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INVITED REVIEW

Danger-associated molecular patterns (DAMPs) in acute lung injury

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Abstract

Danger-associated molecular patterns (DAMPs) are host-derived molecules that can function to regulate the activation of pathogen recognition receptors (PRRs). These molecules play a critical role in modulating the lung injury response. DAMPs originate from multiple sources, including injured and dying cells, the extracellular matrix, or exist as immunomodulatory proteins within the airspace and interstitium. DAMPs can function as either toll-like receptor (TLR) agonists or antagonists, and can modulate both TLR and nod-like receptor (NLR) signalling cascades. Collectively, this diverse group of molecules may represent important therapeutic targets in the prevention and/or treatment of acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS).

Keywords: acute respiratory distress syndrome; acute lung injury; pathogen recognition receptors; fibrosis; chemokines

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Introduction

The lung is continuously exposed to the external environment and the entirety of the circulating blood volume. As a result, this organ is constantly bombarded with potentially injurious agents. These noxious stimuli include exogenous signals, such as microbial and environmental antigens, as well as a plethora of host-derived danger signals. A common final pathway initiated by these exogenous and endogenous triggers is the development of acute lung injury (ALI). ALI and its more severe form, acute respiratory distress syndrome (ARDS), are characterized by an initial exudative phase, which is triggered by the release of inflammatory cytokines [tumour necrosis factor-α (TNFα), interleukin-1β (IL-1β)] and chemokines (CXC, CC and CX_3C) and the influx and activation of inflammatory leukocytes. These events culminate in injury to the alveolar–capillary membrane, resulting in flow of proteinacious fluid and debris from the pulmonary vasculature space into the lung interstitium and ultimately the alveolar space [1]. The proliferative phase of ALI is characterized by organization of interstitial and alveolar exudates and the beginning of alveolar epithelial and endothelial repair. During this phase, fibroblasts migrate to the basement membrane and depose extracellular matrix (ECM) components, including collagen, fibronectin and hyaluronan [2,3]. The majority of patients spontaneously resolve this inflammatory insult. However, nearly one-third of patients will progress to the fibrotic phase of ALI/ARDS. In these patients, chronic respiratory failure is common and mortality is increased [4].

Pathogen recognition receptors

The primary pathway by which the innate immune system is alerted to the presence of noxious stimuli is through pattern recognition receptors (PRRs). Pathogen recognition receptors are activated by both exogenous pathogen-associated molecular patterns (PAMPs) and endogenous danger (or damage)-associated molecular patterns (DAMPs) [5]. Microbial components (eg PAMPs) that are present during infection can induce lung inflammatory responses and injuries that are mediated through Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (*RIG*-I; *RARRES3*) pathways [6]. Activation of these PRRs promotes inflammatory cytokine expression and antimicrobial pathways that are required for microbial clearance. However, inadequately controlled inflammation can result in cell death by necrosis, apoptosis, pyroptosis or autophagy. Of these death pathways, necrosis is the most immunogenic, due to the massive and unregulated release of DAMPs that stimulate both TLR and non-TLR pathways, leading to heightened inflammation and tissue remodelling [7,8]. The DAMP–TLR interaction can be direct, as is the case with fibronectin

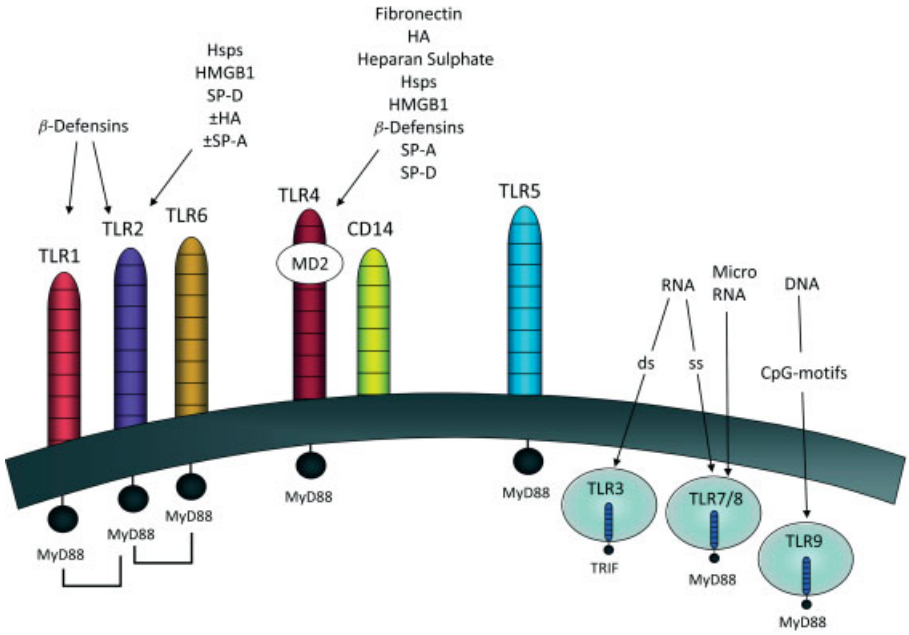

Figure 1. Schematic depicting TLRs and their respective DAMPs: Hsps, heat shock proteins; HA, hyaluronan; HMGB1, high-mobility group box 1; SP-A, surfactant protein A; SP-D, surfactant protein D

