TT-3’, 5’-CTA GGC CAC AGA ATT GAA AGA TCT-3’ and 5’-GTA GGT GGA AAT TCT AGC ATC ATC C-3’. The reaction condition is 94°C for 3 m, 30 cycles of (94°C for 30 s, 51.7°C for 1 m, 72°C for 1 m) followed by 72°C for 2 m. The transgene is about 100 bp and an internal positive control band at 324 bp. All the PCR amplified products were resolved in 1% agarose gel and identified by ethidium bromide staining.

3.6 Quantitative Real-Time PCR

HLMVECs were incubated with either vehicle (DMSO) or actinomycin D (1 µM) for 1 h, or SB 203580 (10 µM) for 30 min or NF-κB inhibitor (5 µM) for 12 h and were then exposed to LPS (1 µg/ml) for indicated time points. Lungs isolated from STIM1EC-/- and STIM1fl/fl mice were minced and homogenized. Total RNA, extracted according to manufacturer’s recommendations with RNeasy kit (Qiagen, CA), from ECs or homogenized tissue, was used to generate first-strand complementary DNA by reverse transcriptase (Invitrogen). cDNA (10 ng), mixed with SYBR green PCR master mix was used for real-time quantitative-PCR. Transcript levels (mRNA) were normalized to β-actin mRNA. Primers used to amplify the genes are:

hSTIM1: Primer 1, 5’- ACCAGCATGAAGTCTTTGAG -3’, Primer 2, 5’-TGAAGATGACAGACCGGAGT -3’; mSTIM1: Primer 1, 5’-GTATCACCTCATCCACAGTCC -3’, Primer 2, 5’-CAATTACCATGACCCAACAGTG -3’;
mTRPC4: Primer 1, 5’- GTCCATTAGATTCCACCAGTCA -3’, Primer 2, 5’-GGTCAGACTTGAAACAGGCAA -3’; mOrai1: Primer 1, 5’-
CTAAAGACGATGAGCAACCCT -3’, Primer 2, 5’- CTACTTAAGCCGCGCAA -3’; β-actin (human or mouse), Primer 1, 5’ TTGCTGACAGGATGCAGAAGGAGA -3’, Primer 2, 5’ACTCCTGCTTGCTGATCCACATCT -3’.

3.7 Immunoblotting (IB)

Endothelial cell lysates or total lung tissue homogenates from C57BL/6 mice were resolved by SDS-polyacrylamide gel electrophoresis on a 10% separating gel under reducing conditions and transferred to Duralose membrane (86). Non specific bands were blocked by incubating the membranes with 5% dry milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) for 1 h at 22°C. Membranes were then incubated with indicated primary antibody (diluted in blocking buffer) overnight. After three washes, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Protein bands were detected by enhanced chemiluminescence (86).

3.8 Cytosolic Ca\(^{2+}\) measurement

The cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in ECs was measured using the Ca\(^{2+}\)-sensitive fluorescent dye Fura-2/AM (10, 21). Cells were grown to confluence on gelatin-coated glass coverslips and then washed two times and incubated for 12 h at 37°C in medium containing 1% FBS. Cells exposed to LPS for the indicated time intervals were washed and loaded with 3 µM Fura-2/AM for 30 min. After loading, cells were washed with HBSS and the coverslips were transferred on a perfusion chamber at 37°C and imaged using a semimotorized microscope (Axio Observer D1; Carl Zeiss GmbH, Jena, Germany) equipped with an AxioCam HSm camera (Carl Zeiss) and a Fluar 40× oil immersion objective. Light was provided by the DG-4 wavelength
switcher (Princeton Scientific Instruments, Monmouth Junction, NJ). A dual excitation at 340 and 380 nm was used, and emission was collected at 520 nm. The AxioVision physiology software module was used to acquire the images at 1-s intervals, and the data were analyzed off-line. In each experiment, 20 to 30 cells were selected to measure change in ([Ca^{2+}]_i).

3.9 siRNA Transfection

ECs grown to ~70% confluence on gelatin-coated culture dishes were transfected with target siRNA or sc-siRNA using siRNA transfection reagent and medium (both from Santa Cruz Biotechnology) according to the manufacturer’s instructions. At 48 or 72 h after transfection, cells were washed with 1% FBS-containing medium and incubated with the 1% FBS-containing medium overnight. Next day they were challenged with LPS (1 µg/ml) in 1% FBS-containing medium for the indicated time points and were used for Ca^{2+} measurements or harvested for Western blot analysis.

3.10 Transendothelial Electrical Resistance Measurement

The real-time change in transendothelial monolayer electrical resistance (TER) was measured (87). Endothelial cells were seeded on gelatin coated 8-well gold-electrodes and grown to confluence. The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier. A constant current of 1 µA was supplied. Voltage between small electrode and large electrode was monitored by lock-in amplifier, stored and processed by a computer. Before starting the experiment, the endothelial cells were incubated with 1% FBS-containing medium, overnight. Next day they were challenged with LPS in 1% FBS-containing
medium. Four hours after LPS exposure, thrombin-induced real-time change in TER was measured. Data are presented in resistance normalized to its value at time 0 (87).

3.11 Mouse Lung Capillary Filtration Coefficient ($K_{f,c}$) Measurement

To test the effects of LPS mice were given a dose of LPS (5 mg/kg; i.p.) for 4 h prior to the $K_{f,c}$ measurements (37). The experiment was performed according to the approved animal protocol of University of Illinois Animal care committee. Mice were anesthetized using 2.5% halothane and anesthesia at 1.5% halothane was maintained through a nose cone. Anesthetic gas mixture was ventilated through the cannulated trachea to maintain a constant pressure. Heparin was administered to the right jugular vein to prevent blood clotting. The heart and the lung were exposed by thoracotomy. A pulmonary arterial cannula was advanced into the pulmonary artery of the right ventricle by an incision. Another polyethylene cannula (PE 60; Becton Dickinson) was introduced into the pulmonary artery and secured by a suture. A catheter (3-mm PE-50 tube) was inserted through the left atrium to drain the pulmonary venous effluent. Next, the lung preparation was perfused in situ with a peristaltic pump. Following perfusion, the anesthetic gas flow was stopped and ventilation with room air was continued. The lung and the heart were rapidly excised and were shifted en bloc to a perfusion apparatus. The lungs were kept suspended from an electronic beam balance and ventilated at 186 breaths/min and end-expiratory pressure of 2 cm H$_2$O. It was perfused with a modified Krebs-Henseleit solution [composition in nmol/L: NaCl 118; KCl 4.7; CaCl$_2$ 1.0; MgCl$_2$ 0.5; HEPES sodium 4.43; HEPES free acid 5.57; NaHCO$_3$ 3; glucose 11; EDTA 0.025; pH 7.4, 3g/100mL of bovine serum albumin (BSA, Fraction V, 99% pure and endotoxin-free; Sigma-Aldrich) at a constant flow of 2 mL/min at 37°C and a venous pressure of +1 cm H$_2$O. The pulmonary arterial pressure was monitored using
a Gould pressure transducer (Gould Instruments Inc.). In experiments testing the effects of PAR-1 agonist peptide on $K_{f,c}$, PAR-1 agonist peptide (30 µM) containing perfusion buffer was infused via a side-port at a rate of 0.2 ml/min. $K_{f,c}$ measurements were made at baseline, and after a 20 min exposure to PAR-1 agonist peptide. The lung wet weight was electronically nulled when it was mounted and subsequent weight changes of the lung (gain or loss) were recorded. All lung preparations underwent 20 min of equilibration perfusion to establish isogravimetric condition. The values are expressed as the ratio of experimental-to-basal $K_{f,c}$ values in the same lung preparation.

3.12 Assessment of Mouse lung Microvessel Permeability in vivo

Mice were given a dose of LPS or saline for 4 h and were then anesthetized (2.5% sevoflurane in room air) for insertion of an indwelling jugular catheter. They were then allowed to recover for 30 min. At 195 min after LPS or saline administration, mice received 100 µl of saline or PAR-1 activating peptide (1 mg/ kg) by retro orbital injection. Fifteen min later the mice received a dose of 100 µl Evans blue dye conjugated with albumin (20 mg/kg; EBA) by retro orbital injection. Thirty min later the mice were sacrificed and the lungs were isolated. The EBA present in the lung tissue was measured as described previously (88).

