Pattern Recognition Receptor–Dependent Mechanisms of Acute Lung Injury

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Acute lung injury (ALI) that clinically manifests as acute respiratory distress syndrome is caused by an uncontrolled systemic inflammatory response resulting from clinical events including sepsis, major surgery and trauma. Innate immunity activation plays a central role in the development of ALI. Innate immunity is activated through families of related pattern recognition receptors (PRRs), which recognize conserved microbial motifs or pathogen-associated molecular patterns (PAMPs). Toll-like receptors were the first major family of PRRs discovered in mammals. Recently, NACHT–leucine-rich repeat (LRR) receptors and retinoic acid–inducible gene–like receptors have been added to the list. It is now understood that in addition to recognizing infectious stimuli, both Toll-like receptors and NACHT-LRR receptors can also respond to endogenous molecules released in response to stress, trauma and cell damage. These molecules have been termed damage-associated molecular patterns (DAMPs). It has been clinically observed for a long time that infectious and noninfectious insults initiate inflammation, so confirmation of overlapping receptor-signal pathways of activation between PAMPs and DAMPs is no surprise. This review provides an overview of the PRR-dependent mechanisms of ALI and clinical implication. Modification of PRR pathways is likely to be a logical therapeutic target for ALI/acute respiratory distress syndrome.

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INTRODUCTION

The lung is an important target organ for systemic inflammatory mediators released after severe infection (1,2) and major trauma (3–5). Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) have been the major causes of morbidity and mortality in intensive care units (6). During the past decade, despite improvements in supportive care, ALI/ARDS still carries high mortality rates between 26% and 35% (7). ALI/ARDS is characterized by noncardiogenic pulmonary edema, and pulmonary and systemic inflammation with a spectrum of increasing severity of lung injury resulting in respiratory failure (8). ALI frequently has systemic components, of which several of the major triggering conditions include sepsis, nonpulmonary trauma and shock. Conversely, diffuse injury and infection of the lung cause the systemic inflammatory response syndrome (SIRS) and sepsis (9,10). Therefore, the interaction of pulmonary and systemic inflammation exaggerates the inflammatory process and the development of ALI.

Recent studies have demonstrated that the pattern-recognition receptors (PRRs) play an important role in the pathogenesis of ALI/ARDS. PRRs are evolutionarily conserved receptors that sense not only pathogen-associated molecular patterns (PAMPs) that derived from invading microbes, but also damage-associated molecular patterns (DAMPs) that are released from dead cells, thereby triggering an inflammatory response to both infectious and noninfectious insults (11). Activation of PRRs results in initiation of several extracellular activating cascades, as well as various intracellular signaling pathways that cause inflammatory responses. Figure 1 summarizes the role of PRRs in mediating infectious- and injury-induced ALI. This review will focus on recent advances in understanding of the role of PRRs in the mechanisms of ALI/ARDS.

BIOLOGY OF PRRS

Three major subfamilies of PRRs have been reported: Toll-like receptors (TLRs), retinoic acid–inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binging oligomerization domain (NOD)-like receptors (NLRs) (12,13). A brief comparison of the three subfamilies of PRRs is shown in Table 1.

Toll-Like Receptors

TLRs are the most extensively studied family of PRRs. To date, 10 TLRs (TLRs 1–10) in humans and 12 TLRs

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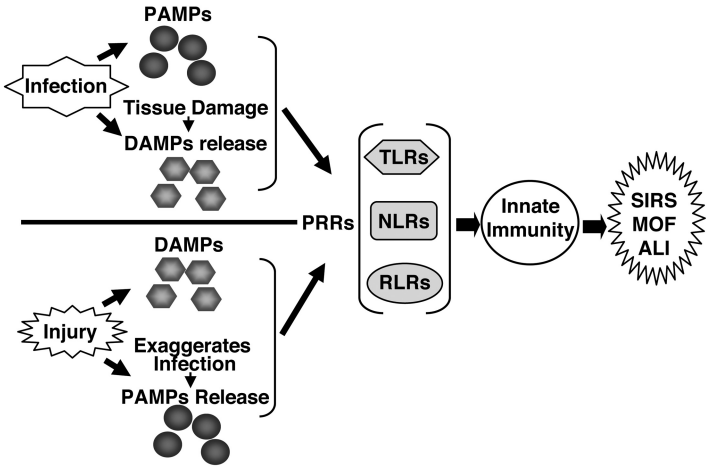

Figure 1. Role of PRRs in mediating inflammation and organ injury. Infection causes PAMP release, but also causes tissue and cell damage and subsequent DAMP release. Similarly, injury caused by trauma or various other factors not only leads to DAMP release but also renders the patient more susceptible to infection and therefore PAMP release. In turn, the PAMPs and DAMPs act through PRRs, which include TLRs, NLRs and RLRs, to activate the innate immune system, yet they can also contribute to persistent and deleterious systemic inflammation and organ injury, including ALI.

(TLRs 1–9 and TLRs 11–13) in mice have been defined (14). TLRs 3, 7, 8 and 9 are expressed intracellularly, whereas TLRs 1, 2, 4, 5, 6 and 10 are expressed on the cell surface. TLRs are expressed on a range of immune cells including macrophages, dendritic cells, B cells and certain types of T cells, as well as on certain nonimmune cells, such as endothelial cells, smooth muscle cells and epithelial cells that lie at potential sites of entry, including the skin and the respiratory, intestinal and genitourinary tracts. The expression of TLRs is modulated by activation, maturation or differentiation of the different cell types (15,16).

TLR proteins are a family of type I transmembrane receptors characterized by an NH2-terminal extracellular leucine-rich repeat (LRR) domain, which mediate the recognition of their respective PAMPs, and a COOH-terminal intracellular tail containing a conserved region called the Toll/interleukin 1 (IL-1) receptor (TIR) homology domain. The TIR domain is the defining motif of the TLR/IL-1 superfamily, and it is likely to be one of the earliest signaling domains to have evolved (17). TLRs can recognize a diverse range of PAMPs, generate inflammatory signals to coordinate innate immune responses and modulate adaptive immune responses. The list of TLR ligands is growing. However, the ligand for TLR10 and mouse TLR8 remains unknown at present. Activation of TLRs initiates two major pathways: the MyD88-dependent pathway, which is used by all TLRs except TLR3, resulting in the activation of nuclear factor (NF)-κB and activator protein-1 (AP-1); and the TRIF-dependent pathway, which is initiated by TLR3 and TLR4, resulting in the activation of type I interferons (IFNs) (13,18,19). Expression of numerous proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-6, IL-12 and IFNs, is one of the major outcomes of the activation of the pathways (15). TLR signaling is summarized and shown in Figure 2.