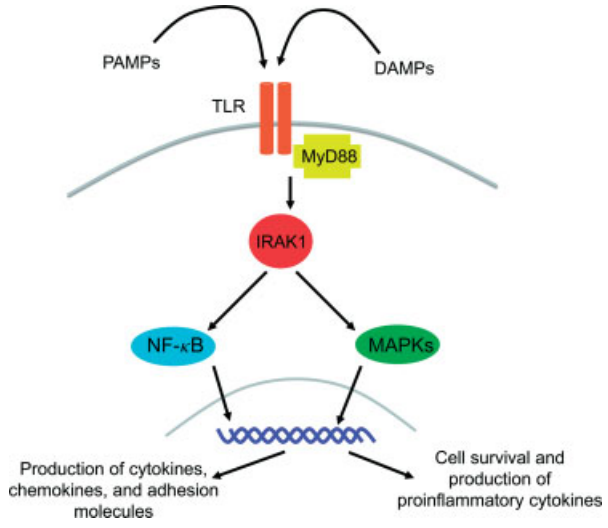

Figure 2. Schematic of MyD88-dependent TLR pathway activation in response to PAMPs and DAMPs

and hyaluronan, which serve as TLR agonists to initiate inflammatory cascades, or indirect, as observed with some heat shock proteins that interact with co-activator molecules to augment TLR signalling [9–11]. High-mobility group box 1 (HMGB1) can act directly with TLR2 and TLR4 to promote inflammation, and can also bind DNA to facilitate co-localization with responding TLRs (eg TLR9) [12–15]. Alternatively, there are some DAMPs, specifically surfactant protein A (SP-A) and possibly surfactant protein D (SP-D), which act to suppress TLR signalling and restore homeostasis through blocking interactions between TLRs and agonist molecules [16,17]. In cases of infectious and non-infectious lung injury, DAMPS can mediate both inflammatory and anti-inflammatory effects [18] (see Figure 1).

TLRs in ALI

TLR signalling pathways play a key, but complex, role in the pathogenesis of ALI. Upon ligand binding, stimulation of the majority of TLRs (except TLR3) promotes myeloid differentiation primary response gene 88 (MyD88)-dependent activation of IL-1 receptor-activating kinase 1/4 (IRAK-1/4), which culminates in mitogen activating protein kinase (MAPK) activation and translocation of nuclear factor *k*B (NF-*k*B) p65 into the nucleus [5]. NF-*k*B promotes transcriptional activation of genes encoding inflammatory cytokines and chemokines, while the MAPK pathway not only induces pro-inflammatory cytokine production but also stimulates cell proliferation and survival [5,19] (see Figure 2). TLRs have been shown to promote inflammatory cell recruitment and activation in both infectious and non-infectious lung injury models, a corollary to the exudative phase of ARDS [6]. However, these same TLRs are also required to maintain the integrity of the alveolar–capillary membrane during ALI. For instance, genetically mutant mice with defective TLR4 signalling [$TLR4^{lps/d}$ (Balb/c) mice] developed increased capillary leak and alveolar epithelial cell (AEC) apoptosis during Gram-negative pneumonia, which was due, in part, to impaired production of the cytoprotective cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) within the airspace [20]. In the non-infectious bleomycin model of ALI, TLR2/TLR4 double-deficient mice had less lung inflammation yet experienced higher mortality, more severe alveolar–capillary leaks and greater AEC apoptosis,

Table 1. Endogenous ligands of Toll-like receptors and Nod-like receptors and their physiological effects

DAMP	Receptor	Effect
ECM components		
Fibronectin	TLR4	Increase NF-*k*B, promote leukotriene synthesis and PMN migration, activate the adaptive immune system
Hyaluronan	TLR4 ± TLR2, NLR3	Induce proinflammatory cytokines, activate DCs and macrophages
Heparan sulphate	TLR4	Increase TNFα expression, activate DCs
Stress-response molecules		
Heat shock proteins	TLR2, TLR4	Induce cytokines and protein kinases, activate PMNs
HMGB1	TLR2, TLR4	Induce NF-*k*B nuclear translocation and cytokine expression
Nucleic acids	TLR3, TLR7, TLR9	Induce cytokine expression, activate DCs, stimulate recruitment of leukocytes and PMNs
microRNA	TLR8	Induce NF-*k*B and cytokines expression
Immunomodulatory proteins		
β-Defensins	TLR1/TLR2, TLR4	Induce NF-*k*B and cytokine expression, activate DCs and monocytes
Surfactant protein A	TLR4 ± TLR2	Reduce NF-*k*B and cytokine expression
Surfactant protein D	TLR2, TLR4	Inhibit cytokine production and recruitment of PMNs
Others		
Uric acid	NLR3	Increase IL-1β expression

