Role of Toll-like Receptor-9 in Lung Injury

Sreehari Panicker, Sanjay Balijepalli, Boya Zhang, Samantha Swamy, Matthew A. Sherman, Krishnan Raghavendran, and Madathilparambil V. Suresh*

Department of Surgery, University of Michigan, Ann Arbor, MI, USA

Toll-like receptors (TLRs) are pathogen associated molecular pattern (PAMP) receptors that are expressed by several cells of the innate and adaptive immune systems. TLR-9, also referred to as CD289, is localized in the endosomal compartment and recognizes specific unmethylated CpG motifs prevalent in microbial but not vertebrate genomic DNA. Activation of TLR-9 results in an inflammatory response, ultimately via a Th1-based pathway. TLR-9 is known to play a critical role in mediating the inflammatory response to lung injury. Due to its significant circulating blood volume and continuous exposure to the external environment, the lung is constantly exposed to potentially injurious agents that may trigger a common final pathway causing acute lung injury (ALI) or its more severe form, acute respiratory distress syndrome (ARDS). Both conditions result in a marked increase in inflammatory cytokines, such as TNFα, IL-1β and IL-6, and can result in significant morbidity and mortality. Prior studies have demonstrated the role of TLR-9 in sepsis and lung injury, focusing on its interactions with artificial CpG DNA and the potential positive immunomodulatory benefits from inhibition. In this review, we discuss the role of TLR-9 in the pathogenesis and treatment of lung injury.

TLR-9 | ALI | ARDS | Macrophages | Cytokines

Introduction
The human immune system functions through the detection of and response to pathological challenges via several families of receptors. Of these detection receptors, Toll-like receptors (TLRs) are the most well understood. TLRs are a family of transmembrane proteins with extra-membrane leucine-rich repeats (LRRs), 10 of which have been identified in humans[1]. TLRs are highly expressed in tissues involved in the immune response and exposed to pathogens, functioning as pattern recognition receptors (PRRs) to activate antigen-presenting cells (APCs)[2-6]. Membrane TLRs identify exogenous antigens mostly derived from the microbial membrane components, whereas endosomal TLRs recognize endogenous products consisting of either single or double stranded DNA/RNA sequences [3, 7-10]. Activating TLRs lead to the expression of inflammatory genes, which have a protective role against infection. TLRs can be classified upon their location within the cell, as each TLR variation deals with a specific subset of pathogenesis[11]. Within the TLR family, TLR-9 is an endosomal TLR that recognizes specific non-methylated cytosine triphosphate deoxyribonucleotide (CpG) motifs. These motifs are typically methylated in an effort to repress genes in vertebrates but can be found unmethylated in viruses, bacteria, and molds [3, 4, 12, 13]. TLR-9 is most extensively expressed in plasmacytoid dendritic cells (pDCs) and B cells. TLR-9 also has been found to be expressed on certain non-immune cells including intestinal epithelium, keratinocytes, and respiratory epithelial cells[14]. Both within immune and non-immune tissues, TLR-9 is vital in mediating the inflammatory response[14]. Immuno-stimulation via TLR-9 occurs when CpG-DNA is internalized and bound with receptor in the endosome [4, 15]. This binding causes a conformational change, resulting in the recruitment of adaptor protein MyD88 and the activation of nuclear factor-kB (NF-kB).

Following injury to the lung, TLR-9 stimulation induces acute inflammation through NF-kB with marked elevation in inflammatory cytokines[2]. Recent studies have suggested that TLR-9 is involved in the development of renal diseases, such as glomerulonephritis and lupus nephritis. These studies also found that TLR-9 triggers pro-tumorigenic signaling in mice[16-18]. Moreover, TLR-9 has been implicated in mediating antigen-specific immune responses, implying downstream activation of adaptive immunity [19]. Recent studies have also demonstrated that other biological molecules, such as malarial hemozoin and RNA/DNA hybrids, are recognized by TLR-9[19-21].

Effects of TLR-9 stimulation on inflammation
The pro-inflammatory effects following activation of TLR-9 has been well established. TLR-9 can recognize both synthetic CpG motif-containing oligodeoxynucleotides (CpG-ODN) and unmethylated CpG motifs containing bacterial or viral DNA. The binding of these motifs to TLR-9 triggers dimerization, leading to the downstream recruitment of MyD88. The interaction between MyD88 and TLR-9 then activates interleukin-1 receptor-associated kinase 1 (IRAK-1) and IRAK-4. IRAK-4 then recruits downstream tumor necrosis factor receptor associated factor 6 (TRAF6). TRAF6 activation ultimately results in the activation of NF-kB, and mitogen-activated protein kinases (MAPKs). MAPKs are responsible for AP-1 activation. Transcription factors NF-kB and AP-1 are primarily responsible for the expression of pro-inflammatory cytokine genes, including IL-6, IL-1β, IL-12 and TNF-α. Upregulation of co-stimulatory molecules CD80 and CD86 is paired with the recruitment of inflammatory cytokines [14].