RIG-Like Receptors

RLRs as DExD/H-containing RNA helicases are expressed in the cytoplasm in a variety of cells, including immune and nonimmune cells. Unlike membrane-bond TLR3, TLR7 and TLR9, which are localized on the endosome and recognize viral double stranded RNA, single-stranded RNA and DNA, respectively, RLRs are cytoplasmic proteins that recognize viral RNA produced as a consequence of viral replication (20,21). RLRs consist of three family members: RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (21,22).

Table 1. Comparison of the three subfamilies of PRRs.

PPRs	Localization	Activators	Adaptor	Signal	Response
TLRs	Cell membrane	Bacteria Viruses Fungi Protozoa	MyD88 Mal Trif TRAM	NF-κB MAPKs IRFs	Cytokines Chemokines Antiviral proteins Pro–IL-1, pro–IL-18
NLRs	Cytoplasm	Bacteria	MyD88	Caspase-1 NF-κB	IL-1, IL-18
RLRs	Cytoplasm	Viruses	IFNβ promoter stimulator 1	IRFs NF-κB MAPKs	Antiviral proteins

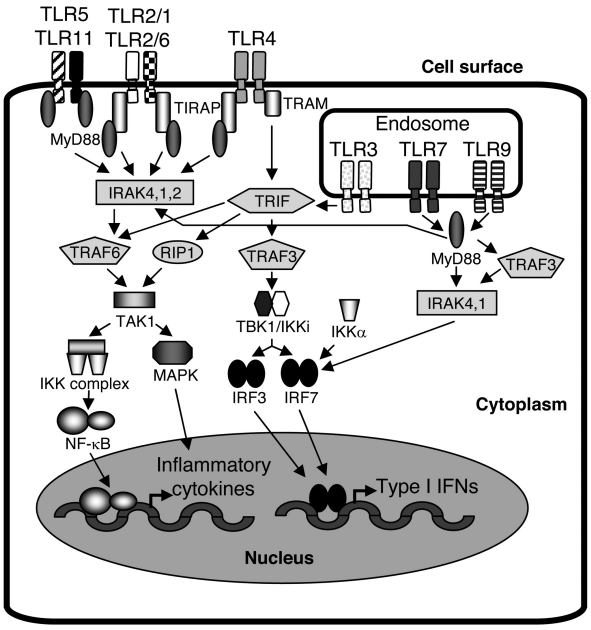

Figure 2. TLR signaling (an example in macrophages and dendritic cells). TLR2 (TLR2 in association with TLR1 or TLR6), TLR4, TLR5 and TLR11 are localized on the cell surface for ligand recognition. TLR3, TLR7 and TLR9 are localized in the endosome for ligand recognition in the lumen of endosome. All TLRs, except TLR3, recruit MyD88, and TLR1, TLR2, TLR4 and TLR6 recruit the additional adaptor TIRAP, which links the TIR domain with MyD88. TLR3 and TLR4 recruit TRIF. TLR4 requires the additional linker adaptor TRAM, which links the TIR domain of TLR4 with TRIF. Stimulation of the cells with TLR1, TLR2, TLR5, TLR6 and TLR11 ligands initiates the MyD88-dependent pathway whereas TLR3 ligands initiate the TRIF-dependent pathway. TLR4 activates both MyD88-dependent and TRIF-dependent pathways. In the MyD88-dependent pathway, MyD88 recruits the IRAK family of proteins and TRAF6. In turn, TRAF6 activates TAK1. The activated TAK1 activates the IKK complex, which activates NF-κB subunits. The activated TAK1 also activates the MAPK pathway. In the TRIF-dependent pathway, TRIF interacts with RIP1 and TRAF6. Activated TRAF6 and RIP1 activate NF-κB and MAPKs. TRIF also interacts with TRAF3 and activates TBK1/IKKi, which activate IRF3 and IRF7. Cells stimulated with TLR7 and TLR9 ligands activate NF-κB and MAPKs via the MyD88-dependent pathway. To induce type I IFNs, MyD88 associates with the IRAK family of proteins. IRAK1 and IKKα activate IRF7. IRAK1 also interacts with TRAF3 and activates IRF7. The activated NF-κB subunits and IRFs are translocated to the nucleus. NF-κB and MAPKs initiate the transcription of inflammatory cytokine genes whereas IRFs initiate the transcription of type I interferons. (The figure is adapted from (13) and used with permission from Elsevier.)

Structurally, RIG-I and MDA5 contain a DExD/H box RNA helicase domain and two caspase-recruiting domain (CARD)-like domains required for eliciting downstream signaling pathways (23,24). The C-terminal region of RIG-I contains a repressor domain (RD), which inhibits downstream signaling. The MDA5 C-terminal region is similar to the RD of RIG-I; however, its function is not clear. LGP2 contains a DExD/H helicase domain and an RD, but lacks the CARD-like region. LGP2 was suggested to play an inhibitory role in virus-induced response, because the LGP2 RD binds the RIG-I RD and suppresses signaling as a consequence of interfering with the self-association of RIG-I (20,25,26).

RIG-I is essential for the recognition of a series of RNA viruses, which include Sendai virus, Newcastle disease virus, influenza virus, vesicular stomatitis virus and Japanese encephalitis virus (27). MDA5 is required for the recognition of other RNA viruses, including picornaviruses such as encephalomyocarditis virus, Mengo virus and Theiler virus (13,27). Thus, RIG-I and MDA5 have specificities in their detection of RNA viruses, presumably through recognition of distinct structures of viral RNA (24,28).

Recent studies revealed a pathway of RLR regulation of NF-κB. RIG-I/MDA5 CARD domains, through a CARD-containing adaptor protein, IFNβ promoter stimulator 1, also known as mitochondrial antiviral signaling protein, and CARD adaptor inducing IFNβ, ultimately activate IRF3 and NF-κB (29,30). The role of RLRs in the mechanism of ALI has not been elucidated.