implying that TLR2 and/or TLR4 promote pro-survival effects on the alveolar epithelium [21]. Similarly, in hyperoxia-induced ALI, TLR4-deficient mice are more susceptible to lung injury, due to increased epithelial and endothelial apoptosis and decreased expression of the antioxidant heme oxygenase-1 [22,23]. Conversely, TLR3 has been shown to mediate lung injury responses during exposure to high oxygen tensions. For instance, TLR3-deficient mice were protected from lung injury and had increased survival after hyperoxic exposure *in vivo*, and TLR3-deficient AECs were less susceptible to hyperoxia-induced ALI [24]. Collectively, this implies that the role of TLRs in ALI is highly contextual and TLR-specific.

DAMPs

DAMPs are host-derived molecules that can function to regulate the activation of PRRs. In general, various DAMPs can be subdivided into three broad categories: (a) ECM components; (b) molecules released during cellular stress; and (c) secreted immunomodulatory proteins. ECM components that can activate TLRs include fibronectin and hyaluronan. Stress-response molecules, including heat-shock proteins (HSPs), HMGB1 and nucleic acids that are secreted by cells or released when cells die compose the second category. The final category includes immunomodulatory proteins, such as β-defensins and surfactant proteins, that are involved in a variety of biological processes, including host defence and lung physiology [25] (see Table 1).

ECM Components

Fibronectin

Fibronectin (MW ~450 kDa) is an extracellular glycoprotein adhesion molecule that exists in soluble (plasma) and insoluble (cellular) forms. The soluble isomer is a major component of blood, whereas the insoluble form is a major component of the ECM [26]. Fibronectin is involved in many physiological processes, but primarily functions in regulating cellular adhesion, migration, proliferation and wound healing [27,28]. This protein has also been shown to regulate immune responses by binding to and activating several TLRs. Specifically, fibronectin has been shown to activate HEK 293 cells transfected with TLR4, but not in non-transfected cells. Stimulation with fibronectin extra domain A (EDA) in TLR4 expressing HEK cells resulted in increased expression of NF-*k*B, similar in magnitude to that observed with lipopolysaccharide (LPS) stimulation [9]. Additional studies have confirmed the ability of fibronectin to activate TLR4 in a variety of cell populations and *in vivo* models [29,30]. Newer data suggest that fibronectin–TLR4 interactions may serve as a positive feedback loop to amplify inflammatory responses [31].

Fibronectin is a major cell-signalling molecule in various forms of acute lung injury. Fibronectin mRNA and protein levels are elevated in bronchoalveolar lavage fluid (BALF) macrophages and alveolar epithelial, interstitial and endothelial cells of animals with hyperoxia-induced lung injury [32–34]. Similarly, fibronectin mRNA and protein are most prominently expressed during the exudative phase of bleomycin-induced ALI [35,36]. In addition, fibronectin has been implicated as a mediator of deleterious inflammation in other ALI models, including oxidant injury, pancreatitis-induced ALI and injury from inhaled pulmonary irritants, such as ozone [37–39]. Consistent with experimental animal data, autopsy studies report elevated amounts of fibronectin in the lungs of patients who have died from ARDS, as compared to patients without underlying lung disease who died of non-pulmonary causes [40]. While there is clear evidence of

robust fibronectin expression in both human and experimental ALI, data implicating TLR4 or other TLRs in mediating fibronectin's biological effects are lacking.

Hyaluronan

Hyaluronan (HA; MW 2000–4000 kDa) is a non-sulphated glycosaminoglycan that resides in the ECM and acts as a moderator of lung water, as well as a matrix glue for other connective tissue components and cells [41]. During homeostasis, HA exists as a high molecular weight (HMW HA) polymer ($1–6 \times 10^6$ Da) that undergoes continuous remodelling, resulting in degradation to lower molecular weight forms ($0.1–0.5 \times 10^6$ Da). The low molecular weight (LMW HA) forms are immunologically active and are associated with diverse inflammatory conditions and/or tissue injury [21,41]. Low molecular weight fragments of HA have been shown to activate dendritic cells (DCs) and macrophages to express inflammatory cytokines, including IL-1β, TNFα and interleukin 12 (IL-12) [42]. The expression of inflammatory cytokines and cell maturation was abrogated in HA-stimulated DCs isolated from Tlr4-deficient but not Tlr2-deficient mice, implying that TLR4 is the effector PRR for HA-induced DC effector responses [10]. Likewise, HA-mediated responses in dermal endothelial cells were shown to be TLR4-dependent [43]. However, other studies suggest that LMW HA is a ligand for both TLR4 and TLR2. For example, reduced expression of the chemokine macrophage inflammatory protein 2 (MIP-2; Cxcl2) was noted in peritoneal macrophages isolated from $Tlr2^{-/-}$ and $Tlr4^{-/-}$ single-deficient mice in response to HA. Chemokine expression was completely abolished in $Tlr2^{-/-}/Tlr4^{-/-}$ double-deficient macrophages in response to HA, indicating that TLR2 and TLR4 serve as the predominant TLRs responding to HA [21].