TLR-9 activation promotes lung injury and inflammation
Following lung injury, TLR-9 has been found to be critical to initiating an inappropriately amplified systemic inflammatory response. Moreover, in multiple studies TLR-9 expression has been found to be associated with poor survival [3, 22-24]. In one study investigating lung contusion (LC), Suresh et al. found that TLR-9 knockout mice developed significantly less injury and inflammation. Compared to wild type mice, there were reductions in bronchoalveolar lavage (BAL) albumin, neutrophil recruitment, and levels of multiple pro-inflammatory markers including IL-1β, IL-6, and TNF-α. Additionally, the degree of histological injury was significantly worse in wild type mice up to 72 hours after injury [3].

Conflict of Interest: No conflicts declared.

* Corresponding Author. Madathilparambil V. Suresh, PhD
Research Assistant Professor
Department of Surgery, University of Michigan, Ann Arbor, MI-48109, USA
Phone: 734-615-7142; Email: madathil@umich.edu
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Studies indicate that CpG-ODN induced lung inflammation can be initiated by both local and systemic TLR-9 activation, contextualizing its importance within lung inflammation [25, 26]. It currently remains unclear, however, how TLR-9 is activated in a post-trauma setting. Lung contusion (LC), a trauma model for inducing ALI/ARDS, has shown great potency as a trigger for TLR-9 expression in the lungs. While the mechanism of activation is not yet understood, the acute inflammatory response that follows is equivalent to that of traditional CpG-ODN-TLR-9 activation [3]. Most importantly, pulmonary inflammation is contingent upon TLR-9 activation, as deficiency or inhibition of TLR-9 is associated with a significantly attenuated inflammatory response.

Shen et al. have demonstrated similar findings in the setting of paraquat associated lung injury, in particular that TLR-9 activation promotes MyD88 and NF-kB expression [27]. In a separate experiment, they found that MyD88 gene knockout reduced the resulting inflammatory response and severity of lung injury [28]. Thus, TLR-9 activation and the downstream pro-inflammatory cascade that follows are crucial to the inflammatory response following lung injury.

**TLR-9 expression in lung injury**

The lungs, which are exposed to all of systemic circulation and serve as a critical interface with the external environment, play an active role in the immune response against potential pathogens [29]. Certain insults such as LC, aspiration, sepsis/LDS, and other exposures can trigger the development of ALI or ARDS. ALI/ARDS is characterized by respiratory failure resulting from rapid onset of inflammation within the lungs and is associated with significant morbidity and mortality [30-34]. TLR-9 has been implicated as a critical component of the inflammatory response to lung injury.

While highly expressed in immune cells, TLR-9 expression has also been discovered in nonimmune cells. Intracellular fluorescence has demonstrated abundant expression of TLR-9 on the surface of human lung epithelial cells [35]. Post-mortem human samples from patients with LC revealed TLR-9 expression in both alveolar epithelial cells and alveolar macrophages [3]. Additionally, it is evident that in an ARDS setting, both myeloid-derived immune cells and stromal cells of the lung express TLR-9 [36].

**The Role of TLR-9 stimulation on sepsis and ALI/ARDS**

Sepsis is an amplified, dysregulated host inflammatory response to infection that can often lead to tissue damage, organ failure, and even death. The lungs are particularly susceptible to sepsis-induced organ failure due to constant exposure to potentially harmful pathogens. Importantly, more than 50% of patients with sepsis develop ALI or ARDS [37-40]. TLR-9 has been implicated in the pathophysiology of sepsis and subsequent lung injury.

Mitochondrial DNA, which contains unmethylated CpG motifs, acts as a ligand for TLR-9 [3, 13, 21, 41, 42]. Zhang et al. reported that TLR-9 triggers acute lung injury and systemic inflammation following intra-peritoneal delivery of mitochondrial DNA [43]. This leads to elevations in the levels of BAL protein and pro-inflammatory cytokines including IL-1β, IL-6, and HMGB1. Additionally, p38 MAPK signaling was found to be elevated following TLR-9 activation [43, 44].

TLR-9 is a key mediator of sepsis-related ALI. Bacterial DNA contains hypo-methylated CpG that activates TLR-9, causing damage to the alveolar-capillary membrane and neutrophil recruitment and activation [29]. Moreover, Itagaki et al. have shown that TLR-9 mediates interactions between neutrophils and endothelial cells causing changes in membrane permeability [22]. Direct lung injury accompanied by bacteria and sepsis trigger overlapping inflammatory pathways, leading to both localized and systemic inflammation, further exacerbating pulmonary damage.

Through TLR-9, direct lung injury caused by bacteria further complicated by sepsis trigger overlapping inflammatory pathways, leading to both localized and systemic inflammation and severe pulmonary damage.

**Therapeutic effects of TLR-9 inhibition**

Recent literature regarding TLR-9 demonstrates the various therapeutic benefits of TLR-9 inhibition and the suppression of a dysregulated acute inflammatory response. In one study, TLR-9 inhibition via antagonist treatment attenuated lung injury [27]. Genetic silencing of TLR-9 was also found to attenuate cell apoptosis in response to paraquat-induced ALI [27].

