

# Lung cell-specific modulation of LPS-induced TLR4 receptor and adaptor localization

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**Abbreviations:** AECl, type I alveolar epithelial cells; AECII, type II alveolar epithelial cells; CF, cystic fibrosis; CFTR transmembrane conductance regulator; IFN, interferon; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; IRF, interferon regulatory factor; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response gene 88; NF- $\kappa$ B, nuclear factor  $\kappa$ B; SP-A, surfactant protein A; SP-D, surfactant protein D; TIRAP, toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TLR4, Toll-like receptor 4; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TRAM, TRIF-related adaptor molecule; TRIF, toll/IL-1R-domain-containing adaptor protein inducing interferon- $\beta$

Lung infection by Gram-negative bacteria is a major cause of morbidity and mortality in humans. Lipopolysaccharide (LPS), located in the outer membrane of the Gram-negative bacterial cell wall, is a highly potent stimulus of immune and structural cells via the TLR4/MD2 complex whose function is sequentially regulated by defined subsets of adaptor proteins. Regulatory mechanisms of lung-specific defense pathways point at the crucial role of resident alveolar macrophages, alveolar epithelial cells, the TLR4 receptor pathway, and lung surfactant in shaping the innate immune response to Gram-negative bacteria and LPS. During the past decade intracellular spatiotemporal localization of TLR4 emerged as a key feature of TLR4 function. Here, we briefly review lung cell type- and compartment-specific mechanisms of LPS-induced TLR4 regulation with a focus on primary resident hematopoietic and structural cells as well as modifying microenvironmental factors involved.

## Introduction

Due to the unique condition of being constantly exposed to air that contains pathogens, allergens, and pollutants, the lung has developed a highly specific local microenvironment where the combined actions of the epithelial barrier, innate defense molecules, and responses of both, the epithelium and professional phagocytes, help to maintain biophysical and immune homeostasis. Key cells mediating lung innate immune responses in the proximal respiratory tract include ciliated cells, goblet cells, Clara cells, and submucosal glands that together

transport particles trapped in secreted mucus from more distally located airways toward proximal, a defense mechanism known as mucociliary escalator. At the alveolar site pulmonary innate immunity is ensured by airway epithelial cells, alveolar epithelial cells (AEC) type I and II and alveolar macrophages that cofunction with surfactant-associated proteins belonging to the C-type lectin superfamily as well as antimicrobial peptides (Fig. 1). Type II AEC synthesize, secrete and reuptake pulmonary surfactant, a lipoprotein complex that reduces surface tension at the air-liquid interface of the lung, thereby allowing normal breathing, and protects the lung from continuous environmental exposures to pathogens and allergens.<sup>1,4</sup> Surfactant is composed of 90% phospholipids (dipalmitoylphosphatidylcholine) and 10% of proteins (SP) SP-A, SP-B, SP-C, and SP-D.<sup>2</sup> The collectins SP-A, the most abundant one in the lung, and SP-D are both soluble pattern recognition receptors and sensing molecules not based on pattern recognition with important functions on lung immune homeostasis *in vivo*.<sup>1,4</sup> The small hydrophobic proteins SP-B and SP-C are essential for the functional structure of the surfactant lipid film at the air-liquid interphase.<sup>3</sup> However, increasing evidence indicates that all surfactant proteins and distinct surfactant lipids regulate innate immune responses of the lung,<sup>5</sup> partly by interacting with alveolar macrophages. Under physiological conditions, resident alveolar macrophages account for approximately 95% of airspace leukocytes, constituting the sentinel phagocytic cell of the innate immune system in the lung. The natural microenvironment of alveolar macrophages is pulmonary surfactant whose lipid and protein elements modulate their functional phenotype in humans, rats and mice *in vivo*.<sup>1,4,6</sup>

Cell surface and endosomal TLRs constitute one of the major cell receptors involved in innate immunity of the lung mediating immediate immune responses against microbial pathogens. TLR4, one of the most extensively studied TLR, is a type I transmembrane protein with an extracellular domain of leucine-rich repeats that provides, dependent on MD2, the