Interestingly, while HA clearly initiates an inflammatory response in manner that is dependent on TLR activation, this molecule appears to play a more complex role in regulating lung injury responses *in vivo*. As might be expected, administration of bleomycin to $Tlr2^{-/-}/Tlr4^{-/-}$ double-deficient mice resulted in reduced influx of BAL neutrophils. Despite reduced lung inflammation, there was increased lung injury and mortality in $Tlr2^{-/-}/Tlr4^{-/-}$ double-deficient mice challenged with bleomycin, which correlated pathologically with enhanced AEC apoptosis in TLR double-deficient mice. AECs from these mice produced less HMW HA compared to control cells, and CC10-driven constitutive expression of HMW HA in lung epithelial cells protected them from apoptosis [21,44]. The mechanism by which HA-induced TLR activation reduced epithelial cell apoptosis was not completely defined, but was thought to be attributable to low-level NF-κB activation and induction of anti-apoptotic molecules, such as B-cell lymphoma 2 (Bcl2). Studies in various animal models have shown that bleomycin-induced ALI causes elevated levels of HA in BALF and alveolar interstitial tissue and that this mirrors the increased amounts of pulmonary oedema seen in ALI/ARDS [45–48].

Similar to the bleomycin model, TLR4 alone or TLR2 and TLR4 in combination mediate protective effects on alveolar epithelium in hyperoxic lung injury. It was noted that $Tlr4^{-/-}$, and especially $Tlr2^{-/-}/Tlr4^{-/-}$ double-deficient, mice exposed to hyperoxic conditions displayed more evidence of diffuse alveolar damage and decreased survival [21]. Increased AEC apoptosis was observed in Tlr4-deficient mice, which was due to an inability to up-regulate the anti-apoptotic molecules Bcl2 and heme oxygenase 1 (HO-1; Hmox1). Although HA is likely functioning as aTLR agonist in this model, the specific contribution of HA or other DAMPs was not definitively established.

It is probable that HA contributes to lung injury responses in patients with ALI/ARDS. For example, an approximately six-fold increase in BALF HA levels has been noted in ARDS patients as compared to control subjects. Moreover, serum levels of HA were 30-fold higher in ARDS patients than in controls. There was an inverse relationship between levels of HA and PaO_2:FiO_2, substantiating a possible protective role of HA in ALI [49]. Whether the potential protective effects of HA in patients with ALI are mediated through TLR2 and/or TLR4 has not been clarified.

Heparan sulphate

Heparan sulphate is a sulphated glycosaminoglycan ECM protein functioning to localize cytokines or modify the activity of cytokines, proteases, growth factors and other ECM proteins within the inflammatory milieu [50]. There is compelling evidence that heparan sulphate can activate DCs in a fashion similar to TLR ligands, such as LPS and CpG DNA. Treatment of bone marrow DCs derived from wild-type mice with heparan sulphate stimulated DC maturation, which was significantly mitigated by co-incubation with a selective pharmacological TLR4 inhibitor [51]. Furthermore, significantly improved survival, associated with abrogated serum TNFα responses, were observed in Tlr4-deficient mice challenged with heparan sulphate intraperitoneally (i.p.), as compared to similarly treated wild-type control animals [52]. Taken together, these findings indicate that heparan sulphate promotes inflammatory responses in manner that is at least partially dependent upon TLR4. There are no data to implicate heparan sulphate as a relevant TLR agonist in experimental models of ALI or patients with ALI/ARDS.