3.13 Promoter Analysis

The 5’-regulatory region of the human (h) STIM1 gene was analyzed utilizing the Genomatix software. The transcription factor consensus binding sites were identified and mapped in the STIM1 promoter.
3.14 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed using ChIP assay kit from Upstate Biotechnology following the manufacturer’s instructions (86). Confluent HLMVECs were washed with serum-free medium and incubated with 1% FBS-containing medium overnight. Next day they were treated with LPS (1 μg/ml) for 0, 30, 60, 120 min. Following treatment, the cells were cross-linked with 1% formaldehyde for 15 min at 22°C. Then they were washed with ice-cold PBS and were lysed in 200 μl of a buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCL, pH 8.1 and 1X protease inhibitor mixture and 1 mM phenylmethylsulfonyl fluoride. Cell lysates collected were then sonicated with three sets of 12 s pulses at 20% maximum power in a VirSonic to produce DNA fragments of 200-600 bp. Sonicated lysates were then diluted to 2 ml with 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM TrisHCl, pH 8.1, with protease inhibitor cocktail. 20 μl of this solution were removed for PCR analysis (input). Next, the lysates were precleared using salmon sperm DNA/protein A-agarose for 1 h at 4°C, indicated Ab (2.5 μg) were added and incubated overnight at 4°C in a rocking platform. Immune complexes were collected with 60 μl of salmon sperm DNA/protein A-agarose (1 h at 4°C), and the agarose beads were washed off. After a final wash in 1 mM EDTA, 10 mM TrisHCl, pH 8.0, bound immune complexes were eluted in a freshly prepared solution of 1% SDS, 0.1 M NaHCO3, and cross-links were reversed in the samples by incubating at 65°C for 4 h. The input samples were also treated similarly to reverse cross-links. Samples were then treated with proteinase K, and DNA was extracted using DNA purification kit (Invitrogen). The primers used for QRT-PCR following ChIP were as follows: NF-κB sites 1-3, forward: 5’- GAG GCT AAC GTC GTG TCC TG -3’; reverse: 5’- AGC TGG ATC CCG GAA TAA CC -3’; AP1 site forward: 5’- GTG TCC TGG GCC TCT GTT TA -3’; reverse: 5’- GTG AAG ACC TCC CCG GAA TC -3’. The DNA-
proteins were isolated from HLMVECs. After LPS exposure, cells were scraped and washed twice with ice-cold Tris-buffered saline (TBS). Cells were then homogenized with 400 µl of solution A (10 mM KCL, 10 mM Hepes pH 7.9, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA, 1 MM DTT, 0.5 mM PMSF, 5 µg/ml Aprotinin and 5 µg/ml Leupeptin). The cells were lysed with 10 strokes using a dounce homogenizer and centrifuging at 14,000 rpm for 1 min. The supernatant was transferred to a new eppendorf tube (the cytoplasmic fraction). Nuclear pellets were then resuspended in 100 µl of solution B (20 mM Hepes pH 7.9, 0.4 Nacl, 1 mM EDTA pH 8.0, 1 MM EGTA, 1 MM DTT, 0.5 mM PMSF, 5 µg/ml Aprotinin and 5 µg/ml Leupeptin) and then incubated on ice for 20 min. The nuclei were pelleted by centrifugation at 14,000 rpm for 1 min. Supernatants containing nuclear proteins were aliquoted in small fractions and stored at -70˚C.

3.16 Electrophoretic mobility shift and supershift assays

NF-κB oligonucleotide containing the NF-κB consensus sequence [NF-κB hSTIM1 or NF-κB1 (-204 to -165): 5' GGC GGG GAT TCC GGG GAG CCG TCT TCA CCG GTT ATT CCG 3'; NF-κB/SP-1 hSTIM1 or NF-κB2 (-216 to -185): 5' - CCG GGA GAG CCC GCT AGG GGC GGG GAT TCC G -3'] was labeled with [γ³²-P]ATP using T4 polynucleotide kinase for 20 min at 37°C in the buffer containing 50 µg poly(dI-dC) and 10 mM Tris-HCl buffer, pH
7.5, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 4% (wt/vol) glycerol, and 1 mM MgCl₂. The nuclear extracts (15 µg protein) were incubated with the radiolabeled NF-κB oligonucleotide (80,000 cpm/reaction) and subjected to electrophoresis on a 6% native gel, dried onto Whatman paper, and then exposed to autoradiography. For supershift assays, nuclear extracts were incubated with anti-p65/RelA Ab for 30 min before the addition of radiolabeled oligonucleotide probe.

3.17 In vivo real-time fluorescent imaging

In vivo real-time fluorescent imaging experiment was performed in collaboration with Dr. Parthasarathi, University of Tennessee. STIM1^{fl/fl} or STIM1^{EC-/-} mice were given a dose of LPS (5 mg/kg; i.p.) for 4 h. PAR-1 peptide was administered by targeted perfusion into the lung capillaries and venules. Microvessels were then loaded with the cell-permeable ratiometric Ca^{2+} indicator Fura2-AM (10 µM) for 30 min. Images of microvessels were captured by exciting sequentially at 340 and 380 nm and collecting fluorescence at 515 nm. Images were obtained at 10s intervals. Baseline Ca^{2+} was recorded for 30 min. After the respective treatment, the response was recorded for more than 60 min. Fluorescence emissions from both 340 and 380 excitations were quantified at a single region along a vessel wall. Changes in Ca^{2+} indicated as changes in the ratio of the fluorescence emissions at 340 and 380 nm (F340/F380). F340/F380 oscillation amplitude in lung microvessels was quantified separately for venules and capillaries. The mean oscillation amplitude from three consecutive oscillations was taken as the amplitude of Ca^{2+} oscillation. Oscillation amplitude was determined separately for baseline and response. Changes from baseline was quantified and plotted.
3.18 Statistical analysis

Data were analyzed by an unpaired two-tailed Student’s $t$-test. Differences in mean values were considered significant at a $p$ value of $\geq 0.05$. 
4. RESULTS

*Portions of the text and figures 9-29 and 31-36 were reprinted with permission from the American Society for Biochemistry and Molecular Biology. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy, Stephen M. Vogel, Dheeraj Soni, Premanand C. Sundivakkam, Asrar B. Malik and Chinnaswamy Tiruppathi. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability.


4.1 LPS induces the expression of STIM1 augmenting PAR-1-induced SOCE and mediating endothelial hyper-permeability in human lung microvessel endothelial cells (HLMVECs)

4.1.1 LPS induces the expressions of STIM1 and store-operated Ca\(^{2+}\) entry (SOCE) components

To determine the effect of LPS stimulation on STIM1 expression, we first measured STIM1 mRNA expression in response to LPS in HLMVECs. LPS induced STIM1 transcript expression in HLMVECs and the expression level was maximal at 4 h [Fig. 9A]. Pretreatment with the transcriptional inhibitor actinomycin D blocked LPS-induced STIM1
transcript expression [Fig. 9A]. We also observed increased STIM1 protein expression in response to LPS challenge [Fig. 9B]. STIM1 protein expression was increased more than 6-fold within 6 h after LPS exposure [Fig. 9B]. However, STIM1 isoform STIM2, localized in ER, was not increased in response to LPS in HLMVECs [Fig. 9B]. We and others have shown that STIM1 activates TRPC1 and TRPC4 (SOC channels) and Orai1 (Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel [CRAC]) (81,89-91) to induce Ca\(^{2+}\) entry in ECs. Increased STIM1 expression alone is not sufficient to augment SOCE, but STIM1 interaction with plasma membrane-localized TRPC or Orai channels is essential to enhance SOCE (79,80); therefore, we determined expression of TRPC1, TRPC4 and Orai1 proteins in HLMVECs in response to LPS challenge. LPS challenge substantially increased the expression of TRPC1, TRPC4, and Orai1 in HLMVECs [Fig. 9B]. These results suggest that TLR4 signaling not only induces STIM1 expression but also induces the expression SOC channel components in HLMVECs.
**A**

![Graph showing Fold Increase over basal (STIM1:β-actin ratio) over LPS exposure time (h).]

- **DMSO Control**
- **Actinomycin D**

**B**

<table>
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<th>LPS (h)</th>
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<th>TRPC4</th>
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<th>Orai1</th>
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Figure 9: LPS induces the expressions of STIM1 and store-operated Ca2+ entry (SOCE) components. A) HLMVECs were treated with LPS (1µg/ml) in the presence and absence of actinomycin D (0.5µM) for 0, 2, 4 and 6 h. After the treatment total RNA was isolated, and QRT-PCR was performed to determine STIM1 mRNA expression. STIM1 mRNA induction fold was calculated by measuring the ratio of STIM1 to β-actin. Results shown are mean ± S.E. of four experiments. *p<0.01, **p<0.001, compared with cells treated with actinomycin D. B) HLMVECs grown to confluence were exposed to LPS (1µg/ml) for 0, 4 and 6 h. After this treatment, total cell lysates were used for immunoblot (IB) to determine STIM1, STIM2, TRPC1, TRPC4, Orai1, and β-actin. Blots were quantified by densitometry and fold increase over basal (FOB) relative to β-actin were shown. Results shown are mean of two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.1.2 LPS-induced expression of STIM1 and store-operated Ca\(^{2+}\) channels (SOCs) augments PAR-1-mediated Ca\(^{2+}\) influx and endothelial permeability

To address the functional relevance of increased expression of STIM1, TRPC1, TRPC4, and Orai1 proteins, we measured the thrombin (Thr)-induced increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). LPS pretreatment, significantly increased the Thr-induced increase in ([Ca\(^{2+}\)]\(_i\)) in HLMVECs [Fig. 10A]. Next we measured Thr-induced ER-stored Ca\(^{2+}\) release and Ca\(^{2+}\) release-activated Ca\(^{2+}\) entry (SOCE) in control and LPS pretreated HLMVECs. In both control and LPS primed cells, Thr-induced ER-store Ca\(^{2+}\) release (initial peak) was similar; however, upon add back of Ca\(^{2+}\), Ca\(^{2+}\) entry (SOCE) was more than 2-fold higher in the LPS primed cells compared with unprimed cells [Fig. 10B].

