# Differential toll-like receptor activation in lung ischemia reperfusion injury

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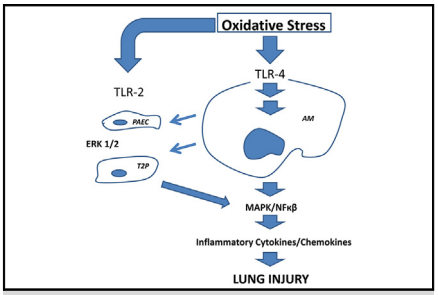
## ABSTRACT

**Objective:** The requirement for toll-like receptors (TLRs) in lung ischemia reperfusion injury (LIRI) has been demonstrated but not fully characterized. Previously, we reported that TLR-4 is required by alveolar macrophages but not pulmonary endothelial or epithelial cells for development of LIRI. Additionally, we demonstrated differential patterns of mitogen-activated protein kinase (MAPK) activation and cytokine release in these cell types during LIRI. Here, we sought to determine whether these differences in activation responses are related to cell-specific TLR activation requirements.

**Methods:** Primary cultures of alveolar macrophages, pulmonary endothelial, and immortalized epithelial cells were pretreated with TLR-2 or TLR-4 short interference RNA (ribonucleic acid) before hypoxia and reoxygenation. Cell lysates and media were analyzed for receptor knockdown, MAPK activation, and cytokine production. Rats were pretreated with TLR-2 or TLR-4 short interference RNA before lung ischemia reperfusion and changes in lung vascular permeability were assessed.

**Results:** Knockdown of TLR-2 in alveolar macrophages did not affect MAPK phosphorylation or cytokine secretion. Conversely, TLR-2 knockdown in pulmonary endothelial and epithelial cells demonstrated significant reductions in extracellular signal-regulated kinase 1/2 activation and cytokine secretion. The lung permeability index in LIRI was decreased by TLR-4 but not TLR-2.

**Conclusions:** Differential TLR signaling and MAPK activation in response to LIRI seem to be cell specific. Short interference RNA provides an outstanding tool for examination of the underlying mechanism. (J Thorac Cardiovasc Surg 2015;149:1653-61)


Overview of toll-like receptor activation in lung ischemia reperfusion injury.

**Central Message**

Lung ischemia reperfusion injury results from the activation of toll-like receptor-4 in alveolar macrophages and toll-like receptor-2 in endothelial and epithelial cells. This discovery allows for a greater understanding of the interactions between these cell types as well as their differential response to ischemia reperfusion.

**Perspective**

The discovery of differential cellular activation in lung ischemia reperfusion injury allows for the exploitation of cellular differences to design precise therapeutics. Ultimately, this work seeks to devise strategies to prevent innate immunity mediated ischemia-reperfusion injury and still allow for the appropriate response to microbial infection.

See Editorial Commentary page 1662.

Despite improvements in organ preservation and donor and recipient management, lung ischemia reperfusion injury (LIRI) remains a significant clinical problem after lung transplantation.[1,2] Up to 25% of lung transplant recipients develop significant reperfusion injury in the hours after implantation.[3] This complication increases the risk of acute rejection, adversely affects early postoperative mortality, and is the strongest acute risk factor for obliterative bronchiolitis.[4,5] Characterization of the cell-specific inflammatory signaling events in LIRI is a crucial step toward identifying strategies and treatments aimed at ameliorating LIRI and improving outcomes.

Work in vitro has demonstrated that, in response to oxidative stress, activation of the mitogen-activated protein kinases (MAPKs), p38, and c-Jun N-terminal kinase, is discretely associated with the alveolar macrophage.[6] Additionally, we have demonstrated the importance of early activation of the alveolar macrophage in the development of LIRI.[7] Conversely, activation of extracellular signal-regulated kinase 1/2, but not p38 or c-Jun N-terminal kinase, occurs in pulmonary artery endothelial cells and

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**Abbreviations and Acronyms**

CINC = cytokine-induced neutrophil chemoattractant
LIRI = lung ischemia reperfusion injury
MAPK = mitogen-activated protein kinase
RPMI = Roswell Park Memorial Institute
siRNA = short interference ribonucleic acid
TLR = toll-like receptor

type 2 pneumocytes (nonalveolar macrophage).[6,7] In addition, these discrete, cell-specific patterns of MAPK activation in vitro have been demonstrated in an in situ model of left LIRI using immunohistochemistry.[6] This differential pattern of MAPK activation in alveolar macrophages versus other cell types suggests alternative requirements for initiation of the proinflammatory signaling cascade in response to oxidative stress. Although MAPK activation is centrally important in LIRI, upstream signaling is likely what renders differentiated cellular responses.