Nucleotide-Binding Oligomerization Domain–Like Receptors

The NLR family is a group of recently identified cytoplasmic PRRs that contain more than 23 members in humans (15). The major role of NLRs is to recognize cytoplasmic microbial PAMPs and/or endogenous danger signals and initiate immunological responses, although the physiological function of most NLRs is poorly understood at present (31). Members of the NLR family are categorized into at least five subfamilies according to their N-terminal structure, including NODs (nucleotide-binding oligomerization domain-1), NALPs (NACHT-, LRR- and pyrin-domain–containing proteins), IPAF (ICE-protease activating factor), NAIPs (neuronal apoptosis inhibitor factors) and class II transactivator (CIITA) (32).

The NLR family shares a domain organization consisting of a C-terminal LRR domain, a central nucleotide-binding NACHT domain, and an N-terminal protein–protein interaction domain composed of a CARD, pyrin domain (PYD) or baculovirus inhibitor of apoptosis repeat (BIR) domain (33). NODs and IPAF contain CARD effector domains, whereas NALPs have PYD domains, and NAIPs possess BIR domains.

The functions of NOD1, NOD2, IPAF and NALP3 are more studied. NOD1 and NOD2 are the first NLRs that are reported

to have a direct function as PRRs in the recognition of peptidoglycan (PGN)-derived peptides. NOD1 senses γ-D-glutamyl-meso-diaminopimelic acid (that is,-DAP) that is derived from Gram-negative bacteria (34), whereas NOD2 senses muramyl dipeptide, which is from both Gram-positive and Gram-negative bacteria (35,36). When NODs bind with PGN-derived peptides, they rapidly form oligomers, which lead to the recruitment of the receptor-interacting protein 2 (RIP2) kinase through CARD–CARD interactions (37). This complex of NOD1-RIP2 or NOD2-RIP2 then recruits the inhibitor of NK-κB kinase complex (IKK), leading to the activation of NF-κB. Activation NOD1 and NOD2 can also initiate an MAPK pathway that leads to the activation of p38 and ERK. In addition, NOD1 signaling can activate JNK as well (38).

NALP proteins are characterized by the presence of an N-terminal pyrin effector domain (39). Several NLRs, namely NALP1, NALP2 and NALP3, have an important role in activation of proinflammatory caspases through formation of inflammasome (37,40). The inflammasome is a multiprotein complex of more than 700 kDa that is responsible for the activation of caspases 1 and 5, leading to the processing and secretion of the proinflammatory cytokines IL-1β and IL-18 (41,42). Martinon *et al*. have presented different caspase activation platforms in which different components constitute the various inflammasomes (43). Two types of NALP inflammasome are better studied: the NALP1 inflammasome that is composed of NALP1, the adaptor protein ASC, caspase-1 and caspase-5 (41), and the NALP2/NALP3 inflammasome that contains NALP2 or NALP3 CARDINAL, ASC and caspase-1 (44).

PRRS AND INFECTION-RELATED ALI

Role of TLRs in Activation of Cellular Inflammatory Responses

TLRs 1–10 are expressed in lung tissue (45), and individual TLRs are differentially regulated in specific lung cell populations in response to microbial stimulation. TLR2, TLR4, TLR5 and TLR9 are the most likely to be involved in recognition of bacteria in the lungs (46–48). A study by Bernard *et al*. has shown that ALI/ARDS induced by lipopolysaccharide (LPS) is a major cause of mortality among humans (49). The LPS membrane receptor complex is composed of several accessory molecules, which include phosphatidylinositol-anchored CD14, TLR4, MD2 and MD1 (50). The LPS-binding protein (LBP) enhances the binding of LPS to its receptor (51). Absence of CD14, MD2 or LBP abrogates most LPS responses (51,52). Expression of functional TLR4 has been found in many cell types in the lung (45), and LPS-induced lethal shock and ALI have been shown to be TLR4 dependent (53–55). Thus, TLR4 plays a critical role in the mechanism of infection-related ALI.

A recent study has shown that respiratory infections in the human lung initiated by TLR2 agonist lipoteichoic acid (LTA, a component of Gram-positive bacteria) and TLR4 agonist LPS (a component of Gram-negative bacteria) exhibit different inflammatory responses (56). The study was performed on healthy subjects with LPS or LTA instillation into the contralateral lung. Alveolar macrophages (AMϕ) isolated from bronchoalveolar lavage fluid were analyzed by multiplex ligation-dependent probe amplification. The results show that whereas both LPS and LTA elicited neutrophil recruitment, only LPS instillation was associated with activation of neutrophils (PMN) (CD11b surface expression and degranulation) and consistent rises of chemokine/cytokine levels. Moreover, LPS but not LTA activated AM, as reflected by enhanced expression of proinflammatory mediators and increased spontaneous cytokine release upon incubation *ex vivo*. Remarkably, only LTA induced C5a release. These data suggest that stimulation of TLR2 or TLR4 results in differential pulmonary inflammation, which may be of relevance for understanding the differences during Gram-positive and Gram-negative respiratory tract infection (56).

TLR and Endothelial Cell Activation

Pulmonary endothelium is a major component of the alveolar-capillary unit, and is susceptible to injury from noxious agents that are either inhaled or delivered to the lung through the pulmonary circulation (57). In ALI, pulmonary endothelium plays a major role by (a) altering metabolic activity to affect pulmonary and systemic homeostasis, (b) mediating polymorphonuclear PMN adhesion to promote PMN infiltration, (c) changing PMN barrier permeability to cause pulmonary edema and (d) secreting cytokines and chemokines to induce lung inflammation (58).

A recent study by Andonegui and colleagues has shown that endothelial cells (ECs) are the key sentinel cells for detecting infection by Gram-negative bacteria and recruiting PMN to peripheral tissues (53,59). Indeed, previous studies have shown that direct activation of circulating PMN with LPS is not sufficient to induce their sequestration within the lung (53). Interactions of PMN with ECs seems important for the process of PMN sequestration into the lung (53,60). LPS stimulates the CD14 and TLR4 complex, which in turn activates NF-κB (61) and increases the expression of adhesion molecule E-selection, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (62,63). The TLR4 signaling also leads to production and release of various bioactive molecules, including IL-1β, IL-6, TNF-α, chemokines and nitric oxide (64), all of which are actively involved in the development of ALI.