To characterize the basis of augmented SOCE in LPS primed cells, we silenced STIM1 expression by transfecting HLMVECs with siRNA specific to STIM1 and then studied the LPS effect. In STIM1-siRNA transfected cells, STIM1 protein expression was blocked compared to control-siRNA (Sc-siRNA) [Fig. 11A]. Also, Thr-induced Ca\(^{2+}\) entry was blocked in STIM1-siRNA transfected cells with or without LPS pretreatment [Fig. 11B], thus indicating the crucial role of STIM1 in activating SOCE in ECs. We have shown previously that Ca\(^{2+}\) overload in ECs signals an increase in vascular permeability (33,37,40,91). To address the functional relevance of LPS priming-mediated SOCE augmentation in response to PAR-1 agonists, we studied the effect of LPS priming on TER, a measure of endothelial monolayer permeability (87). After 4 h of LPS priming, cells were challenged with PAR-1 activating peptide to assess real-time changes in TER. In control cells (not pretreated with LPS), challenge with PAR-1 activating peptide produced a ~55% decrease in TER [Fig. 12] and TER returned to
baseline within ~2 h [Fig. 12]. In the LPS primed cells, the PAR-1 agonist produced a ~75% decrease in TER and there was a prolonged recovery time to the baseline, lasting ~3 h [Fig. 12], indicating that increased SOCE contributes to the potentiated EC monolayer permeability following LPS priming.
Figure 10: LPS augments PAR-1-mediated Ca\(^{2+}\) influx. A) HLMVECs were exposed to LPS (1 µg/ml) for 0 and 4 h were loaded with 3 µM FURA-2 AM and used to measure Thr-induced increase in [Ca\(^{2+}\)], in the presence of extracellular Ca\(^{2+}\) (left). The arrows indicate the time point at which Thr (50 nm) was added. Results shown are representative of four experiments. The bar graph (right) shows quantification of change in fluorescence ratio at peak (100 sec) and at 350 sec. *p<0.01, **p<0.001; compared with control cells. B) HLMVECs pretreated with LPS as above were used to measure Thr (50 nM)-induced ER-stored Ca\(^{2+}\) release and Ca\(^{2+}\) release-activated Ca\(^{2+}\) entry (left). Results shown are representative of three experiments. The bar graph (right) shows the quantification of three experiments. NS = not significant; *p<0.01, compared with control cells. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 11: Silencing of STIM1 blocks PAR-1-mediated SOCE in presence or absence of LPS. A) HLMVECs were transfected with control-siRNA (Sc-siRNA) or STIM1-siRNA (100 nM) and at 48 h after transfection, cells were treated with LPS (1 μg/ml) for 0 and 4 h. After LPS treatment, cells were used to determine STIM1 protein expression by IB. Data shown are representative of two experiments. B) HLMVECs transfected with Sc-siRNA or STIM1-siRNA were exposed LPS as above were used to measure Thr-induced ER-stored Ca²⁺ release and Ca²⁺ release-activated Ca²⁺ entry. Data shown are representative of two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 12: LPS-induced expression of STIM1 potentiates PAR-1-mediated endothelial permeability. HLMVECs were grown to confluence on gold electrode. Cells were washed and incubated in 1% FBS medium for 12 h and then the cells were treated with or without LPS (1µg/ml) for 4 h. After this treatment, the cells were challenged with PAR-1 agonist peptide (TFLLRNPNDK; 25µM). Decrease in resistance, quantified from four independent experiments, are the mean ± S.E. (bottom panel). NS = not significant; *p<0.001, compared with control cells. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.2  LPS induced-STIM1 expression augments lung vascular permeability in mouse lung endothelial cells and potentiates PAR-1-induced increase in lung microvessel permeability, *in vivo*

4.2.1  LPS induces upregulation of STIM1 and store-operated Ca$^{2+}$ channel expressions in Mouse lung endothelial cells (MLECs)

To highlight the relevance of mouse studies to human disease, we studied the effects of LPS on STIM1 protein expression in MLEC. MLEC exposed to LPS for various time periods were used for IB studies to determine STIM1 protein expression. LPS induced STIM1 protein expression in a time-dependent manner (*Fig. 13*A). STIM1 expression was increased ~10 fold over basal after 6 h of LPS challenge (*Fig. 13*A). To address the functional relevance of LPS-induced STIM1 expression in MLEC, we measured Thr-induced as well as pharmacologically-induced SOCE using thapsigargin (TG) in control and LPS primed MLEC. Similar to HLMVEC, LPS priming enhanced SOCE in response to Thr or TG challenge without altering ER-store Ca$^{2+}$ release in MLEC (*Fig. 13*B and 14*).
**Figure 13:** LPS induces upregulation of STIM1 protein expression and augments PAR-1-mediated SOCE in MLECs.  

**A)** MLECs grown to confluence were exposed to LPS (1 µg/ml) for the indicated time points. Total cell lysates were used for IB. Results shown are representative of two experiments. Blots were quantified and fold increase over basal (FOB) relative to β-actin are shown.

**B)** MLECs pretreated with or without LPS (1 µg/ml) for 4 h. Cells loaded with Fura-2 were used to measure Thr (50 nM)-induced ER-stored Ca$^{2+}$ release and Ca$^{2+}$ release-activated Ca$^{2+}$ entry (left). Change in peak fluorescence ratio (340/380) for ER-stored Ca$^{2+}$ release and Ca$^{2+}$ entry over basal was calculated. The bar graph (right) shows the quantification of three experiments. NS = not significant; *p<0.001, compared with control cells.

This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 14: LPS induces augmentation of SOCE in Mouse lung endothelial cells. MLECs pretreated with or without LPS were used to measure TG (1µM)-induced ER-stored Ca\(^{2+}\) release and Ca\(^{2+}\) release-activated Ca\(^{2+}\) entry. TG-induced changes in 340/380 nm ratio color images were shown for control and LPS pretreated cells (top panel). Change in [Ca\(^{2+}\)]\(_i\) is depicted by the colors indicated in the arrow. TG-induced fluorescence ratio (340/380) change over basal for ER-stored Ca\(^{2+}\) release and Ca\(^{2+}\) release-activated Ca\(^{2+}\) entry in control and LPS pretreated cells are depicted in the graph (bottom panel). Results shown are representative two experiments.

This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.2.2 LPS priming *in vivo* increases STIM1 and SOC expressions, potentiating

**PAR-1-induced increase in lung microvessel permeability**

To validate the *in vivo* pathophysiologic relevance of increased STIM1 expression in ECs, we intraperitoneally (i.p.) injected mice (C57BL/6) with LPS, and lungs harvested at different time intervals after LPS injection were used for Western analysis. We observed substantially increased protein expression for STIM1, TRPC1, TRPC4, and Orai1, but not STIM2 in LPS-treated mice compared with control mice injected with saline [Fig. 15]. Further, to address whether the increased expression of STIM1, TRPC1, TRPC4, and Orai1 contributes to lung vascular hyper-permeability, we measured microvessel liquid permeability in isolated intact lung preparations. Here, mice received i.p. saline (control injection) or LPS for 4 h, followed by lung isolation for determining lung vascular liquid filtration coefficient \( K_{f,c} \), a measure of intact lung vascular permeability. We observed that PAR-1 activating peptide induced a ~3-fold increase in lung \( K_{f,c} \) when compared to basal conditions [Fig. 16]. Mice receiving the indicated dose of LPS alone also showed a significant increase in \( K_{f,c} \) [Fig. 16]. Interestingly, lung preparations from LPS-treated mice showed a ~5-fold increase in \( K_{f,c} \), over basal when challenged by PAR-1 activating peptide [Fig. 16].

To further support this observation, we determined PAR-1-induced lung vascular leak *in vivo* by measuring EBA uptake into the lung in control and LPS primed mice (88). Lung EBA uptake in mice receiving the PAR-1 activating peptide alone or LPS priming alone was ~3-fold higher than basal in control mice [Fig. 17], whereas in LPS injected mice, PAR-1 activating peptide administration induced a ~6-fold increased EBA uptake compared to untreated control mice [Fig. 17]. These *in vivo* results further support the notion that LPS-induced expressions of
STIM1 and SOC components, in intact lung microvessels, may contribute to the hyperpermeability response evident during sepsis.
Figure 15: LPS priming in vivo increases STIM1 and SOC expressions. C57BL/6 mice either injected with LPS (5.0 mg/kg, i.p.) or saline. After LPS or saline injection, at the indicated time periods lungs harvested were used for IB analysis to determine STIM1, STIM2, TRPC1, TRPC4, and Orai1. The blots were stripped and re-probed for β-actin antibody, as a loading control. Results shown are representative of three experiments. Blots were quantified and fold increase over basal (FOB) relative to β-actin are shown. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 16: LPS priming in vivo potentiates PAR-1-induced increase in lung microvessel permeability. C57BL/6 mice either injected with LPS (5 mg/kg) or saline as above. At 4 h after LPS or saline injection, lungs harvested were used for isolated lung preparation to determine lung vascular permeability. PAR-1 agonist peptide (30 µM) was included in the perfusion buffer to assess PAR-1-induced liquid filtration coefficient (Kf,c). n=5; *p <0.05, control group vs PAR-1 peptide, LPS treated group vs LPS + PAR-1 peptide group, or PAR-1 peptide group vs LPS + PAR-1 peptide. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and API/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 17: LPS priming in vivo potentiates PAR-1-induced increase in lung microvessel permeability. C57BL/6 mice either injected with LPS (5 mg/kg) or saline as above were used to measure PAR-1-induced EBA uptake in lungs. Top panel shows experimental design. Results shown are mean ± S.E. of changes in lung EBA uptake (n = 5, in each group). *p<0.01, control (saline injected) vs PAR-1 peptide treated, control vs LPS treated, or PAR-1 peptide treated vs LPS + PAR-1 peptide treated. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.3 LPS promotes NF-κB and p38 MAPK activation to induce STIM1 expression in endothelial cells