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recognition of LPS, the invariant virulence factor of Gram-negative bacteria. In recent years some unique regulatory mechanisms of defense pathways intrinsic to the lung have been identified which point at the critical role of resident alveolar macrophages, alveolar epithelial cells, the canonical TLR4 receptor pathway and pulmonary surfactant in initiating and modulating initial immune responses to Gram-negative bacteria and LPS. These normal lung defense mechanisms ensure that most host-microbe interactions do not lead to persistent pathological consequences. However, dependent on pathogen exposure time, load, virulence as well as age- and immune status-related specific factors of individuals being at risk, pneumonia may develop. Pneumonia caused by Gram-negative bacteria is a major cause of morbidity and mortality in humans with an increasing prevalence of community-acquired and early-onset ventilator-associated pneumonia.<sup>7</sup>

Although lung cell-specific regulatory mechanisms of TLR4 signaling are largely unknown, they are expected to maintain defense homeostasis of the lung. LPS-induced cell responses are tightly regulated via distinct pathways including subcellular TLR4 localization and thus ligand sensing, but the role of TLR4 localization specific for the lung is only beginning to be experimentally addressed. In the lung, TLR4 and TLR4 adaptors are expressed by all immune and structural cell types. The purpose of this article is to review lung cell- and context-specific mechanisms of TLR4 regulation with a focus on primary resident hematopoietic and structural cells, represented by alveolar macrophages and alveolar epithelial cells, respectively.

#### TLR4 signaling in gram-negative respiratory infections

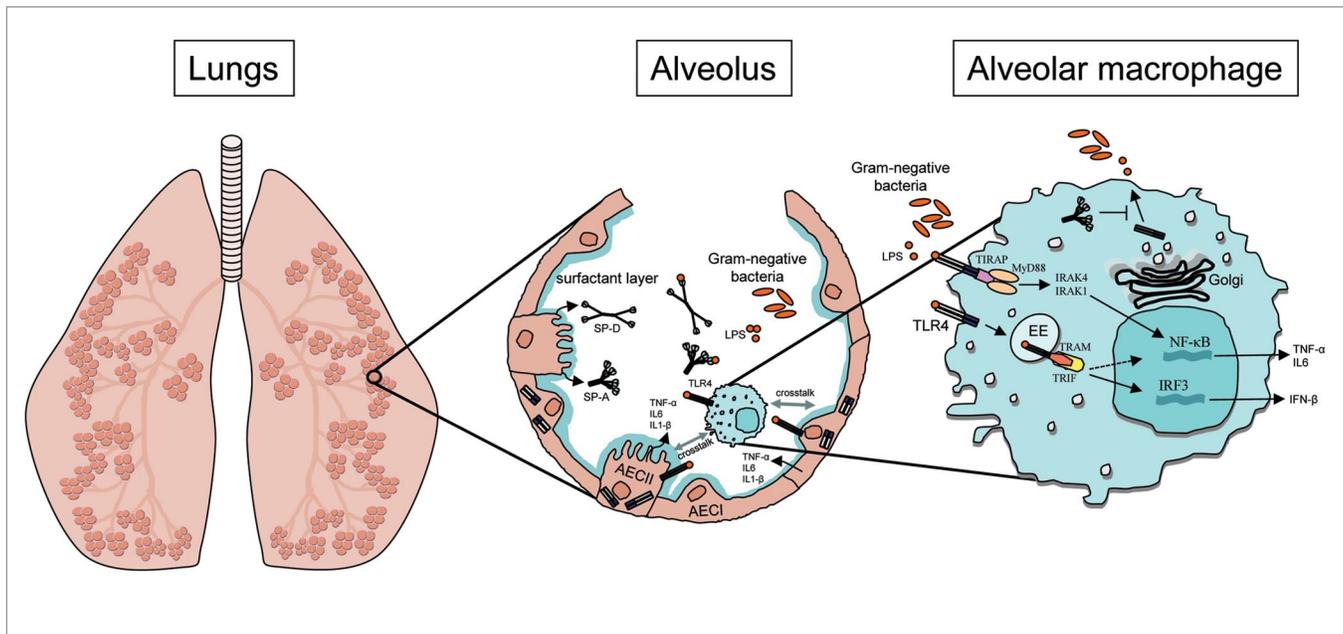
The identification of TLR4 as signaling receptor for LPS is based on studies showing that LPS-resistant C3H/HeJ and C57BL/10ScCr mice have a mutation in the *Tlr4* gene and was confirmed by data showing that cells isolated from TLR4-deficient knockout mice are hyporesponsive to LPS.<sup>8,9</sup> Subsequent in vivo studies in TLR4-deficient mice revealed impaired survival associated with higher bacterial loads, reduced activation of gene expression and diminished production of inflammatory mediators indicating that TLR4 signaling is required to induce a protective pulmonary immune response against common Gram-negative respiratory pathogens, including *Klebsiella pneumoniae*,<sup>10-12</sup> *Pseudomonas aeruginosa*,<sup>13</sup> and *Haemophilus influenzae*.<sup>14</sup> Of note, mice lacking the adaptor protein MyD88, TIRAP, or TRIF also showed strongly reduced clearance of bacteria from the lung due to impaired induction of early immune responses with a much more remarkable phenotype in MyD88-deficient mice infected with *K. pneumoniae*,<sup>15,16</sup> *P. aeruginosa*,<sup>17,18</sup> or *Haemophilus influenzae*<sup>19</sup> indicating that MyD88-dependent signaling is especially important for mounting the initial host response to Gram-negative infections. Additionally, TIRAP has been shown to be critically involved in early lung immune responses against *E. coli* LPS and viable *E. coli*.<sup>20</sup> By adoptive transfer of bone marrow cells from TLR4-deficient mice to wild type mice, it was demonstrated that TLR4 signaling in cells of hematopoietic origin, like lymphocytes and/or macrophages plays an important role in early host