More importantly, TLR4 signaling can also upregulate other TLRs, such as TLR2, and thus amplify the inflammatory responses. Although TLR2 is predominantly expressed in the first-line host defense cells (monocytes, macrophages, dendritic cells and PMN) (65,66), its expression is low in ECs and epithelial cells (67). Studies from our laboratory showed that LPS through TLR4- and MyD88-dependent signaling induced TLR2 upregulation in ECs (68). We have demonstrated that TLR4 signaling, through activating NF-κB,

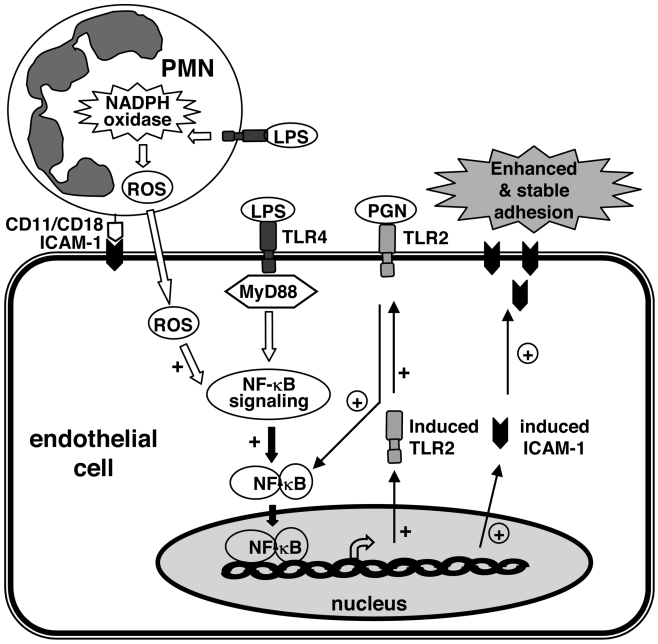

Figure 3. Model of PMN NADPH oxidase-derived oxidant signaling in mediating the TLR4-TLR2 cross-talk in ECs. LPS stimulation induces NAD(P)H oxidase activation and production of ROS in PMN as well as the initiation of MyD88-dependent NF-κB signaling in ECs and the consequent expression of TLR2 and ICAM-1. Adhesion of PMN to ECs is mediated by binding of constitutive ICAM-1 to CD18 integrin and provides the appropriate coupling required for PMN to transmit oxidant signals to ECs. The oxidants augment NF-κB signaling and TLR2 expression (+), which results in the augmented response of the cell to PGN, thereby amplifying ICAM-1 expression (circled +) and promoting stable adhesion of PMN to ECs and increased PMN migration. Thus, the PMN NAD(P)H oxidase-mediated TLR4-TLR2 cross-talk activates a positive feedback signal leading to sustained and amplified endothelial activation in response to invading pathogens. (The figure is adapted from (68) and used with permission from the *Journal of Clinical Investigation*.)

upregulates TLR2 expression in ECs, and this process is enhanced by oxidant signaling generated by PMN NAD(P)H oxidase. The functional relevance of NAD(P)H oxidase in mediating TLR4-induced TLR2 expression in ECs is evident by markedly elevated and stable ICAM-1 expression as well as augmented PMN migration in response to sequential challenge with LPS and peptidoglycan (68). Thus, TLR2 activation, signaled by TLR4 and as regulated by PMN NAD(P)H oxidase, is an important mechanism responsible for amplifying PMN transmigration to sites of infection (Figure 3).

The TLR4-TLR2 interaction suggests a highly coordinated, oxidant-mediated upregulation of TLR2 in response to LPS. When one considers the interactions of the innate immune system as microbes are first encountered, the value of such temporal organization is significant. For example, Gram-negative bacteria persist in tissues and, if they are not immediately killed through the activation of PMN, complement and other antimicrobial factors, they may spill out systemically and result in septic shock. Survival in the face of such infections depends on the innate immune system, which must be able to monitor and respond to pathogens over a prolonged period of time. Given the need for a prolonged response to bacterial infection, it has always seemed somewhat surprising that response to LPS is temporally finite. This endotoxin tolerance means that within hours after exposure to LPS, innate immune cells are incapable of responding again to a rechallenge (69). But it is now clear that as LPS sensitivity wanes, the immune system has at its disposal the capability of marshaling responses via oxidative metabolites and their ability to upregulate other TLRs (70). The subsequent means of responding to bacteria depend on the ability of the innate immune system to destroy microbes and enhance the release of alternative immune stimuli. The TLRs that are utilized are the ones that bind the constituents of degrading bacteria, such as lipopeptides, PGN, heat shock proteins and CpG DNA. It seems plausible that activated PMN may even alter the phenomenon of LPS tolerance, at least in a localized context, by setting into action a positive feedback loop at sites to which PMN are chemoattracted (71). This would enhance inflammatory responses locally and help fight infection. However, in a setting of post-trauma SIRS or ALI, the primed PMN activation serves as an amplifier to cause enhanced PMN infiltration and organ injury.

TLR and PMN Activation

Extensive PMN influx into the lungs is one of the characteristics of ALI. Studies have shown that excessive induction of proinflammatory cytokines in PMN and delayed PMN apoptosis are associated with higher mortality and more severe organ dysfunction in sepsis patients (72,73). Human PMN express all TLR mRNA except TLR3. TLR2 are more abundant than TLR4 on PMN (74,75). PMN recruitment to the lung after LPS inhalation is primarily dependent on TLR4–NF-κB signaling (76,77). The PI3K/Akt pathway was also reported to be involved in TLR4-induced expression of IL-1β, TNF-α and chemokine macrophage inflammatory protein (MIP)-2 (78).

We have reported that TLR4, through regulating G-protein–coupled receptor kinases (GRKs), promotes PMN migration. We demonstrated that MIP-2 induces GRK2 and GRK5 expression in PMNs through phosphoinositide-3-kinase (PI3K)-γ signaling, and LPS-activated