4.3.1 NF-κB regulates STIM1 expression downstream of LPS stimulation

Since STIM1 is crucial for activating SOCE in ECs to induce vascular hyper-permeability, we wanted to elucidate the signaling pathways regulating STIM1 expression downstream of TLR4 activation. It is now well known that signaling via both NF-κB and p38 MAPK pathways contribute to vascular inflammatory responses seen in sepsis (22,92-94). To determine the role of NF-κB, in mediating LPS-induced STIM1 expression, NF-κB activation was inhibited by using a NF-κB specific pharmacological inhibitor. We observed a significant decrease in LPS-induced STIM1 transcript formation in the NF-κB inhibitor pre-treated cells compared to control (vehicle-treated) cells [Fig. 18A]. We first silenced NF-κB protein p65/RelA in HLMVECs by transfecting HLMVECs with siRNA specific to p65/RelA. In this experiment, we observed that STIM1 protein expression in response to LPS was blocked in p65/RelA-siRNA transfected cells compared with control cells or scrambled-siRNA (Sc-siRNA) transfected cells [Fig. 18B, left panel]. Next, we observed that silencing of NF-κB protein p50/NF-κB1 also prevented LPS-induced STIM1 expression in HLMVECs [Fig. 18B, right panel]. Consistent with these results, LPS-potentiation of Thr-induced increase in SOCE was suppressed in HLMVECs after p65/RelA knock down [Fig. 19], indicating that NF-κB signaling is vital for the induction of STIM1 in ECs in response to LPS. These results indicate a critical role of NF-κB in regulating increased expression of STIM1 and subsequently augmented PAR-1-mediated SOCE during LPS induced inflammation or sepsis.
A

![Graph showing relative STIM1 mRNA expression over LPS exposure time.](image)

B

**Control**  Sc-siRNA  p65-siRNA

- **p65**
  - 65 kDa
- **β-actin**
  - 38 kDa

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<th>p65-siRNA</th>
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**STIM1 (SE)**

- 1.0 6.1 1.0 7.0 1.0 1.9

**STIM1 (LE)**

- 1.0 6.3 1.0 7.1 1.0 2.1

- **Control**  Sc-siRNA  p50-siRNA

- **p50**
  - 105 kDa
- **β-actin**
  - 50 kDa
  - 38 kDa

- **LPS exposure time (h)**

- **DMSO Control**
- **NF-κB Inhibitor**
Figure 18: NF-κB regulates STIM1 expression downstream of LPS stimulation. A) HLMVECs were pre-incubated with NF-κB inhibitor (5 μM) for 12 h and then cells were exposed to LPS for the indicated time periods. After LPS exposure, cells were used for QRT-PCR to determine mRNA expression for STIM1 and β-actin. STIM1 mRNA induction fold was calculated by measuring the ratio of STIM1 to β-actin. Results are mean ± S.E. of four experiments. *p<0.01, compared with cells treated with NF-κB inhibitor. B) HLMVECs were transfected with control siRNA (Sc-siRNA) or p65/RelA-siRNA (100 nM) or p50 si-RNA (100 nM). At 72 h after siRNA transfection, cells were exposed to LPS for 0, and 6 h. After LPS treatment, cells were used for IB to determine expression of STIM1, p65/RelA, p50 and β-actin. Abbreviations used: SE- Short exposure, LE- Long exposure. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 19: Silencing of p65/RelA inhibits LPS-induced potentiation of PAR-1-mediated SOCE. HLMVECs transfected with control siRNA (Sc-siRNA) or p65/RelA-siRNA (100 nM) were exposed to LPS for 0, and 6 h after 72 h of siRNA transfection. Following treatment the cells were used to measure Thr-induced ER-stored Ca^{2+} release and Ca^{2+} release-activated Ca^{2+} entry. Results shown are representative of at least two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.3.2 P38 MAPK pathway regulates LPS-induced STIM1 expression

There are several studies that indicate p38 and NF-κB can cooperatively signal proinflammatory gene expression (19,22,95). To determine if p38 MAPK pathway plays a role in mediating LPS-induced STIM1 expression, we inhibited LPS-induced activation of p38 MAPK with a specific pharmacological inhibitor, SB203580. We observed that inhibition of p38 MAPK prevented LPS-induced STIM1 mRNA expression in HLMVECs [Fig. 20A]. Next we investigated the effect of p38 MAPK inhibition on STIM1 protein expression in HLMVECs. We pretreated HLMVECs with p38 MAPK inhibitor SB203580 and challenged control (vehicle) and SB203580 treated cells with LPS for different time intervals; then the cells were used to determine STIM1 protein expression. LPS-induced STIM1 protein expression was markedly reduced in cells pretreated with SB203580 compared with vehicle treated control cells [Fig. 20B]. Next, we examined the effect of inhibition of p38 MAPK on LPS-induced STIM1 expression and subsequent potentiation of Thr-induced SOCE. In this experiment, we pretreated HLMVECs with LPS for 4 h in the presence and absence of SB203580, and then cells were used to measure Thr-induced ER-store Ca^{2+} release and SOCE. We observed that SB203580 blocked LPS induced potentiating of SOCE in response to Thr [Fig. 21].
Figure 20: P38 MAPK pathway regulates LPS-induced STIM1 expression. HLMVECs were pre-incubated with p38 MAPK inhibitor (10 μM) for 30 min and then cells were exposed to LPS for the indicated time periods. A) After LPS exposure, cells were used for QRT-PCR to determine mRNA expression for STIM1 and β-actin. STIM1 mRNA induction fold was calculated by measuring the ratio of STIM1 to β-actin. B) After LPS exposure, cells were homogenized and used to IB for STIM1 protein expression. Results are mean ± S.E. of four experiments. *p<0.01, compared with cells treated with either p38 MAPK inhibitor. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 21: Inhibition of p38 MAPK suppresses LPS-induced potentiation of PAR-1-mediated SOCE. HLMVECs pre-treated with vehicle (DMSO) or SB203580 for 30 min were then challenged with or without LPS for 4 h. After LPS treatment cells were used to measure Thr-induced ER-stored Ca\(^{2+}\) release and Ca\(^{2+}\) release-activated Ca\(^{2+}\) entry. Results shown are representative of two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.3.3 P38 MAPK pathway regulates the expression of STIM1 downstream of LPS stimulation

LPS is known to activate the ERK and JNK signaling pathways (96). Therefore, we investigated the possibility whether ERK and JNK activation also contribute to LPS-induced STIM1 expression in HLMVECs. Here, we used pharmacological approach to prevent LPS-induced activation of ERK and JNK in HLMVECs. We observed that ERK pathway inhibitor PD98059, prevented LPS-induced ERK phosphorylation [Fig. 22A], however, did not have any effect on LPS-induced STIM1 expression in HLMVECs [Fig. 22B]. Next, we observed that JNK pathway inhibitor SP600125, blocked LPS-induced JNK phosphorylation [Fig. 23A] but had no significant effect on LPS-induced STIM1 expression in HLMVECs [Fig. 23B]. These results suggest that LPS-induced activation of ERK and JNK signaling pathways are not involved in the mechanism of STIM1 expression in HLMVECs.

Since we observed only p38 MAPK inhibitor, but not ERK or JNK inhibitor suppressed LPS-induced STIM1 expression in HLMVECs; we determined the in vivo effect of inhibition of p38 MAPK on LPS potentiation of PAR-1-mediated increase in lung vascular permeability. In this experiment, we pretreated mice with and without SB203580, followed by LPS challenge for 4 h, and then lung vascular liquid permeability ($K_{f,c}$) was measured [Fig. 24]. We observed that LPS-induced STIM1 expression was markedly reduced in SB203580 injected mouse lung tissue compared with vehicle treated mouse tissue [Fig. 24A]. Interestingly, SB203580 pretreatment prevented LPS-induced STIM1 expression as well as LPS potentiation of PAR-1-mediated increase in $K_{f,c}$ [Fig. 24B]. These results support key role for p38 signaling in the mechanism of LPS-induced lung vascular leak.
Figure 22: Inhibition of ERK pathway does not alter LPS-induced STIM1 expression. HLMVECs were pre-incubated with PD98059 (ERK pathway inhibitor; 40µM) for 1 h and then cells were exposed to LPS for indicated time points. A) Following stimulation protein lysates collected were used to measure Phospho-ERK, Total ERK and β-actin expressions. Ratio of Phospho-ERK and Total ERK expressions are shown (P/T) as an average value for two experiments. B) After LPS treatments, cells were used for IB to determine STIM1 expression. Blots were quantified by densitometry and fold increase over basal (FOB) relative to β-actin were shown. Results shown are mean of two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 23: Inhibition of JNK pathway does not alter LPS-induced STIM1 expression. HLMVECs were pre-incubated with SP600125 (JNK pathway inhibitor; 20µM) for 30 min and then cells were exposed to LPS for indicated time points. A) Following stimulation protein lysates collected were used to measure Phospho-JNK, Total JNK and β-actin expressions. Ratio of Phospho-JNK and Total JNK expressions are shown (P/T) as an average value for two experiments. B) After LPS treatments, cells were used for IB to determine STIM1 expression. Blots were quantified by densitometry and fold increase over basal (FOB) relative to β-actin were shown. Results shown are mean of two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 24: Inhibition of p38 MAPK pathway prevents LPS-induced STIM1 expression and potentiation of PAR-1-mediated increase in $K_{f,c}$. C57BL/6 mice were either injected with SB203580 (1 mg/kg) or DMSO (vehicle) for 1 h followed by LPS or saline injection. At 4 h after LPS or saline injection, lungs harvested were used to measure STIM1 expression (A) or for isolated lung preparation to determine lung vascular permeability (B). PAR-1 agonist peptide (30 µM) was included in the perfusion buffer to assess PAR-1-induced liquid filtration co-efficient ($K_{f,c}$). n=5; **p <0.01, control group vs LPS + PAR-1 peptide group, LPS + PAR-1 peptide group vs SB 203580 + LPS + PAR-1 peptide group. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.3.4 **Silencing the p38α isoform inhibits LPS-induced STIM1 expression**