Toll-like receptors (TLRs) are an evolutionarily conserved family of pattern-recognition receptors critical to innate immunity. Their ability to sense alarm and initiate inflammation makes them excellent candidates for early signaling in LIRI. Toll-like receptor–4 is activated by a wide array of signals, including stressed, necrotic, or injured tissue, but its response to lipopolysaccharide has been the most thoroughly characterized.[8-10] Activation of TLR-4 in response to lipopolysaccharide stimulation initiates downstream recruitment and activation of specific adaptor proteins, signaling kinases, and transcription factors, ultimately resulting in the transcription and secretion of proinflammatory cytokines and chemokines.[11,12] In addition, TLR-4 has been implicated as a key modulator in several models of ischemia and reperfusion. Deletion or pharmacologic antagonism of TLR-4 has been shown to reduce injury severity in cardiac, hepatic, renal, and cerebral models of ischemia reperfusion.[13-16]

Shimamoto and colleagues and Merry and colleagues[17,18] recently demonstrated that LIRI requires TLR-4, using knockout mice, a finding that supports our work using short interference ribonucleic acid (siRNA) for target molecular deletion of TLR-4. We demonstrated that TLR-4 knockdown in the alveolar macrophage protects against LIRI through decreased MAPK phosphorylation, proinflammatory cytokine production, and a corresponding reduction in the permeability index. However, cytokine production remained elevated in pulmonary artery endothelial cells and type 2 pneumocytes in response to oxidative stress, despite TLR-4 knockdown.[18] This lack of response to TLR-4 knockdown in these cell types, along with the differential MAPK phosphorylation in these cell types, suggests a different initial site or sequence of inflammatory activation.

A similar array of ligands is exhibited by TLR-2, including both bacterial products, such as lipotheichoic acid, and host molecules. Furthermore, TLR-2 is known to have an important role in the initiation of inflammatory responses and the development of ischemic injury in the heart and kidney.[19,20] In the kidney, TLR-2 is strongly expressed within glomerular endothelial cells and epithelial cells of Bowman's capsule. Mice with TLR-2 knockout demonstrate an attenuated injury response to renal ischemia and reperfusion.[19] No reports to date have examined the role of TLR-2 in the mediation of LIRI, but TLR-2 is expressed on bronchial epithelial cells, and has been shown to be involved in various organ-specific models of ischemia reperfusion.[13-16,21,22] Therefore, TLR-2 is a candidate for the initial site of inflammatory signaling activation in pulmonary artery endothelial cells and type 2 pneumocytes in response to oxidative stress.

The purpose of this study was to determine whether the differential responses to oxidative stress among alveolar macrophages, pulmonary artery endothelial cells, and type 2 pneumocytes relate to cell-specific TLR activation patterns leading to differential downstream signaling responses. Previous in vivo studies have demonstrated that intravenous administration of siRNA transfects alveolar macrophages, but not pulmonary artery endothelial cells or type 2 pneumocytes, necessitating the use of in vitro studies to investigate the effects of TLR-2 deletion on these nonalveolar macrophage cell types in response to oxidative stress (unpublished data). Using siRNA to achieve targeted molecular deletion of TLR-2 and TLR-4 in cultured alveolar macrophages, pulmonary artery endothelial cells, and type 2 pneumocytes, we examined the cell-specific requirements for TLR activation in LIRI. Additionally, a well-developed model of LIRI in the rat was utilized for further characterization.

## METHODS

### Short Interference Ribonucleic Acid

The siRNAs used in this study were designed (Invitrogen Corporation, Carlsbad, Calif) and implemented as previously described.[18] A single siRNA duplex targeting TLR-4 was used, based on prior work demonstrating its efficacy of uptake and TLR-4 knockdown, in the cell types of interest and the animal model.[18] Three unique, nonoverlapping siRNA duplexes were designed to target TLR-2. A noncoding, scrambled siRNA sequence was used as a control. For our in vitro experiments, 100 pmol of oligonucleotide was diluted in 50 $\mu$l of growth media, per well, in a 12-well culture plate, incubated for 15 minutes, and combined with either 3 $\mu$l of lipofectamine (Lipofectamine 2000, Invitrogen Corporation, Carlsbad, Calif) diluted in 50 $\mu$l of growth media (for alveolar macrophages), or 1 $\mu$l of lipofectamine diluted in 50 $\mu$l of growth media (for type 2 pneumocytes and pulmonary artery endothelial cells). This siRNA-lipid mixture replaced the cell culture media, and cells were incubated with this mixture for a minimum of 6 hours.

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