defense against *K. pneumoniae*.<sup>21</sup> The combined studies clearly reveal the complexity and redundancy of the TLR4 signaling pathway in inducing initial pulmonary immune responses toward Gram-negative pathogens and suggest a primary role of hematopoietic cells.

#### Cell compartment-specific regulation of TLR4 signaling

First evidence for the mechanistic link between LPS recognition and intracellular transport of LPS from the plasma membrane to intracellular sites of accumulation as well as the dependence of this traffic on the LPS<sup>d</sup> gene was demonstrated by Wright and colleagues.<sup>22-24</sup> Mechanisms of how TLR4 signaling is integrated into the cellular infrastructure have been uncovered in cell line-based studies, murine bone marrow derived macrophages and dendritic cells, murine splenic B-cells and dendritic cells, murine embryonic fibroblasts, as well as human monocytes, neutrophils, and peripheral blood mononuclear cells. In the past decade it has become increasingly evident that subcellular localization of TLR4 and TLR4 adaptors is an important regulatory mode of TLR4 signaling.<sup>25,26</sup>

Under resting conditions, TLR4 cycles between the Golgi and at the plasma membrane and translocates to the cell surface upon LPS exposure.<sup>27</sup> Trafficking of TLR4 from the Golgi to the plasma membrane is regulated by the small TLR4 associated glycoprotein MD2.<sup>28</sup> At the plasma membrane, TLR4 and its co-receptor CD14 are recruited to phosphatidylinositol 4,5-bisphosphate (PIP2)-rich microdomains.<sup>29,30</sup> Within these PIP2-rich microdomains TLR4 oligomerizes and engages MyD88 that is recruited by the sorting adaptor TIRAP.<sup>31</sup> In addition to the MyD88-dependent pathway that leads to early NF- $\kappa$ B activation and production of pro-inflammatory cytokines, TLR4 subsequently activates a second pathway via interaction with TRAM, the sorting adaptor for TRIF. This second pathway is initiated upon receptor endocytosis and, from the endosomal compartment, triggers nuclear translocation of IRF3 and delayed activation of NF- $\kappa$ B and AP-1.<sup>32,33</sup> Both clathrin-mediated endocytosis as well as caveolae and lipid raft-mediated endocytosis of TLR4 are involved in TLR4 signaling<sup>34</sup> and are required for TRIF-dependent interferon expression.<sup>32</sup> From early endosomes, TLR4 is sorted and targeted to a trans-Golgi network pathway<sup>35</sup> and/or to a lysosomal-degradation pathway,<sup>34</sup> required for resolution of the inflammatory response (Fig. 1). Thus, LPS responsiveness is fine-tuned by the levels of TLR4 present on the cell surface membrane which is in turn determined by the amount of TLR4 trafficking from the Golgi to the plasma membrane and the amount of TLR4 internalized into endosomes.<sup>26</sup> Minimal perturbation of any of these steps causes an abnormal inflammatory response. Regulation of the fine-tuning of TLR4-induced signaling by endocytosis and the factors that restrict these processes are only starting to be elucidated. A recent study described an LPS-induced endocytic process that is CD14-dependent but TLR4 signaling independent and involves the tyrosine kinase Syk and its downstream effector PLC $\gamma$ 2.<sup>36</sup> In that study, cell type-specific responses to LPS are conferred by cell type-specific coexpression of TLR4 and CD14.<sup>36</sup> The p110 $\delta$  isoform of phosphatidylinositol-3-kinase has been ascribed to have an important regulatory