We have recently shown that the p38 MAPK isoforms p38α, p38β, and p38γ are expressed in HLMVECs (91). Also, we showed that silencing of p38α suppresses constitutive STIM1 expression in HLMVECs (91). To determine which isoform of p38 is involved in LPS-induced STIM1 expression in HLMVECs, we first silenced p38α in HLMVECs by transfecting HLMVECs with siRNA specific to p38α. In p38α-siRNA transfected cells, expression of p38α but not the other p38 isoforms under study (p38β and p38γ) was blocked [Fig. 25, top panel]. Interestingly, we observed that p38α-siRNA transfection prevented LPS-induced STIM1 expression [Fig. 25, bottom panel]. Next we silenced p38β expression in HLMVECs [Fig. 26A, left] and observed that p38β silencing had no significant effect on LPS-induced STIM1 expression [Fig. 26A, right]. Similarly, we observed that silencing of p38γ had no effect on LPS-induced STIM1 expression in HLMVECs [Fig. 26B]. These results collectively support the notion that p38α signaling plays a critical role in the mechanism of LPS-induced STIM1 expression in HLMVECs.
Figure 25: Silencing the p38α isoform inhibits LPS-induced STIM1 expression. HLMVECs were transfected with control siRNA (Sc-siRNA) or p38α-siRNA (200 nM). At 72 h after siRNA transfection, cells were exposed to LPS for 0, 2, 4, 6 h. After LPS treatment, cells were used for IB to determine STIM1 protein expression. The blot was stripped and re-probed with anti-p38α pAb or anti-β-actin mAb (bottom panel). Cell lysates were immunoblotted with antibodies specific to p38α, p38β, and p38γ (top panel). Results shown are representative of at least two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 26: Silencing either p38β or p38γ isoform does not alter LPS-induced STIM1 expression. A) HLMVECs were transfected with indicated concentrations of Sc-siRNA or p38β-siRNA. Left, after transfection, cells were used for IB to determine expression of p38β and β-actin. Right, after transfection, cells exposed to LPS were used for IB to determine expression of STIM1 and β-actin. B) HLMVECs transfected with indicated concentrations of Sc-siRNA or p38γ-siRNA. Left, after transfection, cells were for IB to determine expression of p38γ and β-actin. Right, after transfection, cells exposed to LPS were used for IB to determine expression of STIM1 and β-actin. In B and C, results shown are representative of at least two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.4 LPS induces the binding of the NF-κB component, p65/RelA, and the AP1 component, c-Fos, to the STIM1 promoter

4.4.1 Analysis of the 5’-regulatory sequence of human and mouse STIM1 gene

To gain insight into the transcriptional mechanism of LPS-induced STIM1 expression, we analyzed the 5’-regulatory region of the human (h) STIM1 gene utilizing the Genomatix software. We observed the presence of three putative NF-κB binding sites at -172, -198 and -216 bp (NF-κB1, 2, 3) and one AP1 site, -347 bp upstream of TSS in the hSTIM1 promoter (Fig. 27A). Interestingly, we also observed the presence of binding sites for the transcription factors NF-κB and AP1 in the 5’-regulatory region of the mouse (m) STIM1 gene (Fig. 27B).
Figure 27: Analysis of the 5'-regulatory sequence of human and mouse STIM1 gene. The putative nucleotide sequence identified in the regulatory region of human (A) and mouse (B) STIM1 genes is shown. Nucleotides are numbered relative to the transcription start site as +1. Potential consensus sequences for the transcription factors NF-κB and AP1 are indicated boldface and underlined. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.4.2 LPS-induces time-dependent binding of the transcription factors NF-κB and AP1 to their consensus binding sites on STIM1 promoter

To address whether LPS induces the binding of transcription factors NF-κB and AP1 to the hSTIM1 promoter, we performed ChIP assays. HLMVECs exposed to LPS for different time intervals were used for ChIP assays. We observed that LPS induced time-dependent binding of the NF-κB protein p65/RelA to the hSTIM1 promoter in HLMVECs [Fig. 28]. The binding of p65/RelA to the hSTIM1 promoter was ~6-fold higher than basal 60 min after LPS challenge and returned to basal 120 min after LPS challenge [Fig. 28]. Also, we observed the binding of the AP1 components c-Fos [Fig. 29A] and c-Jun [Fig. 29B] to the hSTIM1 promoter in response to LPS challenge in HLMVECs. The maximal binding for both the components were observed at 30 min after LPS stimulation [Fig. 29 A, B].

NF-κB being the major proinflammatory transcription factor downstream of LPS, EMSA was performed to further confirm the previous observation. Oligonucleotides mimicking the NF-κB binding consensus sequences in the STIM1 promoter, NF-κB1 and NF-κB 2 & 3 were designed (Details in “Methods” section). Nuclear extracts from HLMVECs treated with LPS for 0, 30, 60, and 120 min were incubated with radiolabelled probes/oligonucleotides. We observed maximal binding at 120 min and 60 min after LPS treatment for NF-κB1 and NF-κB 2 & 3 respectively [Fig. 30]. To confirm specificity of the band nuclear extracts were pre-incubated with p65/RelA antibodies. Super-shift was observed only for the p65/RelA antibody and not for p52 antibody. These results support the notion that LPS activates transcription factors NF-κB and AP1 to induce STIM1 transcription in ECs.
Figure 28: LPS-induces time-dependent binding of the transcription factor p65/RelA (NF-κB component) to its consensus binding sites on STIM1 promoter. ChIP assay of the interaction of p65/RelA with hSTIM1 promoter. Primers specific to the NF-κB binding sites 1-3 were used to perform quantitative PCR. HLMVECs exposed to LPS (1 µg/ml) for 0, 30, 60 and 120 min were used for ChIP assay. Results are mean ± S.E. of four experiments. Results were normalized to those of input DNA and are presented relative to basal values. *p<0.01, 0 min vs 30 min; **p<0.001, 0 min vs 60 min. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
A  

**c-Fos**

Fold increase over Basal (Arbitrary units)

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B  

**c-Jun**

Fold increase over Basal (Arbitrary units)

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* and ** indicate statistical significance.
Figure 29: LPS-induces time-dependent binding of the transcription factors c-Fos and c-Jun (AP1 components) to its consensus binding site on STIM1 promoter. ChIP assay of the interaction of the AP1 components c-Fos (A) and c-Jun (B) with hSTIM1 promoter. Immunoprecipitation was performed with specific c-Fos and c-Jun antibodies. Primers specific to the AP1 binding site was used to perform quantitative PCR. HLMVECs exposed to LPS (1 µg/ml) for 0, 30, 60 and 120 min were used for ChIP assay. Results are mean ± S.E. of four experiments. Results were normalized to those of input DNA and are presented relative to basal values. **p<0.001, 0 min vs 30 min; *p<0.01, 0 min vs 60 or 120 min. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 30: LPS-induces time-dependent binding of the transcription factor p65/RelA (NF-κB component) to its consensus binding sites on STIM1 promoter. HLMVECs grown to confluence were incubated overnight in 1% serum medium followed by LPS (1 µg/ml) stimulation for the indicated time points. Next, nuclear extracts were prepared and EMSA was performed (bottom panel) using 32P-labelled double stranded oligos containing the STIM1-specific NF-κB binding sites (top panel), NF-κB1 (Panel1), NF-κB2 (Panel2) and NF-κB2 (Panel3). P65/RelA and p52 antibodies were used to perform supershift assay (Panel3). The experiment was repeated three times and results shown are from representative experiments.
4.5 **P38α signaling downstream of TLR4 mediates c-Fos expression to induce STIM1 expression in endothelial cells**

4.5.1 **P38α-mediated c-Fos expression is required for LPS-induced STIM1 expression**

We showed above that silencing of NF-κB proteins (p65/RelA or p50) or p38α prevented LPS induced STIM1 expression in HLMVECs. Further, we showed that LPS induced the binding of the NF-κB protein p65/RelA and the immediate-early gene c-Fos, a component of AP1, to the STIM1 promoter in HLMVECs. To determine whether c-Fos signaling is essential for LPS induced STIM1 expression, we silenced c-Fos expression in HLMVECs by transfecting HLMVECs with c-Fos-siRNA. In c-Fos-siRNA transfected cells, basal as well as LPS-induced c-Fos expressions were markedly reduced in HLMVECs compared with control or Sc-siRNA transfected cells [Fig. 31]. Interestingly, we observed that LPS-induced STIM1 expression was also prevented in c-Fos-siRNA transfected cells [Fig. 31]. Next, we silenced c-Jun expression and results showed that in c-Jun-siRNA transfected cells, c-Jun expression was largely reduced compared with control cells or cells transfected with Sc-siRNA [Fig. 32]. Surprisingly, we observed that LPS-induced STIM1 expression was only partially prevented in c-Jun-siRNA treated cells compared with controls [Fig. 32]. These observations suggest that c-Fos, but not c-Jun signaling plays a dominant role in LPS-induced STIM1 transcription in HLMVECs.