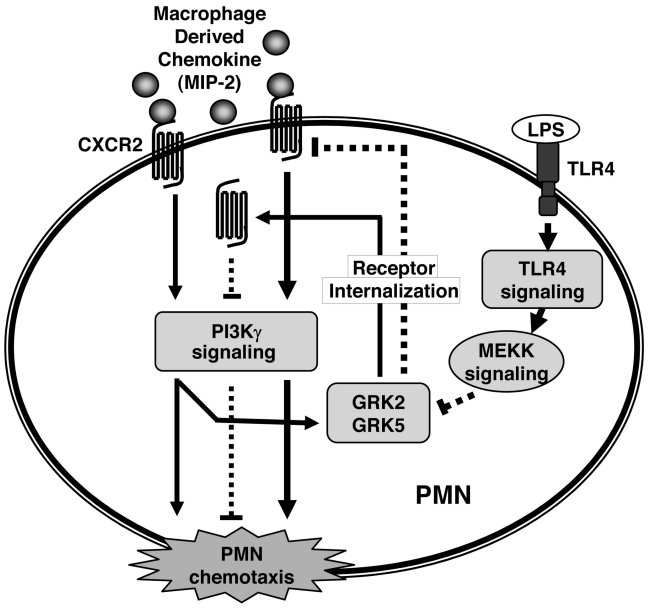

Figure 4. Model of TLR4 and chemokine receptor cross-talk. MIP-2 binding to CXCR2 induces PMN migration, as well as GRK2 and GRK5 expression, through PI3K-γ signaling. Increased GRK2 and GRK5 expression results in chemokine receptor internalization and desensitization, thereby negatively regulating PMN migration. Thus, PI3K-γ–activated signaling is postulated to be a feedback mechanism regulating PMN migration. Because persistent infection requires continued PMN infiltration, LPS acting through the TLR4 signaling pathway transcriptionally downregulates expression of GRK2 and GRK5 in response to MIP-2. This decreases chemokine receptor desensitization by preventing CXCR2 internalization and thus augments PMN migration. MEK kinase is involved in mediating the cross-talk between TLR4 and chemokine receptors. Dashed lines indicate inhibitory signals. (The figure is adapted from (79); and for it, J Fan acknowledges first publication in *Nature Medicine*.)

signaling through the TLR4 pathway transcriptionally downregulates the expression of GRK2 and GRK5 in response to MIP-2. The reduced expression of GRKs lowers chemokine receptor desensitization and markedly augments the PMN migratory response. These data indicate that TLR4 modulation of PMN surface chemokine receptor expression after the downregulation of GRK2 and GRK5 expression is a critical determinant of PMN migration (79) (Figure 4).

A recent study explored a novel role of mTOR complex 1 (mTORC1) in TLR2- and TLR4-induced PMN activation (80). Administration of rapamycin, an inhibitor of mTORC1, decreased the severity of lung injury after intratracheal LPS or PAM (a TLR2 ligand) administration, as determined by diminished neutrophil accumulation in the lungs, reduced interstitial pulmonary edema, and diminished levels of TNF-α and IL-6 in bronchoalveolar lavage fluid. These results indicate that mTORC1 activation is essential in TLR2- and TLR4-induced PMN activation, as well as in the development and severity of ALI.

The E3 ubiquitin ligase Cblb has a crucial role in the prevention of chronic inflammation and autoimmunity. However, a recent study showed that Cblb also has an unexpected function in acute lung inflammation (81). Cblb attenuates the sequestration of PMN in the lungs after administration of LPS. In a model of polymicrobial sepsis in which acute lung inflammation depends on TLR4, the loss of Cblb expression accentuates acute lung inflammation and reduces survival. Cblb controls the association between TLR4 and the intracellular adaptor MyD88. Expression of WT Cblb, but not expression of a Cblb mutant that lacks E3 ubiquitin ligase function, prevents the activity of a reporter gene for NF-κB in monocytes that have been challenged with LPS. The downregulation of TLR4 expression on the cell surface of PMN is impaired in the absence of Cblb. These data reveal that Cblb regulates the TLR4-mediated acute inflammatory response that is induced by sepsis (81).

PMN apoptosis is a crucial injury-limiting mechanism of inflammatory resolution. Circulating PMN undergo constitutive apoptosis that results in the shutdown of secretary capacity and allows PMN recognition and removal by macrophages (82,83). Several inflammatory agents, such as LPS, TNF, IL-8, IL-6, IL-1 and granulocyte colony-stimulating factor (G-CSF), can delay apoptotic response, providing PMN with a longer life span, which in turn allows the PMN to accumulate at local tissue sites of inflammation/infection (84,85). Protein 53 (p53) is a transcription factor that is important in multicellular organisms, where it regulates the cell cycle and promotes apoptosis. Modulation of p53 by nutlin-3α diminished the response of PMN and macrophages to stimulation through TLR2 or TLR4 as well as attenuated LPS-induced ALI. NF-κB has been reported as a modulator of apoptosis in inflammatory cells (86). p53 can negatively regulate NF-κB activity by decreasing binding of NF-κB to the promoters of genes for proinflammatory cytokines. In $p53^{-/-}$ mice, the inflammatory process and severity of ALI in response to LPS are enhanced (87).

TLR and AMϕ Activation

AMϕ account for approximately 95% of airspace leukocytes (88). Tissue damage induced by LPS is mediated mainly by inflammatory products released from AMϕ (89,90), thus activated AMϕ play a critical role in the development of ALI (91). LPS inhalation induces AMϕ to produce and release inflammatory mediators TNF-α, IL-1β and MIP-2 in a TLR4-dependent manner (92), which further result in the recruitment of PMN into the lower respiratory tract and activate other

cell types, including epithelia and endothelia (93).

TLR4 is constitutively expressed in AMϕ, and TLR2 can be induced in response to LPS or proinflammatory cytokines. The inducible TLR2 expression might be important in responding to other bacterial components from Gram-positive bacteria (48).

The role of CD44 in the regulation of LPS-TLR signaling in macrophages has recently been reported (94). CD44 is a transmembrane adhesion molecule and hemopoietic CD44 has an essential role in hyaluronan clearance and resolution of noninfectious lung injury. Following intratracheal LPS treatment, $CD44^{-/-}$ mice demonstrated an exaggerated inflammatory response characterized by increased inflammatory cell recruitment, elevated chemokine expression in bronchoalveolar lavage fluid and a marked increase in NF-κB DNA-binding activity in lung tissue *in vivo* and in macrophages *in vitro*. Furthermore, $CD44^{-/-}$ mice were more susceptible to LPS-induced shock. The study further found that the induction of the negative regulators of TLR signaling IL-1R–associated kinase-M, Toll-interacting protein and A20 by intratracheal LPS *in vivo* and in macrophages *in vitro* was significantly reduced in $CD44^{-/-}$ mice. Collectively, these data suggest that CD44 plays a role in preventing exaggerated inflammatory responses to LPS by promoting the expression of negative regulators of TLR-4 signaling (94).