**Figure 1.** Simplified overview of TLR4 signaling in primary lung cells. Within the alveolus LPS is recognized by the TLR4 receptor complex that is constitutively expressed by primary alveolar macrophages and epithelial cells. Upon receptor binding TLR4 sequentially activates the MyD88-dependent pathway from the plasma membrane resulting in NF- $\kappa$ B activation and the TRAM/TRIF-dependent pathway from the endosomal compartment leading to activation of IRF3. Cell type-specific regulation of LPS-induced TLR4 signaling in the lung is modified by microenvironmental factors, including the pulmonary surfactant lipids and proteins that partly regulate signaling through modulation of subcellular TLR4 localization. AECI, type I alveolar epithelial cells; AECII, type II alveolar epithelial cells; IFN, interferon; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; IRF, interferon regulatory factor; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response gene 88; NF- $\kappa$ B, nuclear factor  $\kappa$ B; SP-A, surfactant protein A; SP-D, surfactant protein D; TIRAP, toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TLR4, Toll-like receptor 4; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TRAM, TRIF-related adaptor molecule; TRIF, toll/II-1R-domain-containing adaptor protein inducing interferon- $\beta$ .

function in TLR4 endocytosis by promoting the transfer of TLR4 to endosomes thereby controlling the balance between TIRAP-MyD88-dependent pro-inflammatory and TRIF-dependent anti-inflammatory cytokine expression.<sup>37</sup> Besides spatiotemporal localization of TLR4 itself, signaling is also dictated by subcellular localization of the sorting adaptors TIRAP and TRAM. An example for how adaptor localization can regulate TLR4 signaling was provided by recent data showing that TAG, a splice variant of TRAM, is directed to late endosomes, unlike TRAM that localizes to early endosomes, and thus, in association with a recently identified protein called TMED7, negatively regulates TLR4-induced IFN production.<sup>38,39</sup> These seminal cell biological studies have revolutionized the understanding of compartmentalized TLR4 signaling and will pave the way for increasing the knowledge on regulation of TLR4 signaling in primary immune cells and identifying cell- and context-specific factors involved.

#### Rab-regulated TLR4 trafficking

Only recently, Rab (Ras related in brain) GTPases, known as pivotal regulators of phagocytic, endocytic, and exocytic trafficking in eukaryotic cells<sup>40</sup> have been described to regulate receptor trafficking and thus signaling. Rab GTPases involved in regulating subcellular TLR4 localization are Rab7b, Rab10, and Rab11. Rab7b controls trafficking between endosomes and the TGN<sup>41</sup> and has been suggested to negatively regulate TLR4 signaling by promoting TLR4 lysosomal degradation in RAW264.7 cells.<sup>42</sup> Rab10 is essential for optimal macrophage activation

upon LPS stimulation by promoting continuous replenishment of TLR4 to the plasma membrane of RAW264.7 cells and, when overexpressed in macrophages, enhances LPS-induced acute lung injury in mice.<sup>43</sup> Rab11a is essential for the trafficking of TLR4 and TRAM to *Escherichia coli*-enriched phagosomes in human monocytes and thereby controls IRF3 activation from this compartment.<sup>44</sup> The regulation of Rab-mediated TLR4 trafficking in primary lung-specific cells is only beginning to be understood.