P38 MAPK signaling activates the AP1 transcription factor (97-100). Since we observed that suppression of p38α expression in HLMVECs prevented LPS-induced STIM1 expression, we investigated the possible role of LPS-induced p38α activation in mediating the
induction of c-Fos expression. In this experiment, we silenced p38α expression in HLMVECs and then measured the expression of the AP1 components c-Fos and c-Jun. Interestingly, silencing of p38α inhibited LPS-induced c-Fos expression; however, it increased LPS-induced c-Jun expression [Fig. 33]. This suggests that p38α signaling might be regulating LPS-induced STIM1 expression via c-Fos but not c-Jun. Since we observed that NF-κB signaling is required for LPS-induced STIM1 expression, we determined whether NF-κB signaling is required for LPS-induced c-Fos expression in HLMVECs. We observed that silencing of p65/RelA had no effect on c-Fos expression in HLMVECs [Fig. 34], indicating that p38α but not NF-κB signaling controls c-Fos expression in HLMVECs.

Next we measured the effect of p38α knockdown on LPS induced phosphorylation of c-Fos at Thr-325 and observed significant inhibition of c-Fos activation compared to control and Sc-siRNA transfected HLMVECs [Fig. 35]. Since the basal level of c-Fos expression was inhibited in the p38α knockdown cells, we wanted to confirm the role of p38α in regulating c-Fos activation. We treated the cells for a brief period, 10 min with the p38 inhibitor, SB203580, followed by LPS stimulation for 0, 5, 10 and 15 min. We observed no significant alteration of the constitutive c-Fos expression. However, activation of c-Fos was significantly inhibited in the SB203580 treated cells compared to untreated (Control) cells [Fig. 36]. These results collectively support the notion that p38α-mediated c-Fos expression is required for STIM1 expression in endothelial cells.
Figure 31: Silencing of c-Fos suppresses LPS-induced STIM1 expression. HLMVECs transfected with control siRNA (Sc-siRNA) or c-Fos-siRNA (100 nM). At 72 h after siRNA transfection, cells were exposed to LPS (1 µg/ml) for 0, 2, 4, 6 h. After LPS treatment, cells were used for IB to determine STIM1, c-Fos, and β-actin expression. Experiment was repeated three times and results shown are mean ± S.E. *p<0.05; **p<0.001; significantly different from control cells not stimulated with LPS. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 32: Silencing of c-Jun does not significantly alter LPS-induced STIM1 expression. HLMVECs transfected with control siRNA (Sc-siRNA) or c-Jun-siRNA (100 nM). At 72 h after siRNA transfection, cells were exposed to LPS (1 µg/ml) for 0, 2, 4, 6 h. After LPS treatment, cells were used for IB to determine STIM1, c-Fos, and β-actin expression. Experiment was repeated three times and results shown are mean ± S.E. *p<0.05; **p<0.01; significantly different from control cells not stimulated with LPS. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
Figure 33: Knockdown of p38α suppresses c-Fos expression. HLMVECs transfected with control siRNA (Sc-siRNA) or p38α-siRNA. At 72 h after siRNA transfection, cells were exposed to LPS (1 μg/ml) for 0, 2, 4, and 6 h. After LPS exposure, cells were for IB to determine c-Fos, c-Jun and β-actin expression. Results shown are mean ± S.E. of two experiments. *p<0.05; **p<0.001; significantly different from control cells not stimulated with LPS. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyperpermeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051. © the American Society for Biochemistry and Molecular Biology.
Figure 34: Silencing p65 does not inhibit c-Fos expression. HLMVECs transfected with control siRNA (Sc-siRNA) or p65/RelA-siRNA (100 nM). After transfection, cells exposed to LPS as above were used for IB to determine c-Fos and β-actin expression. Results shown are representative of at least two experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051. © the American Society for Biochemistry and Molecular Biology.
Figure 35: Knockdown of p38α suppresses c-Fos activation. HLMVECs transfected with control siRNA (Sc-siRNA) or p38α-siRNA (200 nM). At 72 h after siRNA transfection, cells were exposed to LPS (1 μg/ml) for 0, 15, 30, and 60 min. After LPS exposure, cells were for IB with antibodies specific to phospho-c-Fos or total c-Fos. Experiments were repeated three times and results shown are mean ± S.E. (top and middle panels). *p<0.01, significantly different from control cells not stimulated with LPS. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051. © the American Society for Biochemistry and Molecular Biology.
Figure 36: Pharmacological inhibition of p38α suppresses c-Fos activation. HLMVECs were pre-incubated with SB203580 (p38 MAPK inhibitor) for 10 min and then cells were exposed to LPS for indicated time periods. After LPS treatments, cells were used for IB to determine phospho-c-Fos and total c-Fos expressions. Ratio of Phospho-c-Fos and Total c-Fos expressions are shown (P/T). Results are a mean of three experiments. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy et al. Endotoxin-induced STIM1 expression in endothelial cells requires cooperative signaling of transcription factors NF-κB and AP1/c-Fos and mediates lung vascular hyper-permeability. In press, published on July 11, 2014 as doi: 10.1074/jbc.M114.570051 © the American Society for Biochemistry and Molecular Biology.
4.6 Generation of endothelial cell-restricted STIM1 knockout mice

Previously, we observed that prevention inhibition of LPS-induced STIM1 expression in the endothelial cells suppressed LPS-induced microvascular leak. To validate the in vivo role of endothelial cell-expressed STIM1 in regulating endothelial permeability, we generated endothelial cell-restricted STIM1 knockout mice [STIM1$^{EC-/-}$] mice.

4.6.1 STIM1$^{EC-/-}$ mice was generated by crossing STIM1$^{fl/fl}$ and Tie2$^{Cre+/+}$ mice

Endothelial cell-restricted STIM1 knockout [STIM1$^{EC-/-}$] mice was generated by crossing B6.Cg-Tg(Tek-cre)1Ywa/J (mouse endothelial-specific receptor tyrosine kinase, Tie2 promoter driving the expression of Cre recombinase; a.k.a. Tie2Cre mice) with STIM1$^{fl/fl}$ mice. Breeding was set up as depicted [Fig. 37]. Evidences suggest Tie2Cre transgenic mice although predominantly deletes floxed-gene expression from ECs, it also deletes gene expression in the hematopoietic cells (101). We also generated another STIM1$^{EC-/-}$ mice using the Ve-Cadherin driving Cre gene expression, transgenic mice, and STIM1$^{fl/fl}$ mice to confirm our observations.
Figure 37: Diagrammatic representation of the breeding scheme followed to generate endothelial cell-restricted STIM1 knockout (STIM1EC/-) mice. Mice having loxP sites flanking STIM1 (STIM1fl/fl) were crossed with Tie2Cre+ transgenic mice. The first generation, heterozygous male, STIM1fl/+Cre+ mice were crossed with female STIM1fl/fl mice to generate second generation offsprings which were STIM1fl/fl.Cre+ or STIM1EC-/- mice.
4.6.2 **Deletion of STIM1 in the lungs of STIM1\textsuperscript{EC-/-} mice**

To confirm the genotype of the mice PCR was performed using the DNA isolated from the mice tail snips. Bands corresponding to 348 bp (WT) and 399 bp (STIM1\textsuperscript{fl/fl}) were observed. Mice expressing 399 bp and 580 bp gene products were the STIM1\textsuperscript{fl/fl}.Cre\textsuperscript{+} (a.k.a. STIM1\textsuperscript{EC-/-}) mice [**Fig. 38**]. To confirm the deletion of STIM1 in lung ECs of STIM1\textsuperscript{EC-/-} mice, STIM1 mRNA expression was measured in whole lung tissue. We observed significant decrease in STIM1 mRNA levels in STIM1\textsuperscript{EC-/-} mice lungs compared to STIM1\textsuperscript{fl/fl} mice lungs [**Fig. 39A**]. Next, protein expression of STIM1 was measured in the lung obtained from STIM1\textsuperscript{fl/fl} and STIM1\textsuperscript{EC-/-} mice. Significantly decreased STIM1 protein expression was observed in STIM1\textsuperscript{EC-/-} mice [**Fig. 39B**]. Cre protein expression was observed in the STIM1\textsuperscript{EC-/-} lungs; however, Cre expression was absent in STIM1\textsuperscript{fl/fl} lungs [**Fig. 39B**].
Figure 38: Genotyping of STIM1<sup>EC−/−</sup> mice. DNA isolated from mice tail snips was used to perform PCR. 348 bp, 399 bp and 580 bp products. Primer details are provided in “Methods”.
Figure 39: Deletion of STIM1 in STIM1<sup>EC<sup>-/-</sup></sup> mouse lungs. A) Total RNA isolated from the lungs of STIM1<sup>fl/fl</sup> and STIM1<sup>EC<sup>-/-</sup></sup> mice was used for QRT-PCR to determine STIM1 mRNA expression. Results were normalized with β-actin mRNA expression. B) Lungs from three mice in each group (indicated as 1, 2 and 3) were homogenized and immunoblotted with antibodies specific to STIM1, Cre and β-actin (left). Quantification of the STIM1 expressions in STIM1<sup>fl/fl</sup> and STIM1<sup>EC<sup>-/-</sup></sup> mice are shown (right). Results shown are at least repeated twice, n=3 for each experiment.
4.6.3 Deletion of STIM1 in lung endothelial cells of STIM1^{EC/-} mice

Further, to specifically test if STIM1 expression was disrupted in the endothelial cells, lung ECs were freshly isolated from STIM1^{EC/-} and STIM1^{fl/fl} mice. Immunoblot was performed with anti-STIM1 antibody to test STIM1 protein expression. We observed almost complete loss of STIM1 protein expression in STIM1^{EC/-} lung ECs (LECs) compared to STIM1^{fl/fl} LECs [Fig. 40A]. To test the functional relevance of STIM1 deletion, freshly isolated LECs were used to measure PAR-1-mediated SOCE. SOCE was completely blocked in the STIM1^{EC/-}-LECs when compared to STIM1^{fl/fl}-LECs [Fig. 40B]. Thus these observations confirm the critical role of STIM1 in mediating PAR-1-mediated SOCE in LECs.
Figure 40: STIM1 was deleted in lung endothelial cells of STIM1<sup>EC−/−</sup> mice. Lung endothelial cells (LECs) were freshly isolated from STIM1<sup>EC−/−</sup> and STIM1<sup>fl/fl</sup> mice A) were used to perform IB using anti-STIM1 and anti-β-actin antibodies. B) Freshly isolated LECs were used to measure PAR-1-mediated SOCE. Arrow indicates the time at which Thr was added. These experiments were repeated at least twice and for each experiment LECs collected from five mice were pooled together.
4.6.4 **STIM1 deletion in endothelial cells down-regulates expression of the SOC channel components**

Since we observed complete inhibition of SOCE in STIM1\textsuperscript{EC/-} -LECs, we wanted to measure the expression levels of TRPC4 (dominantly expressed TRPC isoform in mice) and Orai1 in these mice. Surprisingly, we found significant decrease in TRPC4 and Orai1 protein expression in STIM1\textsuperscript{EC/-} lungs basally [Fig. 41].