In searching for novel ways to inhibit this inflammatory pathway, studies have uncovered further details regarding TLR-9 inflammatory mechanisms. Hu et al. revealed that the inhibition of TLR-9 in a sepsis model suppressed the inflammatory response and improved survival [23]. In a model of cecal ligation and puncture (CLP) induced polymicrobial sepsis, TLR-9 knockout (KO) mice had significantly better survival rates compared to WT mice. This was further attributed to reduced p38 MAPK activation, suggesting regulation by TLR-9. The levels of cytokines including IL-6, IL-10, IFNγ, and TNFα were all attenuated in TLR-9 knockout mice [23]. Suresh et al. showed similar therapeutic effects of TLR-9 KO in the context of ALI/ARDS [3]. TLR-9 KO were found to have lower levels of pro-inflammatory cytokine expression, including keratinocyte chemotractant and MCP-5. The TLR-9 KO also demonstrated decreased BAL neutrophils but increased macrophage phagocytic activity. Inhibition of TLR-9 is a valid approach to limit both the local and systemic inflammatory activation associated with ALI/ARDS [3].

Inhibition of inflammatory mediators downstream of TLR-9 has further confirmed the therapeutic benefits from TLR-9 pathway inhibition. MyD88 is responsible for further amplification of the acute inflammatory response. Multiple studies have demonstrated that MyD88 KO leads to attenuation of ALI/ARDS and reduced levels of proinflammatory cytokines, specifically NF-kB [28] [45]. Ketamine has been found to be one such inhibitor of the TLR-9 pathway [46]. Within an LPS-induced ALI model, ketamine inhibited the receptor for advanced glycation end products (RAGE) and TLR-9. Inhibition of the RAGE-TLR-9-NF-kB pathway led to reduced inflammatory mediators and decreased lung myeloperoxidase activity [24, 47].

Chloroquine has also been found to be a potent inhibitor of TLR interactions with CpG DNAs as well as an inhibitor of endosomal acidification. Chloroquine has been shown to inhibit bacterial-DNA induced permeability in signaling between polymorphonuclear neutrophils and endothelial cells (PMN-EC). This discovery implicated TLR-9 as being crucial to promoting PMN-EC interactions, leading to increased endothelial cell permeability. These findings were further confirmed with a human specific TLR-9 inhibitor, ODN TTAGGG [21]. Moreover, in a sepsis model inhibition of TLR-9 through either specific inhibition or chloroquine limited the pathologic expression of adherence molecules on EC and PMN [22].

**TLR-9 polymorphisms**

The TLR-9 gene is located on human chromosome 3 [48]. There have been numerous studies regarding TLR-9 polymorphisms in the context of various infectious and autoimmune diseases, but studies regarding ARDS and sepsis offer a better understanding of TLR-9’s functionality in both systemic and localized
inflammation. One study sought to distinguish the role of TLR-9 polymorphisms within the context of sepsis. The two gene polymorphisms compared were TLR-9 (-1486 T>C) and TLR-9 (C>T). These findings were further corroborated by discovering that serum levels of both polymorphisms had been reduced due to sepsis-related immunosuppression. This further implies a lack of differentiation in both gene polymorphisms [49].

Pulmonary tuberculosis can also lead to ALI. Interestingly, this study found that higher expression of the single-nucleotide polymorphism (SNP) rs187085 (T-1486C) increased transcriptional activity of TLR-9, which in turn further worsened the effects of pulmonary tuberculosis[50]. However, polymorphisms that retained the original T allele had increased expression of protective cytokines (IFNγ and TNFα). This implicates the T-1486C TLR-9 polymorphism having greater susceptibility to pulmonary tuberculosis [50].

Conclusion

Through its various mechanistic activation and downstream mediators, TLR-9 expression is an important prognostic indicator in lung injury. Expressed by both immune and stromal cells, TLR-9 is densely populated in lung tissue. Combined with constant pathogen exposure and contact with systemic circulation, TLR-9 appears to be a key agent in inducing an immune response, both innate and adaptive. The inflammatory response, however, is dysregulated and inappropriate, often leading to host tissue damage. This dysregulated immune response is what implicates TLR-9 as a negative prognostic factor in ALI/ARDS and sepsis.

Future studies should focus on the identification of other ligands capable of activating TLR-9. It is possible that other DNA fragments from damaged pulmonary cells could interact with TLR-9. Other biological molecules, such as hemozoin and histone proteins have also shown the capacity to activate TLR-9. These mechanisms should be further explored, as TLR-9 inhibition has various therapeutic benefits in regulating inflammation and reducing mortality.

Acknowledgements

The authors gratefully acknowledge the support of National Institutes of Health Grants - R01 GM111305-01 (KR).

Author Disclosure

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Author contributions

Drafting the manuscript SP; SB; BZ; SS; MAS; KR; and MVS.


34. Knight PR, Holm BA: The three components of hyperoxia. Anesthesiology 2000, 93:3-5.


