The *in vivo* and *in vitro* Roles of Epithelial Pattern Recognition Receptors in Pneumococcal Infections

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*Streptococcus pneumoniae*, also called pneumococcus, is a major cause of infectious disease in human. Pneumococcus resides in the nasopharynx as an upper respiratory commensal, and most of pneumococcal colonizations are asymptomatic in immunocompetent individuals. When nasopharyngeal mucosal homeostasis is disrupted, pneumococcus migrates into middle ear and lower respiratory tract and causes detrimental colonization. In this regard, the epithelial cells of middle ear and lung act as first line of defense against pneumococcus to prevent invasive pneumococcal diseases. Respiratory epithelial cells express various cell-surface and intra-cellular receptors sensing microbial pathogens and respond to sensed pathogens by triggering intra-cellular signaling pathways and inducing pathogen-specific innate immune responses. Various epithelial cell-surface and intra-cellular receptors, such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), intracellular DNA sensing receptors, and scavenger receptors (SRs), participate in sensing of pneumococcus, and the activation of these receptors by pneumococcal components induces anti-pneumococcal innate immune responses including epithelial apoptosis and inflammatory cytokine/chemokine expressions. Epithelial sensing of pneumococcus is a critical step for setting an early defense against pneumococcal infection, and also is required to recruit and activate innate immune cells and trigger adaptive immunity.

**Key Words:** *Streptococcus pneumoniae*, Pneumococcus, Epithelial cell, Pattern recognition receptor, Inflammation

**INTRODUCTION**

*Streptococcus pneumoniae*, also called pneumococcus, is a gram-positive, facultative anaerobic bacterium, which was first isolated by Louis Pasteur in 1881 (1). Since the first identification of pneumococcus, pneumococcal pathogenicity has been extensively studied over the past 100 years, resulting in developing somewhat effective therapeutics and vaccines. Despite a significant progress in understanding the microbiological and immunological characteristics of pneumococcal infections, it causes about 1.6 million deaths each year, including 0.7 to 1 million deaths in young children under the age 5 (2). Quickly growing resistance to effective antibiotics and limited efficacy of currently available vaccines may in part account for such a high mortality.

Some pneumococci are surrounded with complex polysaccharide capsule, and over 90 different serotypes have been identified based on the antigenic differences in their capsular polysaccharides (3, 4). Further studies found that capsular polysaccharide acts as a key pathogenic factor during invasive pneumococcal diseases (IPDs), but only a
limited number of serotypes contribute to develop IPDs (5, 6). Although there are geographical variations, the 10 most common serotypes are responsible for most IPDs worldwide, and it makes possible to develop pneumococcal conjugate vaccine (PCV), which is effective at preventing IPDs in children (7–10). The use of PCV-7, which contains 7 the most prevalent IPD serotypes in young children, was effective in reducing overall prevalence of and mortality due to IPDs. This effect, however, was temporary and prevalence of overall pneumococcal disease has not been decreased over time mainly due to increased pneumococcal infections with non-vaccine serotypes (11–13). Although PCV-7 has been upgraded by adding 6 newly emerging serotypes, namely PCV-13, it still contains only 13 out of over 90 serotypes (14, 15). Anyone can expect new serotypes to emerge as pathogenic strains in near future. Therefore, extensive researches are ongoing in developing universally effective vaccines for all the serotypes and also even effective for un-encapsulated, nontypeable pneumococcus (16–18). Even if an effective vaccine is available, there is still a big concern that vaccination will not be effective in immunocompromised patients, who are highly susceptible to IPDs and who grow dramatically in number along with advances in medical science (19–21). There is also a concern that underlying mechanism responsible for an emerging of serotype switching has not been fully understood yet.

In the meantime, although many people carry pneumococcus in their nasopharynx, most of the people carry pneumococcus as part of their normal flora and are generally asymptomatic (22, 23). However, some people develop detrimental pneumococcal colonization. Although many risk factors causing detrimental colonization of pneumococcus have been reported including pneumococcal pathogenic factors and host factors down-regulating anti-pneumococcal defense responses, the underlying molecular mechanisms by which pneumococcus develops detrimental, life-threatening colonization has also not been fully understood (24–27). Taken together, it is required to develop novel therapeutic strategies against pneumococcal infections based on the understanding of immunophysiology of pneumococcal infections.