Stress response molecules

Heat shock proteins

Heat shock proteins (HSPs) are a group of molecules that directly mediate protein folding or serve as molecular chaperones that bind to nascent polypeptides and partially folded intermediates, preventing aggregation

and misfolding [53]. These molecules are primarily intracellular proteins whose expression is constitutively repressed under homeostatic conditions. During times of physiological stress (i.e. inflammation, infections, malignancy, etc.), gene expression is increased and HSPs are released into the extracellular compartment. HSPs are also released from necrotic cells and promote inflammation via the induction of multiple pro-inflammatory cytokines [53,54]. Stimulatory effects on cytokine production requires TLR4, as stimulation of bone marrow macrophages derived from Tlr4 mutant C3H/HeJ mice stimulated *in vitro* with heat shock protein 60 (Hsp60; Hspd1) displays reduced production of TNFα and nitric oxide (NO), as compared to the robust response observed in wild-type C57BL/6 cells [55]. Additional studies performed in human embryonic kidney fibroblasts transfected with TLR2 or TLR4 indicate that Hsp 60, in combination with the co-stimulatory molecule MD-2, uses TLR2 and TLR4 to activate the toll–IL-1 receptor (TIR) signalling pathway to induce inflammatory responses mediated by multiple protein kinases [11]. Similarly, heat shock protein 70 (Hsp70) induces inflammatory cytokine production via MyD88–NF-*k*B–TIR pathways, utilizing both TLR2 and TLR4 for signal transduction in human peripheral blood monocytes and RAW264.7 macrophages [56,57]. Heat shock protein Gp96 (Gp96) has been shown to mediate NF-*k*B activation through a TLR2–TLR4 pathway in bone marrow-derived DCs that were lacking TLR2, TLR4 or both, whereas heat shock protein 72 (Hsp72) induced the cytokines IL-8 and TNFα through a TLR4-dependent mechanism, as shown in human HL-60 cells treated with TLR4 neutralizing antibody and neutrophils isolated from C3H/HeJ mice [58,59]. Relevant to lung inflammatory responses, Hsp72 induced IL-8, TNFα and KC in mouse lung epithelial cells, and this induction was negated when assessed in TLR4-deficient cells or in the presence of NF-*k*B inhibition [60].

In infectious models of ALI, the inflammatory properties of HSPs seem to have a protective role if administered before infectious challenge but become cytotoxic if elevated or exogenously administered after microbial exposure [61]. The role of HSPs in regulating non-infectious lung injury is less clear, as there are limited studies in experimental models of lung injury, including bleomycin. Data exist to suggest that Hsp70 protects against bleomycin-induced inflammation and injury. For example, bleomycin challenge in transgenic mice that over-express Hsp70 have reduced leukocyte recruitment to the lung, less parenchymal cell apoptosis and reduced expression of IL-1β, TNFα and IL-6 in BALF compared to wild-type mice [62]. Similarly, Hsp70 protects human respiratory epithelial cells from injury in the setting of hyperoxia, as cells over-expressing Hsp70 were shown to be resistant to hyperoxia-induced cell death *in vitro* [63]. It has not been established in the bleomycin model whether Hsp70 is signalling through TLRs or other receptors, and whether the protection observed might be attributable to direct TLR-dependent pro-survival effects on the respiratory epithelium. Conversely, Hsp27 expression was decreased in respiratory epithelial cells after exposure to 95% FiO2 and was linked with less hyperoxia-induced apoptosis when compared to controls, implying that there are disparate effects of the various HSPs [64].

As a human correlate, airway epithelial cells isolated from healthy patients inhaling 100% oxygen show increased expression of genes encoding Hsp70. Induction of Hsp70 genes has also been observed in human airway epithelial cells exposed to > 98% oxygen *in vitro* [65]. In patients with ARDS, levels of Hsp72 were higher in pulmonary oedema fluid as compared to those with hydrostatic pulmonary oedema, suggesting that Hsp72 is produced or released within the alveolar space in ALI [66]. There are no data to suggest that levels of Hsp72 correlate with morbidity or mortality in ALI patients. By comparison, serum Hsp60 levels correlated positively with the development of ALI in severe trauma patients [67]. This suggests that selected HSPs may not only regulate lung injury responses in a TLR-dependent fashion, but could also serve as a marker for ALI development in at-risk patients.

High-mobility group box 1 (HMGB1)

HMGB1 is a highly conserved intranuclear protein with the primary function of flexing the DNA double helix to allow for proper DNA orientation, so that transcription factors can efficiently bind DNA [68]. The two principle ways by which HMGB1 is released from cells is through disorganized necrosis and active secretion from a variety of immune and structural cells [69]. Release of HMGB1 into the extracellular space is known to promote inflammation via stimulation of monocytes to release TNFα and other inflammatory cytokines [70]. It was initially believed that HMGB1-induced inflammation was mediated by the receptor for advanced glycation end products (RAGE). However, subsequent studies in other cells or cell lines suggested that signalling through RAGE may be a less relevant pathway of inflammatory cytokine expression [14,71–73]. For instance, the mechanism of action for HMGB1-related inflammation in both neutrophils and macrophages is mediated through a direct interaction with TLR2 and TLR4, leading to increased TLR signalling and nuclear translocation of NF-κB as well as enhanced expression of pro-inflammatory cytokines [12,14,74]. These observations provide support for the notion that HMGB1 is a central component of a positive feedback loop, via TLRs, to amplify and sustain inflammation [75,76]. In total, the controversy regarding the relationship between HMGB1 and TLRs may be fuelled by the differential utilization of TLRs in different tissues, species and cell lines [77].