Emerging evidence indicates that lung-specific microenvironmental factors such as surfactant play a critical role in regulating subcellular membrane trafficking. In 2006 Ferguson et al. were the first demonstrating that pulmonary C-type lectins can modulate Rab-regulated intracellular membrane trafficking. In that study, coating of *Mycobacterium tuberculosis* with SP-D modified phagosome-lysosome fusion in human monocyte-derived macrophages.<sup>45</sup> Furthermore, both SP-A and SP-D significantly increase the number of *Legionella pneumophila* co-localized with lysosome-associated membrane protein-1 in THP-1 cells.<sup>46</sup> Using primary rat alveolar macrophages, we could show that SP-A specifically and transiently modulates endocytic/phagocytic membrane trafficking via regulation of Rab GTPases thereby functionally enhancing the lysosomal delivery of GFP-labeled *Escherichia coli* in these cells.<sup>47</sup> Together, these studies provide evidence for lung-specific mechanisms in modulating Rab-regulated receptor trafficking.

### **Constitutive and LPS-modulated TLR4 gene and protein expression in primary alveolar macrophages**

TLR4 signaling outcomes are partly generated through differences in TLR4 expression patterns by distinct cells. LPS-induced cytokine release by primary murine alveolar macrophages depends on TLR4, MyD88, and TRIF.<sup>48</sup> Constitutively expressed TLR4 mRNA and protein by primary murine and rat alveolar macrophages are significantly and transiently regulated by LPS treatment in vitro and in vivo intranasal, inhalative, or intratracheal challenge depending on LPS dose and exposure time.<sup>49-53</sup> Using chimeric mice separately expressing TLR4 on hematopoietic or structural lung cells, Hollingsworth et al. demonstrated a critical role of TLR4 expression on specifically alveolar macrophages for the biological response to inhaled LPS.<sup>54</sup> Since the expression of TLR4 on structural lung cells is essential for neutrophil recruitment after systemic LPS exposure, the authors suggested the existence of lung-specific mechanisms for inhaled but not systemic exposure to LPS.<sup>54</sup> Furthermore, the inflammatory trafficking of monocytes into the alveolar space is associated with a significantly increased expression of TLR4 and CD14 mRNA supporting the assumption that freshly recruited alveolar phagocytes substantially contribute to acute immune responses of the lung.<sup>55</sup> By comparing the constitutive and ligand-induced expression of TLR4 on human alveolar macrophages and autologous blood monocytes, it was demonstrated that the constitutive cell surface expression on alveolar macrophages is either significantly lower than on monocytes<sup>56</sup> or equally low on both cell types.<sup>57</sup> Comparably, the constitutive TLR4 mRNA expression is lower in alveolar macrophages than in autologous monocytes.<sup>57</sup> Taken together, the TLR4 expression profile of autologous human alveolar macrophages and monocytes is not identical and may thus provide specificity of immune responses to TLR4 ligation by LPS both in the lung and systemically. Exposure to LPS enhances TLR4 surface expression already after 10 min and TLR4 mRNA after 1 h on both cell types with a subsequent decrease of TLR4 mRNA in both cell types after 24 h.<sup>57</sup> Similarly, the low constitutive TLR4 cell surface expression on human alveolar macrophages is significantly increased after LPS treatment at the same concentration with staining of TLR4 being most distinct at the cell surface after 30 min and located more intracellularly after 3 h as shown by confocal microscopy.<sup>58</sup> The combined data demonstrate that constitutive TLR4 expression in freshly isolated primary human alveolar macrophages is low, but quickly and transiently upregulated at the gene and protein level by LPS in vitro. Inhalation of LPS by healthy humans decreases TLR4 mRNA expression in alveolar macrophages after 6 h,<sup>59</sup> whereas lung subsegmental instillation of LPS in healthy humans does not influence the cell surface expression of TLR4 or CD14 on alveolar macrophages recovered after the same time,<sup>60</sup> suggesting that LPS application procedures in humans differentially affect TLR4 abundance in alveolar macrophages.