TLR and Epithelial Cell Activation

Impairment of the alveolar epithelial barrier is important in the development of ALI. Under physiologic conditions the epithelial barrier is less permeable than the endothelial barrier; thus, destruction of epithelial barrier integrity prompts a progressive influx of protein-rich fluid into the alveoli (95). On the other hand, the loss of epithelial integrity represents an impairment of physiologic transepithelial fluid transport and further inhibits the reabsorption of alveolar edema (96). TLRs are critical for airway epithelial cell recognition of inhaled pathogens and for innate immune signaling. In cultured human lung epithelial cells, mRNA of all TLRs has been detected (97,98). TLR2, TLR3, TLR5 and TLR6 have the highest expression, and the ligands for these TLRs increased IL-8 and vascular endothelial growth factor (VEGF) production in normal human bronchial epithelial cells (99). TLR2 is a heterodimer with TLR1 and TLR6, and each of these is present on the airway epithelial surface. TLR3, which recognizes dsRNA or poly(I:C), is located in endosomes in unstimulated human bronchial epithelial cells (100). TLR5 is also present on the airway epithelial surface where it can interact with epidermal growth factor receptor (EGFR). Studies have shown that TLR ligands stimulated IL-8 and VEGF production via EGFR and the downstream signaling that might include MAP kinases and NF-κB (101,102). Interestingly, heat shock proteins (Hsp), such as Hsp72 and Hsp90, appear to be intimately involved in the recognition of LPS. Extracellular Hsp72 released from virally infected airway epithelial cells induces IL-8 express in human bronchial epithelial cells, resulting in the recruitment and activation of PMN via TLR4 (103,104). Type II alveolar epithelial cells can also be activated by LPS mediated through TLR4 signaling and in turn promote pulmonary inflammatory processes (105,106).

A study by Togbe *et al*. of whether *TLR* gene dosage contributes to infection has demonstrated that overexpression of TLR4 augmented an LPS-induced bronchoconstrictive effect, as well as TNF-α and CXC chemokine ligand 1 (keratinocyte-derived chemokine) production (55). The study further showed that PMN recruitment, microvascular and alveolar epithelial injury with protein leak in the airways, and damage of the lung microarchitecture were dependent on *TLR4* gene dose. Therefore, the TLR4 expression level determines the extent of acute pulmonary response to inhaled LPS, and TLR4 may thus be a valuable target for immunointervention in acute lung inflammation as a result of infection.

NLRs and ALI

In addition to the TLRs, NLRs are critically involved in the sensing of bacterial pathogens. NOD1 senses diaminopimelic acid–containing peptidoglycan present in Gram-negative bacteria, whereas NOD2 senses the muramyl dipeptide present in most organisms. Because of the apparent lack of direct effects on cell signaling induced by activators of NLRs, it is suggested that their role in pathogen sensing is one of cooperation with the TLRs (107). However, studies suggested that the actions of NOD1 vary between cell types and, unlike those seen with LPS, the *in vivo* effects may be independent of leukocyte activation. For instance, Cartwright and colleagues have shown that although selective activation of NOD1 in macrophages has no apparent effect, in vascular cells NOD1 activation results in the profound induction of NOSII and shock *in vivo* (108).

NOD2 is thought to be important in the maintenance of a healthy gut barrier because individuals who carry a defective NOD2 have an increased risk of Crohn disease and other intestinal disorders (109).

PRRS AND NONINFECTIOUS ALI

Although the importance of TLR family in sensing pathogens is well recognized, it is also plausible that they may function in noninfectious diseases because TLR expression is also regulated in conditions other than infection. Indeed, growing evidence has shown that PRRs play a central role in the mechanisms of noninfectious ALI.

Several TLRs not only have the ability to recognize more than one ligand, but often recognize ligands with completely different chemical structures. Such TLR activation does not occur under normal circumstances but only when there is a change in the environment that either leads to the release of endogenous ligands from a cellular compartment, or

leads to the modification of endogenous mediator that gives them the ability to activate TLRs (110,111). Because of the association of many endogenous ligands with tissue injury, the nomenclature of DAMPs has been suggested. The best characterized DAMPs include those products released from cells in response to stress or undergoing abnormal death, including Hsp60, Hsp70, the extra domain A of fibronectin, oligosaccharides of hyaluronic acid and high-mobility group box 1 (HMGB1). Most of these ligands act as agonists of TLR2 or TLR4, or both receptors (112).

THE ROLE OF TLR IN HEMORRHAGIC SHOCK–PRIMED ALI

Resuscitated hemorrhagic shock (HS) often promotes the development of lung injury by priming the immune system for an exaggerated inflammatory response to a second, often trivial, stimulus, the so-called "two hit hypothesis" (113).

We used a simplified animal model of the two-hit paradigm to address the mechanisms of HS-primed PMN migration and lung inflammation (114). In this model, animals are subjected to a nonsevere resuscitated HS (hypotension at 40 mmHg for 1 hour, followed by a small dose of intratracheal LPS. Although neither shock nor LPS alone induces injury, the combination caused lung PMN accumulation and increased ^{125}I-albumin transpulmonary flux. Findings from this model have suggested that the mechanisms underlying the priming of PMN and inflammation involve a complicated receptor cross-talk process and interaction between PMN and AMϕ, which is described below.

HS-Activated PMN Mediate TLR4 Signaling Upregulation of TLR2 in AMϕ

We demonstrated that LPS-TLR4 signaling upregulates TLR2 expression in AMϕ, and HS-activated PMN play a critical role in the mechanism of TLR2 upregulation (115). This cross-talk between TLR4 and TLR2 in AMϕ results in the amplification of expression of cytokines and chemokines in response to the bacterial products LPS and PGN, and subsequently leads to enhanced PMN sequestration in the lung. These findings reveal a novel mechanism underlying HS-primed lung injury, namely that HS-activated PMN that were initially sequestered into the alveoli can instruct AMϕ to upregulate TLR2, thereby sensitizing AMϕ to TLR2 ligands and promoting enhanced lung inflammation.

How does HS-activated PMN enhance TLR4 upregulation of TLR2 in AMϕ? We found that reactive oxygen species (ROS) derived from PMN NAD(P)H oxidase play an important role in amplifying the TLR2 upregulation (116). Studies have also shown that lack of endogenous NAD(P)H oxidase in the AMϕ caused a decrease in TLR2 expression in response to LPS stimulation; however, the decrease was restored when the AMϕ was coincubated with PMN isolated from wild-type mice subjected to HS. These results indicate that although the endogenous NAD(P)H oxidase in AMϕ is also involved in the signaling, the exogenous oxidants from PMN NAD(P)H oxidase are essential for inducing amplified TLR2 expression in AMϕ in response to LPS.