There is strong evidence that HMGB1 promotes pulmonary inflammation during ALI. The intratracheal (i.t.) instillation of purified HMGB1 in mice resulted

in alveolar oedema and neutrophil influx in association with elevated levels of IL-1β, TNFα and MIP-2 in BALF. Histologically, these samples demonstrated diffuse alveolar damage identical to the pathological findings in ALI/ARDS [78]. Inflammatory effects in this model were not entirely TLR4-mediated, as inflammatory changes in response to HMGB1 were still apparent in C3H/HeJ mice. The stimulation of rat alveolar macrophages with HMGB1 has also been shown to dose-dependently stimulate the expression of inducible nitric oxide synthase (iNOS; Nos2). Elevated levels of iNOS promoted further inflammation in rat lungs after HMGB1-induced ALI [79]. In a bleomycin model of ALI, increased levels of HMGB1 were found in the BALF of wild-type mice, peaking 7–14 days post-bleomycin. When compared to wild-type mice, RAGE-deficient mice challenged i.t. with bleomycin had similar numbers of BALF inflammatory cells but reduced BALF protein levels, suggesting that the HMGB1–RAGE pathway likely contributes to the high-permeability state observed in bleomycin-induced ALI [80]. Several other animal models of non-infectious lung injury have also been associated with elevated levels of HMGB1, such as acute pancreatitis and haemorrhage [81,82]. In haemorrhagic shock, HMGB1-induced inflammatory effects on lung endothelial cells and PMNs was abrogated in mice deficient in Tlr4 [83,84].

Our understanding of HMGB1 biology in patients with ALI is limited. One small study of patients with sepsis-induced ALI noted that HMGB1 was higher in epithelial lung fluid and plasma in ALI patients compared to controls. These findings persisted during both the acute and subacute phases of ARDS. Levels of HMGB1 did not correlate with lung injury score or mortality [85]. Additional studies are needed to better define the contribution of HMGB1 and receptors involved in this patient population.

Nucleic acids

Multiple studies have implicated nucleic acids from bacterial and viral genomes as potent activators of TLRs [86–89]. Human nucleic acids are located intracellularly but are released into the extracellular environment during cell necrosis and late apoptosis [90].

Once within the extracellular environment, these nucleic acids can be modified and recognized as foreign, eliciting an innate immune response. Human nucleic acids were first shown to be ligands for TLR9 (DNA), TLR7 [single-stranded (ss) RNA] and TLR3 [double-stranded (ds) RNA] when DNA and RNA immune complexes were found to directly activate plasmacytoid pre-dendritic cells, resulting in interferon-α (IFNα) production. Treatment with TLR7- and TLR9-blocking antibodies dramatically decreased INFα production in response to DNA and RNA complexes, respectively [91]. Endogenous mRNA can stimulate TNFα and TLR3 gene expression from human DCs, as well as cell surface expression of the activational markers CD83 and HLA-DR. Moreover, endogenous mRNA induced DCs to secrete elevated levels of IL-12, IL-8 and IFNα, which was reversed in the presence of TLR3 blocking antibody [92]. Finally, using genetically deficient mice, TLR3 was shown to mediate many of the pathophysiological events in experimental abdominal sepsis, an effect that was mediated by the presence of cellular debris from necrotic PMNs (presumably nucleic acids) [93]. Thus, it appears that both endogenous mRNA and DNA can stimulate the effector functions of DCs and potentially other cells, further amplifying the innate immune response.

New research has provided additional insights into the mechanism by which 'self' DNA can activate TLR9. Due to its high binding affinity for nucleic acids, HMGB1 readily binds to host DNA and modulates TLR-dependent inflammation [15,94,95]. HMGB1 may do this by facilitating DNA:TLR9 trafficking to the endosome and enhancing TLR9-dependent signal transduction [96]. Thus, the ability of endogenous DNA to stimulate TLR9 is not only enhanced by chemical modifications to the DNA (eg changes in CpG methylation) but also by complexing with proteins that promote DNA:TLR9 co-localization.

MicroRNAs are host-derived single-stranded RNA species that modulate the expression of many genes during inflammation, including during ALI [97,98]. MicroRNAs are induced by TLR activation and post-transcriptionally regulate TLR-dependent gene expression and signalling [99,100]. Emerging data, primarily in cancer model systems, suggest that microRNAs can also bind and functionally activate certain TLRs, such as TLR8 [101]. These observations support the notion that microRNAs may function as relevant TLR signalling modifiers in the setting of pulmonary inflammation and injury. However, there are no data implicating DNA, RNA or microRNA as relevant TLR agonists in experimental models of ALI or patients with ALI/ARDS.