### **Constitutive and LPS-modulated TLR4 gene and protein expression in human (AECI and AECII)**

Together with alveolar macrophages, alveolar epithelial cells are the first to encounter LPS. Recently, distinct roles of AECI

and AECII in immunomodulation begin to emerge and additionally point to positive or negative impacts of both alveolar macrophages and surfactant on the functional status of AECs. Primary rat AECI, which have been shown to express TLR4,<sup>61</sup> produce more pro-inflammatory cytokines upon LPS treatment than AECII and, equally important, AECI-released cytokines are significantly enhanced by co-cultured alveolar macrophages and decreased by co-incubation with surfactant.<sup>62</sup> Confirming and extending previous data,<sup>63,64</sup> Guillot et al. demonstrated the constitutive expression of TLR4 mRNA and protein in human alveolar (A549) and bronchial (BRAS-2B, CFT-2, and NT-1) epithelial cell lines as well as MD2 mRNA expression in A549 and BEAS-2B cells<sup>65</sup> and provided first evidence for an intracellular compartmentalization of TLR4 in lung epithelial cells. LPS treatment does not alter TLR4 mRNA or protein expression or localization in BEAS-2B cells though it induces a strong cytokine release.<sup>65</sup> The constitutive expression of TLR4 mRNA and protein by primary human AECII is significantly upregulated by LPS treatment accompanied by an enhanced cytokine release.<sup>66</sup> Likewise, LPS induces a marked increase in TLR4 protein expression in primary human AECII after 30 min which increases further after 3 h of exposure.<sup>58</sup> However, in contrast to the staining pattern of TLR4 in autologous primary alveolar macrophages, TLR4 staining in AECII is most prominent intracellularly. LPS-induced increases of TLR4 expression are functionally relevant as determined by enhanced TNF $\alpha$  release by both cell types. Thorley et al. suggested that this differential expression of TLR4 by alveolar epithelium and macrophages is important in coordinated responses to inhaled pathogens.<sup>58</sup> In addition, the LPS co-receptor CD14, that facilitates TLR4 responsiveness to very low levels (< 1 ng/ml) of LPS<sup>67</sup> and controls the LPS-induced endocytosis of TLR4,<sup>36</sup> is constitutively expressed on the cell surface of primary human AECII and immortalized AECI.<sup>58</sup>

### **Lung cell-specific microenvironmental regulation of TLR4 receptor and adaptor localization**

In general, the role of lung cell-specific microenvironmental factors in regulating expression and intracellular TLR4 localization in response to LPS is only beginning to be experimentally addressed. The so far best-studied microenvironmental factor modulating TLR4 signaling in the lung is pulmonary surfactant. Pulmonary SP-A modulates the alveolar macrophage threshold of LPS activity both in vitro and in vivo through various mechanisms. SP-A does not affect constitutive TLR4 surface expression on human monocyte-derived macrophages as determined by flow cytometry.<sup>68</sup> Likewise under basal conditions, SP-A has very little effect on TLR4 mRNA expression during monocyte differentiation into macrophages.<sup>68</sup> We observed that SP-A alone transiently decreases TLR4 staining at early time points (15 min and 30 min) of exposure and antagonizes LPS-enhanced TLR4 protein expression in primary rat alveolar macrophages.<sup>53</sup> The combined data suggest that SP-A, under resting conditions, only transiently affects TLR4 abundance, but can persistently decrease TLR4 expression levels in the presence of LPS. First evidence for specifically coordinated TLR4 positioning and thus functioning