The TLR2 gene promoter contains multiple binding sites for transcriptional factors, which include NF-κB, CCAAT/enhancer binding protein, cAMP response element–binding protein and STAT (signal transducer and activator of transcription) (117). Of these, NF-κB has been reported to regulate TLR2 expression in response to cytokines and mycobacterial infection (117,118). It has been demonstrated that LPS-TLR4–induced TLR2 upregulation in AMϕ is largely mediated through the NF-κB signaling pathway, because the NF-κB inhibitor IKK-NBD significantly decreased LPS-induced TLR2 expression in AMϕ (115). Although oxidants are involved in the NF-κB signal transduction pathway (119–121), their molecular targets have not yet been defined. The contribution of redox regulation and location of potential redox-sensitive sites within the NF-κB activation pathway are the subjects of controversy (119).

HS Augments Lung EC Activation: Role of Temporal Alterations of TLR4 and TLR2

Recently we reported that HMGB1/TLR4 signaling mediates the HS-induced increase in TLR2 surface expression and decrease in TLR4 surface expression in the lung as well as in mouse lung vascular ECs (MLVEC) (122). These alterations in TLR4 and TLR2 expression result in HMGB1-mediated activation of NAD(P)H oxidase and expression of ICAM-1 in MLVEC that is TLR4 dependent in the early phase and switches to being TLR2 dependent in the late phase following HS. More importantly, the HS-induced surface expression of TLR2 contributes to an enhanced activation of MLVEC and augmented pulmonary PMN infiltration in response to the TLR2 agonist PGN. Thus, the study demonstrates a novel mechanism underlying HS-augmented lung inflammation, namely that induction of increased TLR2 surface expression in lung endothelial cells, which is induced by HS/R and mediated by HMGB1 activation of TLR4 signaling, is an important mechanism responsible for EC-mediated inflammation and organ injury following HS (122).

HMGB1-TLR4 Signaling Mediates HS-Induced NAD(P)H Oxidase Activation in PMN

We have shown that HS-induced PMN NAD(P)H oxidase activation is mediated by HMGB1-TLR4 signaling. HMGB1 was originally defined as a nuclear protein that functions to stabilize nucleosome formation, and it also acts as a transcription factor that regulates the expression of several genes (71). HMGB1 can be secreted by innate immune cells in response to microbial products or other inflammatory stimuli (123,124). HMGB1 is also released by injured cells and is known as one of the main prototypes of the emerging DAMPs (125–127). HMGB1 was initially identified as an inflammatory cytokine that is a late mediator of

lethality in sepsis (123,124). However, recent studies suggest that HMGB1 acts as an early mediator of inflammation, contributing to the development of ALI after hemorrhage (128), and hepatic injury after liver ischemia-reperfusion (129).

We found in our study that HS/R activates the TLR4-MyD88-IRAK4 signaling pathway through HMGB1, and further activates p38 MAPK and Akt pathways to initiate PMN NAD(P)H oxidase activation. PMN NAD(P)H oxidase–derived oxidants, in turn, mediate TLR4-TLR2 cross-talk in AMϕ and sensitize AMϕ response to TLR2 ligands, which act in a positive feedback manner to amplify pulmonary PMN infiltration and inflammation (130).

Multisystem Interaction Mediates HS-Induced Lung Inflammation

We have also addressed a fundamental question regarding how HS globally regulates PMN infiltration in the lungs. We have shown that HS, through alarmin HMGB1, induced IL-23 secretion from macrophages in an autocrine and TLR4 signaling–dependent manner. In turn, IL-23, through an IL-17–G-CSF–mediated mechanism, induced PMN egress from bone marrow. Therefore a sustained and HS-primed migration of PMN was maintained. We have also shown that β-adrenergic–receptor activation by catecholamine of macrophages mediated the HS-induced release of HMGB1. These data indicate that HS, a global ischemia/reperfusion stimulus, regulates PMN mobilization through a series of interacting pathways that include neuroendocrine and both innate and acquired immune systems (131).

Hyaluronan-TLR Signaling–Induced ALI

Hyaluronan (HA) is a massive sugar polymer in the extracellular matrix. Under physiologic conditions, HA exists as a high–molecular-weight polymer (>106 D) and undergoes dynamic regulation resulting in accumulation of lower molecular weight species (10–500 kD) after tissue injury. HA fragments can trigger innate immune responses in a manner that overlaps with both Gram-positive and Gram-negative organism recognition pathways (132). It has been demonstrated that fragmented HA accumulates during tissue injury (133–135). CD44 is required to clear HA during tissue injury, and impaired clearance of HA results in unremitting inflammation. Additionally, fragmented HA stimulates the expression of inflammatory genes by inflammatory cells at the injury site (136). Recently, Jiang *et al*. demonstrated that HA fragments require both TLR2 and TLR4 to stimulate mouse macrophages to produce inflammatory chemokines and cytokines. In a noninfectious lung injury model, mice deficient in both TLR2 and TLR4 showed an impaired transepithelial migration of inflammatory cells, increased tissue injury, elevated lung epithelial cell apoptosis and decreased survival (132). Lung epithelial cell overexpression of high molecular mass HA protected mice against ALI and apoptosis, in part through TLR-dependent basal activation of NF-κB. The exaggerated injury in TLR2- and TLR4-deficient mice appears to be due to impaired HA-TLR interactions on epithelial cells. These studies demonstrate that host–matrix component HA and TLR interactions provide signals that initiate inflammatory responses, maintain epithelial cell integrity and promote recovery from ALI (136).

Role of TLR in ALI Induced by MechanicalVentilation

Mechanical ventilation (MV) provides life-saving support for many patients with respiratory failure (137). However, mechanical stresses produced by MV can induce lung injury, termed ventilator-induced lung injury (138). Evidence from animal experimental studies has demonstrated that MV *per se* can induce inflammatory responses (139–141). A recent report has demonstrated that TLR4, but not TLR2, played a role in development of the inflammatory response after short-time MV (142). MV not only causes ventilator-induced lung injury in healthy animals, but also exacerbates damage in the injured lung (143). TLR4 blockade reduces pulmonary inflammation caused by the combination of LPS and MV (144). In the mechanism of hyperoxia-induced ALI, TLR3 expression and activation seems impotent. Exposure of human epithelial cells to hyperoxia in the absence of an exogenous viral pathogen significantly increased TLR3 expression. *In vivo* studies showed that both the absence of TLR3 via gene deletion in mice and the presence of an anti-TLR3 antibody in wild-type mice conferred significant protection in a hyperoxia-mediated lung injury model (145).