Since the expressions of the SOC channels, TRPC4 and Orai1, were decreased constitutively, we wanted to elucidate if STIM1 was somehow involved in regulating TRPC4 and Orai1 expression transcriptionally. We performed QRT-PCR using RNA isolated from the lungs of STIM1\textsuperscript{fl/fl} and STIM1\textsuperscript{EC/-} mice to test TRC4 and Orai mRNA expressions. The level of transcript formation for TRPC4 and Orai1 remain unaltered in STIM1\textsuperscript{fl/fl} and STIM1\textsuperscript{EC/-} mice [Fig. 42]. Thus, we speculate the possibility that STIM1 might be involved in stabilizing the Orai1 and TRPC4 proteins.
Figure 41: Deletion of STIM1 in the endothelial cells suppresses the expressions of TRPC4 and Orai1. Lung tissues from STIM1^{fl/fl} and STIM1^{EC-/} mice were used for IB (top panel). Orai1 and TRPC4 expression in STIM1^{fl/fl} and STIM1^{EC-/} lungs were normalized with the β-actin expression levels and quantified (bottom panel).
Figure 42: TRPC4 and Orai1 transcript levels remain unaltered in STIM1^{fl/fl} and STIM1^{EC-/-} lungs. RNA was isolated from the lungs of STIM1^{fl/fl} and STIM1^{EC-/-} mice. QRT-PCR was performed to determine TRPC4 (top panel) and Orai1 (bottom panel) expression levels. β-actin transcript levels were used to normalize the results. N.S. = not significant.
4.7 LPS-mediated potentiation of PAR-1-induced increase in lung microvessel permeability is abrogated in STIM1$^{EC/-}$ mice

4.7.1 LPS-induced ICAM1 expression prevented in STIM1$^{EC/-}$ mice

STIM1$^{EC/-}$ mice, we observed deletion of STIM1 in the endothelial cells decreased expression of the store-operated Ca$^{2+}$ entry components, TRPC4 and Orai1. Previous studies from our laboratory have shown PAR-1-mediated Ca$^{2+}$ influx through TRPC cannels is required for NF-κB activation (60). It is also well known that NF-κB up-regulates the expression of adhesion molecules such as ICAM-1 and VCAM-1, in the endothelium during inflammation aiding leukocyte migration and vascular permeability (102,103). Thus, we determined the effect of deletion of STIM1 in the endothelial cells during inflammation by measuring the expressions of the adhesion molecule, ICAM1 in response to LPS in STIM1$^{fl/fl}$ and STIM1$^{EC/-}$ mice. We administered LPS, i.p., to STIM1$^{EC/-}$ and STIM1$^{fl/fl}$ mice and after 6 h the lungs were isolated. The lungs were homogenized and used for IB. We see significant inhibition of LPS-induced ICAM1 expression after 6 h of LPS administration in STIM1$^{EC/-}$ mice compared to STIM1$^{fl/fl}$ mice [Fig. 43]. Similar observation was also reported by a paper published by Gandhirajan et al. (8).
Figure 43: Suppression of LPS-induced ICAM-1 expression in STIM1EC/- mice. STIM1fl/fl and STIM1EC/- mice received LPS, i.p., for 0 and 6 h. Next, lungs were isolated, homogenized and used for IB. Anti-STIM1, anti-ICAM-1 or anti-β-actin antibodies were used. Experiment was repeated twice and each experiment was performed with at least 2 mice in each time point and each group.
4.7.2 Sepsis mediated lung vascular leak is abrogated in STIM1$^{EC/-}$ mice

Next, we wanted to test the pathophysiological *in vivo* relevance of deleting STIM1 from the endothelial cells. We measured microvessel liquid permeability in isolated intact lung preparations in response to LPS in STIM1$^{EC/-}$ mice and STIM1$^{fl/fl}$ mice. Lung vascular liquid filtration coefficient, K$_{f,c}$, is a measure of intact lung vascular permeability (31,37). Here, mice received an i.p. dose of saline (control injection) or LPS for 4 h, and then lungs isolated were used for K$_{f,c}$ measurement. We observed that PAR-1 activating peptide induced increase in lung K$_{f,c}$ in STIM1$^{fl/fl}$ lungs [Fig. 44]. Interestingly, PAR-1-mediated increase in K$_{f,c}$ was completely abrogated in STIM1$^{EC/-}$ lungs [Fig. 44]. Lungs isolated from LPS-treated STIM1$^{fl/fl}$ mice showed a ~6-fold increase in K$_{f,c}$ over basal when perfused by PAR-1 activating peptide [Fig. 44]. Surprisingly, LPS potentiation of PAR-1-mediated increase in K$_{f,c}$ was completely blocked in STIM1$^{EC/-}$ mice [Fig. 44]

These results comprehensively suggest that endothelial-STIM1 might play a critical role in mediating lung vascular leak during sepsis.
Figure 44: Sepsis mediated lung vascular leak is abrogated in STIM1EC−/− mice. STIM1fl/fl and STIM1EC−/− mice either received an i.p. dose of LPS (5 mg/kg) or saline. At 4 h after LPS or saline injection, lungs harvested were used for isolated lung preparation to determine lung vascular permeability. PAR-1 agonist peptide (30 µM) was included in the perfusion buffer to assess PAR-1-induced liquid filtration co-efficient ($K_{f,c}$). n=5; *p <0.05
4.7.3 **Suppression of LPS-induced augmentation of PAR-1-mediated SOCE in intact venules and capillaries of STIM1*EC/-* mice**

To characterize the effect of STIM1 deletion on the permeability of venules versus capillaries in response to LPS or PAR-1 peptide in STIM1*EC/-* and STIM1*fl/fl* mice, we performed *in vivo* real-time fluorescence imaging in collaboration with Dr. Kaushik Parthasarathi (The University of Tennessee Health Science Center Memphis, Tennessee). We observed that in response to targeted infusion of PAR-1 peptide into lung capillaries and venules, cytosolic Ca$^{2+}$ concentration remained low in the lungs of STIM1*EC/-* mice when compared STIM1*fl/fl* mice [Fig. 45A].

Next, STIM1*fl/fl* of STIM1*EC/-* mice received an i.p. dose of LPS or saline (control) for 4 hrs. Real-time fluorescent imaging revealed lower cytosolic Ca$^{2+}$ concentration in the lung venules and capillaries of STIM1*EC/-* mice compared to STIM1*fl/fl* mice [Fig. 45B]. These results collectively support the notion that endothelial STIM1 is a critical regulator of PAR-1-induced lung vascular leak during sepsis.
Figure 45: Suppression of LPS-induced augmentation of PAR-1-mediated SOCE in intact venules and capillaries of STIM1<sup>EC/-</sup> mice. A) Targeted infusion of PAR-1-activating peptide was performed in intact capillaries (left) and venules (right) of STIM1<sup>fl/fl</sup> and STIM1<sup>EC/-</sup> mice. B) STIM1<sup>fl/fl</sup> and STIM1<sup>EC/-</sup> mice received an i.p. dose of LPS. Microvessels were then loaded with the cell-permeable ratiometric Ca<sup>2+</sup> indicator Fura2-AM. After the respective treatment, the response was recorded for more than 60 min. Fluorescence emissions from both 340 and 380 excitations were quantified at a single region along a vessel wall. The mean oscillation amplitude from three consecutive oscillations was taken as the amplitude of Ca<sup>2+</sup> oscillation. Oscillation amplitude was determined separately for baseline and response. Changes from baseline was quantified and plotted. n=5; *p <0.05
5. DISCUSSION

*Portions of the text were reprinted with permission from the American Society for Biochemistry and Molecular Biology. This research was originally published in The Journal of Biological Chemistry. Auditi DebRoy, Stephen M. Vogel, Dheeraj Soni, Premanand C. Sundivakkam, Asrar B. Malik and Chinnaswamy Tiruppathi.