through surfactant protein-mediated alterations in membrane trafficking was provided by a study showing that Survanta, a clinical surfactant extract that contains SP-C, inhibits LPS signaling in vitro by blocking translocation of TLR4 to lipid rafts in A549 cells.<sup>69</sup> Data from our group demonstrated that SP-A modulates the spatiotemporal compartmentalization of LPS-induced TLR4 in primary alveolar macrophages in vitro and in a mouse model of intratracheal LPS challenge. SP-A reduces the LPS-induced colocalization of TLR4 with early endosomes and promotes TLR4 localization with the Golgi thereby inhibiting LPS-induced TLR4 signaling.<sup>53</sup> Furthermore, the lack of direct SP-A/TLR4 co-localization, the SP-A-mediated upregulation of  $\beta$ -arrestin 2 protein expression and the SP-A-enhanced  $\beta$ -arrestin 2/TLR4 interaction suggest that SP-A-modulated cellular distribution of TLR4 in primary alveolar macrophages is mediated indirectly by integrating  $\beta$ -arrestin 2 scaffolding interactions. However, the underlying mechanisms are unknown but one could speculate that SP-A either decelerates the LPS-induced transport of TLR4 from the Golgi to the plasma membrane or accelerates the retrograde transport of TLR4 from endosomes to the Golgi.<sup>53</sup> The combined studies provide first evidence for a critical role of surfactant lipids and proteins in regulating TLR4 subcellular localization thereby fine tuning the quantity of LPS-induced immune responses of the lung. Among numerous negative TLR4 regulators is IL-1R-associated kinase-M (IRAK-M) that is highly expressed in resting human alveolar macrophages.<sup>70</sup> IRAK-M inhibits TLR-induced NF- $\kappa$ B activity by binding to MyD88 and TRAF<sup>71</sup> and can suppress sepsis-induced lung innate immunity.<sup>72</sup> Both SP-A and surfactant lipids (Survanta) upregulate IRAK-M expression in human alveolar macrophages over 24 h leading to reduced LPS-stimulated cytokine release.<sup>73</sup> The host defense peptide cathelicidin LL37 inhibits TLR4 responses in myeloid cells<sup>74</sup> but enhances the LPS-induced activation of primary human bronchial epithelial cells by facilitating the delivery of LPS to TLR4-containing intracellular compartments.<sup>75</sup> The combined data provide evidence for lung cell type-specific effects of LL37 on TLR4 activation. Likewise, the host defense peptide CLP-19 inhibits LPS-induced microtubule-dependent translocation of TLR4 from the endoplasmic reticulum to the cell surface in the mouse macrophage cell line RAW264.7 functionally inhibiting TNF release and phosphorylation of I $\kappa$ B- $\alpha$ .<sup>76</sup> The tetraspanin CD9 prevents LPS-induced TLR4 localization at lipid-enriched membrane microdomains in RAW264.7 cells and pro-inflammatory cytokine release by alveolar macrophages after in vivo LPS challenge thereby preventing LPS-induced lung inflammation.<sup>77</sup> In vivo oxidative stress induces the formation of a surface TLR4 receptor complex within lipid rafts of rat alveolar macrophages resulting in an augmented responsiveness to LPS.<sup>78</sup> This was the first study showing that oxidative stress alters subcellular TLR4 distribution in vivo. Actin depolymerization is required for multiple steps of endocytic receptor trafficking.<sup>79</sup> The actin depolymerization protein glia maturation factor- $\gamma$  (GMFG) is highly expressed in endothelial cells derived from human lung.<sup>80</sup> Recent data demonstrated that GMFG functions as a negative regulator of LPS-induced TLR4 signaling

by promoting TLR4 trafficking from early endosomes to late endosomes in primary human macrophages without affecting TLR4 expression levels.<sup>81</sup> Activated adenylyl cyclases mediate the conversion of ATP to cAMP whose compartmentalized signaling regulates central cellular processes in the lung.<sup>82</sup> The AC6 isoform, expressed in lung structural and immune cells,<sup>82</sup> promotes a shift of TLR4 endocytosis via the clathrin-dependent pathway toward the lipid-raft-mediated pathway resulting in an accelerated degradation of TLR4 with subsequently suppressed downstream signaling in RAW264.7 cells and bone-marrow-derived macrophages.<sup>83</sup> The combined studies provide evidence for a critical role of lung-specific microenvironmental factors in TLR4 trafficking and thus signaling.