Immunomodulatory proteins

β-Defensins

β-Defensins are small antimicrobial and cytotoxic peptides that are produced during infection due to a variety of microbial organisms, including Gram-positive and Gram-negative bacteria, mycobacteria, fungi and enveloped viruses [102]. In addition to being a key component of innate immunity, β-defensins have been shown to be endogenous ligands for toll-like receptors and function to augment the innate response. Specifically, murine β-defensin 2 can stimulate bone marrow-derived DCs to express cell surface CD11c and the co-stimulatory molecule CD40. The inflammatory cytokine profile expressed in response to β-defensin 2 was similar to that expressed by DCs in response to LPS. In macrophages, the stimulatory effect of

β-defensin 2 was TLR4-dependent, as neither LPS nor β-defensin 2 were able to activate macrophages obtained from Tlr4-deficient mice [103]. In addition to stimulation of inflammatory cytokines in DCs, β-defensin 2 may also promote early cell death, a process mediated by TLR4/MyD88-dependent activation of NF-κB. The implication of this work is that endogenous β-defensin 2 may have a dynamic role in inflammation; initially serving as a signal for immune cell activation and later driving the elimination of activated cells to prevent the long-term consequences of persistent inflammation [104].

Human β-defensin 3 has also been shown to induce cell surface co-stimulatory molecules on human peripheral blood monocytes *in vitro*. In experiments using HEK 293 cells that were mutated to express various TLRs, there was no interaction between any single TLR in isolation with human β-defensin 3. However, when these cells co-expressed TLR1 in combination with TLR2, stimulation with human β-defensin 3 induced NF-κB and this effect was blocked by antibodies to TLR1 and TLR2 [105]. These intriguing findings suggest that TLR1 and TLR2 can cooperatively respond to human β-defensin 3, whereas neither in isolation is sufficient to initiate this process.

A possible protective role of β-defensins has been noted in sepsis-induced ALI. The over-expression of β-defensin 2 in lung prior to an i.t. challenge with *Pseudomonas aeruginosa*, or undergoing experimental abdominal sepsis, resulted in reduced alveolar damage, interstitial oedema and BALF neutrophilia [106]. In a rat intestinal ischaemia and reperfusion-induced ALI model, high levels of β-defensin 2 were noted and levels were inversely correlated with pulmonary permeability [107]. Whether this effect was mediated by direct TLR activation was not addressed.

Pulmonary surfactant proteins

SP-A

Pulmonary surfactant is a lipoprotein complex that functions to reduce surface tension within the alveoli and prevent alveolar collapse at end expiration. Surfactant contains four specific proteins, surfactant protein A (SP-A), SP-B, SP-C and SP-D. These proteins play unique but complementary roles in surfactant lipid metabolism, lipid membrane organization and host defence [108]. SP-A and SP-D are members of the collectin family, and have been shown to directly interact with microbes such as bacteria and viruses to inhibit proliferation and microbicidal activity and facilitate microbial uptake by macrophages [109]. Early studies performed in a human acute monocytic leukaemia cell line demonstrated that SP-A dose-dependently activated the NF-κB pathway in a similar manner to LPS, suggesting they may share a common receptor [109]. *In vitro* stimulation of bone marrow-derived macrophages from a Tlr4-competent strain mice (C3H/HeOuJ) with SP-A dose-dependently increased the expression of TNFα and the anti-inflammatory cytokine IL-10 [110]. Conversely, SP-A-induced inflammatory responses were abrogated in macrophages isolated from an isogenic mutant Tlr4-deficient (C3H/HeJ) mouse strain, indicating that TLR4 is a receptor for and is activated by SP-A [110].

While SP-A appears to be capable of directly activating TLR4, accumulating data suggest that this protein can actually attenuate the activation of macrophages in response to PAMPs, including LPS, peptidoglycan and zymosan, and that this process is mediated through the TLR2 or TLR4 pathways [17,111,112]. For example, pre-treatment of alveolar macrophages with SP-A blunted subsequent NF-*k*B activation and TNFα production in response to either TLR2 or TLR4 ligands [16,113]. The mechanism for this suppressive effect has not been fully elicited, but one theory is that SP-A binding to TLRs may block the ability of other PAMPs to bind and initiate TLR-mediated inflammation [114].

Studies using non-infectious models of lung injury have demonstrated that SP-A can reduce type II alveolar cell apoptosis, although this effect does not appear to be TLR-mediated [115]. The administration of bleomycin to SP-A-deficient mice results in enhanced recruitment of leukocytes, expression of inflammatory cytokines (IL-1β, TNFα and KC), oedema formation and apoptosis, as compared to wild-type mice [116]. This is consistent with the premise that SP-A acts to reduce inflammation in acute lung injury.