**Epithelial sensors of pneumococcus**

Microbial pathogens, which migrate to the lung, first come in contact with alveolar epithelium. In this regard, alveolar epithelial cells play critical roles for an early defense against respiratory microbial pathogens by sensing pathogens and providing pathogen-specific anti-microbial defense (28–30). Since the first discovery of Toll-like receptor 4 (TLR4) as a receptor for lipopolysaccharides (LPS), a diverse family of pattern recognition receptors (PRRs) for pathogen associated molecular patterns (PAMPs) have been found, which includes membrane associated TLRs, cytoplasmic NOD-like receptors (NLRs) and RIG-I like receptors (RLRs), and scavenger receptors (SRs) (31–33). Pneumococcus is also sensed by alveolar epithelial cells via multiple PRRs, and sensing of pneumococcus by these cells is a critical step to combat against pneumococcal infections. There is a growing body of research data suggesting specific PRRs as epithelial sensors for pneumococcus (34, 35).

Type 1 transmembrane protein TLRs sense a diversity of microbial products, PAMPs. To date, 10 human Toll-like receptors (TLR1 to 10) and 12 mouse TLRs (Tlr1 to 9, and Tlr11 to 13) have been identified (31, 33). Human respiratory tract epithelium expresses all 10 known human TLRs (TLR1-10) on the apical surface (TLR1, 2, 4, 5, 6, 9), on the basolateral surface (TLR4, 5), and also on the endosomal membrane (TLR3, 4, 7, 8) (36–39). It is interesting to know that expression of TLR5 has been known to be exclusively limited to the basolateral surface of epithelial cells in gut, but alveolar epithelial TLR5 seems to be also expressed on the apical surface following stimulation of cells with its ligand flagellin (40, 41). Moreover, expression of TLR9 is limited to the cytoplasm in immune cells, but apical TLR9 expression was also found in alveolar epithelial cells (37, 42, 43). Such distinctive findings on the experssional pattern of TLRs in alveolar epithelial cells suggest a possible existence of alveolar epithelial specific regulation and role of PRRs.

Multiple pneumococcal components have been found as
ligands for TLRs, including peptidoglycan (PGN), lipoteichoic acid (LTA), pneumococcal capsular polysaccharides (PCP), RgaA oligomer of type 1 pilus, pneumolysin (PLY), ClpP, and unmethylated CpG DNA (44–50). Among many alveolar epithelial TLRs, TLR2 seems to be a main receptor for pneumococcal PAMPs, but the in vivo function of TLR2 in regulating pneumococcal infections is still under debate. Pneumococcal cell wall, which contains LTA and PGN, is recognized by TLR2 with help from co-receptors and adaptors, TLR1, LPS binding protein (LBP), CD14, and CD36 (45–47, 49, 50). Treatment of cell wall active antibiotics, such as β-lactam antibiotics, thus was known to increase pneumococcal cell wall-mediated activation of TLR2 signaling pathways, probably by releasing active components of PGN and LTA (48, 51). There is no doubt in the concept that TLR2 senses pneumococcal LTA. The idea that TLR2 is a receptor for PGN, however, has been challenged by the study of Travassos et al., which showed that the activation of TLR2 by PGN is due to contaminated gram-negative lipoprotein or gram-positive LTA (52). Further studies by multiple research groups, however, showed clear role of TLR2 for sensing PGN. TLR2 also has been found to serve as a receptor for pneumococcal capsular polysaccharide and RgaA oligomer of pneumococcal type 1 pilus (45, 49).

Following finding of TLR2 as a major receptor for pneumococcal PAMPs, various degrees of animal studies have been conducted in TLR2 knock out (KO) mice to seek the in vivo roles of TLR2 in pneumococcal infections. In mice models of pneumococcal pneumonia, TLR2 KO mice showed modest or no defect on the responses to pneumococcal infections, which results in no differences on pneumococcal clearance or survival rate between TLR2 KO and wild-type (WT) mice following pulmonary pneumococcal infections (53–55). However, in mice models of meningitis TLR2 KO mice showed severe defects in controlling pneumococcal growth, which causes development of more severe forms of meningitis and enhancement in lethality (56–59). Such an indispensable role of TLR2 in defending against pneumococcal infections was also found in mice models of otitis media. TLR2 KO mice showed enhanced tissue damage, uncontrolled pneumococcal outgrowth, enhanced dissemination of pneumococcus to the circulation, and higher lethality during pneumococcal otitis media (60, 61).