#### **Dysregulation of TLR4 spatiotemporal localization associated with Gram-negative human lung diseases**

Two frequently occurring<sup>84</sup> single nucleotide polymorphisms (SNPs) in the human TLR4 ecto-domain, the missense mutations Asp299Gly (D299G) and Thr399Ile (T399I), are associated with LPS hyporesponsiveness and enhanced susceptibility to infection with Gram-negative bacteria.<sup>85</sup> Another study on human individual genetic variations revealed a combination of D299G and T399I within the TLR4 and Ser180Leu within the TIRAP/MAL gene is associated with a significantly higher risk for developing sepsis and pneumonia.<sup>86</sup> Recent data provide structural and biological evidence of the functionality of the mutant TLR4 carrying T399I and D299G, respectively.<sup>87,88</sup> First evidence for a mechanism by which a TLR4 polymorphism alters TLR4 signaling was provided by Figueroa et al. demonstrating that the D299G polymorphism impairs the recruitment of MyD88 and TRIF to TLR4 and subsequent pathway-specific signaling.<sup>88</sup>

Studies on TLR4 subcellular localization and signaling employing primary alveolar macrophages from patients suffering from lung diseases induced by or associated with Gram-negative infection are limited. One disease, for which first data relevant to this subject are provided, is cystic fibrosis (CF). Uncontrolled inflammatory response to infection with *Pseudomonas aeruginosa* is associated with morbidity and mortality in patients with CF.<sup>89</sup> Abnormal trafficking and degradation of TLR4 in macrophages results in elevated inflammatory responses in both murine CF models and patients suffering from CF.<sup>90</sup> Untreated CF transmembrane conductance regulator CFTR<sup>-/-</sup> bone-marrow derived macrophages display significantly higher levels of plasma membrane-associated TLR4 than wild-type macrophages.<sup>90</sup> Upon LPS stimulation, the functionally hyperresponsive phenotype of CFTR<sup>-/-</sup> macrophages is associated with a prolonged retention of TLR4 in early endosomes.<sup>90</sup> Similarly, differentiated human monocytes from CF patients are hyperresponsive to LPS.<sup>90</sup> Kelly et al. examined the intracellular trafficking of TLR4 in primary nasal epithelial cells from non-CF controls and CF patients after LPS challenge. They found a sustained gene expression of Rab10 in CF epithelium and an abolished TLR4 targeting to the lysosome in CF airway epithelial cells.<sup>91</sup> The combined data imply that in pulmonary and immune cells from CF patients TLR4 spends

a prolonged time in the early endosome resulting in enhanced signaling.

Because pneumonia induced by Gram-negative bacteria frequently develops postoperatively,<sup>92</sup> Chalk et al. recently started a pilot study to characterize lung-cell specific alterations of the immune profile after cardiac surgery.<sup>93</sup> Patients who postoperatively developed pneumonia revealed a stronger reduction of TLR4 expression on alveolar macrophages than patients who did not<sup>93</sup> suggesting that a local cell-mediated immunosuppression in the lung might be a risk factor for postoperative pneumonia. The expression of TLR4 on alveolar macrophages from patients with ARDS is suppressed and, compared with control cells, does not change after *ex vivo* stimulation of the cells with LPS.<sup>94</sup> Comparably, though employing blood monocytes, decreased TLR4 expression on these cells is associated with mortality in elderly patients with severe pneumonia.<sup>95</sup> Together, these studies suggest that TLR4 expression profiles on hematopoietic cells are linked to pulmonary diseases induced by or associated with Gram-negative bacteria.

## Conclusions

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Emerging evidence demonstrates the functional significance of subcellular TLR4 receptor and adaptor localization in regulating cellular responses to LPS. It is, however, unknown whether cell type-specific differences in TLR4 compartmentalization are associated with individual responses to TLR4 ligation by LPS and thus LPS signaling specific for the lung is only beginning to be experimentally addressed. Pulmonary surfactant-modulated trafficking of the TLR4 receptor in primary alveolar macrophages provides first evidence for lung-specific regulation of TLR4 signaling. Future studies are likely to focus increasingly on whether defined surfactant compositions can switch subcellular TLR4 localization in response to LPS. Further investigations on lung cell type-specific TLR4 responses and the impact of unique pulmonary microenvironmental factors on intracellular TLR4 positioning and thus ligand sensing will help to potentially improve the qualitative and quantitative outcome of innate immune responses of the lung to Gram-negative bacteria.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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