Serum SP-A levels are elevated in patients with ARDS, as compared to healthy controls and mechanically ventilated control patients, and circulating levels of SP-A may be of prognostic significance in this disease. In a smaller series, serum SP-A levels were inversely correlated with oxygenation and lung compliance [117,118]. Conversely, a trial investigating the influence of ARDSnet ventilation strategy on biomarkers of lung injury failed to find any correlation between plasma SP-A levels and clinical outcomes [119]. It is unclear whether levels of SP-A in circulation simply reflect the magnitude of permeability change across the alveolar capillary membrane, or rather that SP-A may have some causative role in modulating the lung injury response.

SP-D

SP-D, like SP-A, can interact with TLRs. SP-D has been shown to directly bind to TLR2 and TLR4, although the biological impact of this interaction was not described [120]. Importantly, pre-incubation of rat alveolar macrophages with SP-D mitigated LPS-induced inflammatory response [121]. Consistent with anti-inflammatory effects of SP-D, SP-D-deficient mice developed enhanced influx of BALF neutrophils, increased total protein, and higher levels of inflammatory cytokines (IL-1β, IL-6 and MIP-2) in response to i.t. LPS administration. The exogenous administration

of SP-D resulted in marked suppression of the inflammatory response. Similar results were noted when the TLR2 agonist lipoteichoic acid (LTA) was used as the inciting cause of ALI [122,123]. These findings indicate that SP-D plays a protective and anti-inflammatory role in LPS and LTA induced ALI.

Similar anti-inflammatory properties of SP-D have been described in the bleomycin model of ALI. After the i.t. instillation of bleomycin, serum levels of SP-D were elevated, peaking on day 7 and coinciding with the amount of inflammation seen in histological lung samples [124]. There was a dose-dependent increase in morbidity and mortality in SP-D-deficient mice challenged with bleomycin, compared to wild-type. Conversely, mice over-expressing SP-D in the lung were highly resistant to bleomycin-induced morbidity and mortality [125]. The mechanism of protection conferred by SP-D in these model systems has not been precisely defined. One can speculate that SP-D binding to TLRs might antagonize interactions with other TLR agonists in a fashion analogous to SP-A; however, this hypothesis requires experimental validation.

In patients with ARDS, higher plasma SP-D levels have been associated with increased risk of death, fewer ventilator-free days and fewer organ failure-free days.[119] In contrast, reduced pulmonary oedema fluid SP-D in patients with ARDS was associated with worse clinical outcome, substantiating a potential protective role of SP-D in ALI/ARDS [117].

Nucleotide oligomerization domain (NOD)-like receptors (NLRs)

Another class of PRRs is the NOD-like receptors. These receptors exist intracellularly and serve to activate caspase-1 and initiate an inflammatory response triggered by intracellular PAMPs [126]. NLRs are known to respond to a variety of PAMPs, including the bacterial cell wall components peptidoglycan and muramyl dipeptide (MDP), bacterial flagellin and other bacterial toxins [126–128]. NLRs have also been shown to respond to two classes of non-infectious stimuli, DAMPS and environmental insults [126]. Environmental insults such as silica and asbestos have been shown to induce inflammation in a NLR3-dependent manner [129]. The strongest evidence for DAMP-induced NLR activation comes from a study of uric acid and NOD-like receptor 3 (NLR3). Uric acid is known to be released from dying cells [130]. Researchers noted a brisk IL-1β response when monocytes were incubated with monosodium urate (MSU). This interaction was dependent on ASC (a necessary NLR co-factor), providing compelling evidence that the IL-1β response was mediated through NLR pathway signalling. Moreover, the inflammatory response induced by MSU was abrogated in NLR3 (Nlrp3)-deficient mice [131]. Similarly, uric acid appears to mediate lung inflammation in bleomycin-induced lung injury, as reduced lung injury is observed in mice with decreased uric acid production (by xanthine oxidase administration) or in mice genetically lacking NLR3 [132]. Hyaluronan, which was previously shown to activate the TLR pathway, has more recently been discovered to be a NLR3 ligand, including causing IL-1β release in peritoneal macrophages [133]. This is the first description of a DAMP that is capable of activating both the TLR and NLR pathways.

Conclusions

TLRs are central mediators of the pathophysiological events that occur in both infectious and non-infectious lung injury. While much investigation has focused on external triggers of ALI, emerging data illuminate the critical role of endogenous danger signals in modulating the lung injury response. These DAMPs originate from multiple sources, such as injured and dying cells or the ECM, or exist as immunomodulatory proteins within the airspace and interstitium. DAMPs can function as either TLR agonists or antagonists, and can modulate both TLR and NLR signalling cascades. Additional research is needed to define the contribution of DAMPs in fibroproliferative processes, including the proliferative phase of ALI. Regardless, DAMPS may represent an important therapeutic target in the prevention and/or treatment of ALI/ARDS.

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Author contributions

Both authors contributed equally to this review.

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