Role of NLR in Danger Signal–Induced ALI

The inflammasome is a multiprotein complex that mediates the activation of caspase-1, which promotes secretion of the proinflammatory cytokines IL-1β and IL-18, as well as pyroptosis, a form of cell death induced by bacterial pathogens. Members of the NLR family, including NLRP1, NLRP3 and NLRC4, and the adaptor ASC are critical components of the inflammasome that link microbial and endogenous danger signals to caspase-1 activation.

The role of NALP3 in mediating noninfectious ALI has been revealed by two recent studies. Gasse and colleagues reported that uric acid locally produced in the lung upon bleomycin (BLM)-induced DNA damage and degradation triggers NALP3 inflammasome activation, and in turn causes lung injury (146). Reduction of uric acid levels using the inhibitor of uric acid synthesis allopurinol or uricase leads to a decrease in BLM-induced IL-1β production, lung inflammation, repair and fibrosis. Local administration of exogenous uric acid crystals recapitulates lung inflammation and repair, which depend on the NALP3 inflammasome, MyD88 and IL-1R1 pathways and TLR2 and TLR4 for optimal inflammation but are independent of the IL-18 receptor (146). Babelova *et al*., however, reported the role of biglycan, a ubiquitous LRR proteoglycan of the extracellular matrix, in mediating ALI through interacting

with TLR2 and TLR4 on macrophages (147). The study showed that in macrophages soluble biglycan induces the NLRP3/ASC inflammasome and subsequent activation of caspase-1 and release of mature IL-1β without need for additional costimulatory factors. This is caused by the interaction of biglycan with TLR2/4 and purinergic P2 × 4/P2 × 7 receptors, which induces receptor cooperativity. Furthermore, ROS formation is involved in biglycan-mediated activation of the inflammasome. By signaling through TLR2/4 biglycan stimulates the expression of NLRP3 and pro–IL-1β mRNA. These results provide evidence for direct activation of the NLRP3 inflammasome by biglycan and suggest a fundamental paradigm of how tissue stress and injury are monitored by innate immune receptors detecting the release of the extracellular matrix components and turning such a signal into a robust inflammatory response (147).

CLINICAL IMPLICATIONS

Identification of TLR Genetic Variations for Predicting Disease Susceptibility

Susceptibility and response to infectious disease is, in part, heritable. Potential associations between clinical outcome from sepsis and many inflammatory cytokine gene polymorphisms, innate immunity pathway gene polymorphisms and coagulation cascade polymorphisms have been observed. We may yet be able to tease out the complex influence of genetic variation on susceptibility and response to infectious disease (148).

In 2000, Arbour and colleagues reported that two polymorphisms of the *TLR4* gene were present in a higher proportion of individuals who are hyporesponsive to inhaled LPS (149). This finding led to a number of studies investigating the potential impact of these TLR4 polymorphisms on the course of infectious diseases and the development of septic shock and TLR4 polymorphisms (150–152). These polymorphisms do not seem to confer susceptibility to all Gram-negative infections, because other groups (153,154) have shown no correlation in other infectious diseases such as meningococcal disease, and again one should be mindful that very significant hypofunctioning TLR4 alleles are likely to have a very strong negative selection pressure across the generations (155). TLR-2 polymorphisms have also been linked to susceptibility to staphylococcal infection (156) and lepromatous leprosy (157,158). Although some of these studies are still relatively small in scale, they reinforce the important role of TLRs in pathogen recognition and immune response in humans. CD14 polymorphisms, key accessory molecules for TLR signaling, have been associated with increased prevalence of positive bacterial culture findings and sepsis attributed to Gram-negative infections in a critically ill population (159), as well as the susceptibility to chronic *Chlamydia pneumoniae* infection in patients with coronary artery disease (160).

Targeting PRRs for ALI Therapies

The discovery of the importance of PRRs in the pathogenesis of SIRS and organ injury, including ALI, has led to a therapeutic strategy targeting PRRs. TLRs are the most extensively studied family of PRRs, and thus recently developed new drugs mainly target TLRs and are either agonists of TLRs to enhance immune responses against infectious agents or antagonists designed to reduce inflammation due to infection or autoimmune responses (161). The approaches to modulating TLR activity have focused on the following aspects: (a) ligands or analogues such as Eritoran (E5564) from Eisai (Woodcliff Lake, NJ, USA), a synthetic analogue of bacterial lipid A that inhibits LPS from activating cells through the TLR4/CD14/MD2 complex (162–164); (b) monoclonal antibodies, soluble receptors and other accessory proteins, such as a natural soluble form of TLR2, found in mouse plasma and breast milk, which acts to block TLR2 ligand stimulation (165), and a member of the TLR/IL-1 receptor family (TIR8 or SIGIRR) that inhibits NF-κB signaling and may be an endogenous inhibitor of the TLR system (166); (c) signal transduction blockers; many of the key molecules in the signaling pathways for each TLR have been identified and are considered potential drug targets (167–169). The structural bases of TIR domain interactions between TLRs and adapters such as MyD88, Mal, TRAM and TRIF have been modeled, and small peptidic sequences based on the TIR domain BB loop or peptidomimetics of this region have been made that can block the interactions (110,169,170). (d) siRNA and antisense; studies with knockout mice suggest that deficiency in individual TLRs has limited consequences for animals under normal conditions, but exhibits impact under conditions of specific infectious challenge (110,171,172). However, siRNA sequences themselves may be ligands for intracellular TLRs (173); thus attention to the design of appropriate sequences is necessary.

Drugs targeting TLRs have not yet been clinically applied to the treatment of ALI, but because of the critical role of PRRs in the development of ALI, targeting of PRRs has opened up a productive area for the therapy of ALI.

CONCLUSION

Current pharmacotherapy has not been highly successful in increasing patient survival in cases of ALI/ARDS. Since PRRs were recognized, their significance in the mechanisms of ALI has been quickly identified. The combined activation of these different receptors may result in complementary, synergistic or antagonistic effects that modulate the process of ALI. Therefore, modification of PRR pathways is likely to be a logical therapeutic target for ALI/ARDS. However, a complete understanding of the role of PRRs in the mechanism of ALI requires further "decoding" of these multiple receptor interactions.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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