Sepsis associated with acute lung injury (ALI) is one of the most common causes of death in hospitalized patients (4,34,104,105). Lung vascular leak and protein-rich pulmonary edema are the hallmarks of ALI (34,106). However, the signaling cascade mediating lung vascular leak during sepsis is still not clear. Previous studies from our laboratory have shown that Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels (SOCs) regulates lung vascular barrier function (33,37). In addition, we have shown Thr, which is generated in abnormal levels in the vascular system during sepsis (26,107-110), can induce Ca\(^{2+}\) entry through activation of transient receptor potential canonical channels (TRPC1 and TRPC4) in the endothelial cells to cause vascular barrier dysfunction (35,37,40,81,91). Further, we have shown that inflammatory mediators, via the activation of the proinflammatory transcription factor NF-κB, induce the expression of TRPC1 in human lung vascular ECs (35,36). Also, the increased TRPC1 expression was
associated with augmented SOCE and increased permeability in human ECs. Recent studies from our laboratory have shown the critical role of the endoplasmic reticulum (ER)-localized Ca\(^{2+}\) sensing protein STIM1 in activating SOCE in the endothelial cells (81,91). Importantly, last year a study was published which showed endotoxin-induced lung vascular leak and injury responses were suppressed in endothelial cell-restricted STIM1 knockout mice (8). This suggests endothelial STIM1-mediated Ca\(^{2+}\) entry (i.e. SOCE) to play a vital role in the mechanism of sepsis-induced lung vascular leak. To elucidate the mechanism by which STIM1 regulates lung vascular hyper-permeability, we questioned whether the expression of STIM1 is increased during sepsis and further if its level of expression directly correlates with the abnormal lung vascular leak.

We observed the expression of STIM1 to be induced in response to endotoxin in both human and mouse lung endothelial cells. Interestingly, we also found the expressions of the SOC components TRPC1, TRPC4, and Orai1 channels to be induced under similar conditions. In addition, we show the increased expressions of STIM1 and SOC components was associated with augmented PAR-1-mediated SOCE and further, PAR-1-mediated endothelial permeability. Several studies have shown endotoxin (LPS) and TNF-\(\alpha\), induce the up-regulation of inflammatory genes by activating the major proinflammatory transcription factor NF-\(\kappa\)B and p38 MAPK signaling pathways, and thereby triggering an inflammatory response (22,93-95). To test whether both NF-\(\kappa\)B and p38 MAPK pathways downstream of TLR4 activation plays a role in regulating STIM1 expression, we pharmacologically inhibited either of them in endothelial cells. Interestingly, we observed blocking either NF-\(\kappa\)B or p38 MAPK prevented LPS-induced STIM1
expression and also the subsequent increase in PAR-1-mediated SOCE. These observations were recapitulated by knocking down either NF-κB protein p65/RelA or the p38 MAPK isoform, p38α, in endothelial cells. These findings support the hypothesis that cooperative signaling of both NF-κB and p38α regulates STIM1 expression in response LPS challenge in endothelial cells.

To further dissect the transcriptional mechanism of STIM1 expression in response to inflammatory stimuli in endothelial cells the STIM1 gene promoter was analyzed. We found consensus binding sites for the transcription factors NF-κB and AP1 in the 5′-regulatory regions of both human and mouse STIM1 genes. Since both of these transcription factors are proinflammatory, we speculated they might be both involved in regulating STIM1 expression downstream of LPS. To determine binding of these factors to the STIM1 promoter in response to LPS, ChIP was performed. We indeed observed that LPS induced the binding of the p65/RelA (NF-κB protein) and c-Fos (AP1 component) to the hSTIM1 promoter in a time-dependent manner in HLMVECs. Consistent with this result, we observed that silencing of either p65/RelA or p50 (NF-κB components) or c-Fos (AP1 component) prevented LPS-induced STIM1 expression in HLMVECs. However, knocking down c-Jun only partially inhibited LPS-induced STIM1 expression. These results collectively support the notion that LPS induce the expression of STIM1 via the proinflammatory transcription factors NF-κB and AP1/c-Fos.

There are four different p38 MAPK isoforms (MAPK14 [p38α]; MAPK11 [p38β], MAPK12 [p38γ], and MAPK13 [p38δ]) in mammalian cells (19). Recently we reported
the expression of p38α, p38β, and p38γ isoforms in HLMVECs (91). We also show that SOCE-mediated p38β activation induced STIM1 phosphorylation which consequently inhibited SOCE in endothelial cells (91) and silencing of p38 MAPK isoform p38α, significantly reduced constitutive STIM1 expression in endothelial cells (91). Here, we observed that endotoxin-induced STIM1 expression was markedly reduced in p38α knocked down HLMVECs, suggesting that p38α signaling is required for endotoxin-induced STIM1 transcription. Several evidences suggest that p38 MAPK signaling might regulate inflammatory response in more than one way. It can control inflammatory gene transcription through chromatin remodeling or post-transcriptionally regulating mRNA stability. In this event we speculated that p38 could increase the accessibility of transcription factors (c-Fos/AP1) to their binding sites in the STIM1 promoter regions (22,98). Thus, to elucidate the role of p38α signaling in the mechanism of LPS induced STIM1 expression; we measured c-Fos expression in response to LPS in p38α knockdown HLMVECs. We observed p38α knockdown significantly inhibited c-Fos expression under basal conditions as well as in response to LPS challenge. Whereas, p38α knockdown increases c-Jun expression upon LPS challenge, indicating it is not playing a major role in LPS-induced STIM1 expression. This suggests that p38α signaling may induce STIM1 expression via c-Fos (AP1 component) in endothelial cells [Fig. 46].
Figure 46: Signaling pathway downstream of TLR4 activation inducing STIM1 transcription. Upon LPS binding to the TLR4 receptor on the endothelial cell membrane, activated TNF receptor (TNFR)–associated factor 6 (TRAF6) induces TAK1 activation, which in turn is responsible for stimulating p38 MAPK and IKK activation (111). Specifically p38α induces c-Fos expression and activation leading to AP1 heterodimer (c-Fos/c-Jun) translocation to the nucleus (22,98). On the other hand, IKK activation induces NF-κB (p65/p50) translocation to the nucleus (16-18). Based on our results, we propose that cooperative signaling of the transcription factors NF-κB and AP1 are required for STIM1 transcription during inflammation.
To validate our findings in an *in vivo* model, endothelial-restricted STIM1 mice [STIM1\(^{EC/-}\) mice] was generated by crossing B6.Cg-Tg(Tek-cre)1Ywa/J (mouse endothelial-specific receptor tyrosine kinase, Tie2 promoter directing the expression of Cre recombinase) with STIM1\(^{fl/fl}\) mice for two generations. We confirmed the successful deletion of STIM1 expression from the endothelial cells. Interestingly, we found constitutive down-regulation of SOC components expression, TRPC4 and Orai1 in STIM1\(^{EC/-}\) mice. We further tested the transcript formation levels of TRPC4 and Orai1 in STIM1\(^{EC/-}\) lungs and found no significant difference when compared with STIM1\(^{fl/fl}\) mice. Thus, we speculate the possibility of STIM1 being involved in maintaining protein stability of the SOC protein components. To determine the functional relevance of STIM1 deletion in the endothelial cells we freshly isolated endothelial cells from the lungs of STIM1\(^{EC/-}\) mice, and measured PAR-1-mediated SOCE in these cells. Interestingly, we find complete blockade of PAR-1-mediated store-operated Ca\(^{2+}\) entry. These results are consistent with our *in vitro* observations, where knockdown of STIM1 using specific STIM1-siRNA inhibited SOCE in HLMVECs. Published studies indicate that Ti2Cre transgenic mice delete the expression of floxed-gene dominantly in ECs and to some extent in cells of hematopoietic origin (101). Therefore, we only used blood-free lungs and lung-ECs for our studies, thus the effect of the lack of expression of STIM1 in the myeloid cells can be ignored in our observations. We also generated STIM1\(^{EC/-}\) mice using the Ve-Cadherin driven Cre transgenic mice to confirm our observations.

A recent study has shown that reactive oxygen species (ROS) can induce STIM1-mediated Ca\(^{2+}\) entry through Orai1 channels in ECs by activating STIM1 (8), implicating a
crucial role of STIM1 during inflammation. In this study, we show that deletion of STIM1 in the endothelial cells results in complete blockade of PAR-1-mediated SOCE in freshly isolated ECs from STIM1^EC-/- lungs and protects the mice from PAR-1-mediated endothelial permeability. These results comprehensively suggest a critical role for endothelial-STIM1 in mediating microvascular leak associated with sepsis. Thus, an innovative and unique approach to eliminate vascular leak can be pursued by generating a very specific peptide drug to interrupt TRPC channel gating by STIM1 in endothelial cells, blocking augmented PAR-1-mediated SOCE during sepsis and subsequent PAR-1-mediated endothelial barrier dysfunction.

In summary, we have shown that the bacterial endotoxin, LPS, induced the expression of STIM1 and its interacting calcium channel proteins, TRPC1, TRPC4, and Orai1, in both human and mouse endothelial cells. The increased expression of these proteins was associated with augmented PAR-1-mediated permeability responses. Importantly, we have shown that NF-κB activation and p38α-mediated c-Fos expression in response to endotoxin contribute to STIM1 transcription in vascular endothelial cells, setting the stage for endothelial Ca^{2+} overload and resulting vascular leak induced by mediators such as thrombin. We further demonstrate that STIM1^EC-/- mice are completely protected from PAR-1-mediated endothelial barrier dysfunction. These observations validate the critical role for endothelial cell-expressed STIM1 in mediating microvascular leak during sepsis.
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APPENDICES
APPENDIX A

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Department of Pharmacology and Center for Lung and Vascular Biology
Tel # 312-355-0249
Email: tiruc@uic.edu
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Author: Jiaping Xue, Prabhakar B. Thippegowda, Guochang Hu, Kurt Bachmaier, John W. Christman, Asrar B. Malik, Chinnaswamy Tiruppathi
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