Human studies on the role of TLR2 in pneumococcal infections also showed such controversial results. The impact of three most common TLR2 polymorphisms (R557H, P631H, and R753Q) on pneumococcal infections was studied to seek the in vivo role of TLR2. Study by Telleria-Orriols JJ et al. found a significant association of TLR2-R753Q polymorphism with pneumococcal meningitis, but studies by other groups found no association of these 3 polymorphisms of TLR2 with pneumococcal diseases (62–66). However, Berenson CS et al. showed that chronic obstructive pulmonary disease (COPD) patients, who are commonly prone to pneumococcal infections, showed reduced TLR2 expression (67). Studies by Fallah MP et al. and Boyd AR et al. also suggest that enhanced pneumococcal pathogenesis by aging is due to defect in TLR2 signaling, which results in increased pneumococcal growth and mortality (68, 69). Such controversial findings from the in vivo studies on the role of TLR2 in pneumococcal infections may be explained in part by coordinated sensing of pneumococcal PAMPs by multiple PRRs and by complex activation of multiple cellular pathways by TLR2.

Although some of animal and human studies did not find exclusive roles of TLR2 in regulating pneumococcal growth and mortality, these studies still observed defects in early inflammatory responses in TLR2 KO mice (70). Sensing of pneumococcal components by TLR2 activates multiple intracellular signalings, resulting in expression of pro- and anti-inflammatory mediators, activation of cellular apoptosis, and modulation of epithelial barrier function (71–73). Although deficiency of TLR2 seems to be compensable in not severe forms of pneumococcal infections, we still observe the requirement of TLR2 for controlling severe pneumococcal infections, such as meningitis (56–59). Thus, it is suggested that TLR2 acts as a receptor for pneumococcal PAMPs, and sensing of pneumococcus by TLR2 is more likely to control severe forms of pneumococcal infections not only by regulating epithelial immune/
inflammatory responses but also by preventing pneumococcal dissemination into circulation.

Since the observation of reduced survival in pneumococcus-infected TLR4-defective C3H/HeJ mice (74), the roles of TLR4 in sensing pneumococcal PAMPs and regulating pneumococcal infections have been studied. As results, PLY, one of the most well characterized pneumococcal pathogenic factors, and pneumococcal HSP100/ClpP were found to interact with TLR4 (46, 47). Alveolar epithelial cells were found to respond to PLY in a TLR4 dependent manner (47, 75–77). It is, however, suggested that the expressions of TLR4 adaptor molecules, such as MD2 and CD14, are not enough to sense TLR4 ligands in the alveolar epithelial cells. Alveolar epithelial cells thus show unresponsiveness to LPS when serum is not supplied (78). Based on these observations, it has been suggested that alveolar epithelial cells may activate TLR4 signaling by sensing endogenous TLR4 ligand, which is induced by or released from host cells following pneumococcal infections, rather than directly sense PLY. Our study in alveolar epithelial A549 cells also found that PLY-induced TLR4 signaling activations are abrogated by addition of cholesterol or serum or by depleting cholesterol from plasma membrane (data not shown). These data suggest that plasma membrane cholesterol, which was originally found as a membrane-binding partner for PLY, plays critical role in activating TLR4 signaling pathways. Results of human studies on the role of TLR4 in pneumococcal infections are more skeptical. Human studies found that the two most common TLR4 polymorphisms (D299G and T399I) showed no contribution of these polymorphisms to the prevalence of IPDs (62–65), and even higher frequency of TLR4 polymorphisms were found in healthy controls compared to IPD groups (66). Thus, it is not conclusive if PLY directly interacts with and activates TLR4 in alveolar epithelial cells and if TLR4 is required for the protection against pneumococcal infections.

Pneumococcal regulatory function of TLR9 also has been investigated, and epithelial sensing of pneumococcus by TLR9 has been found (44, 79–81). These studies found that TLR9 responds to pneumococcal infections by sensing unmethylated CpG DNA of pneumococcus, and TLR9 KO mice fail to control pneumococcal pneumonia (44, 79). In addition, study by Ripoll VM et al. also showed that susceptibility to pneumococcal infections is coincide with the expression level of TLR9 in mice as pneumococcal susceptible strain CBA/Ca expresses lower level of TLR9 compared to that of resistant strain BALB/c (80). These findings suggest a possible critical implication of TLR9 in regulating pneumococcal infections in vivo in human patients. However, by considering relatively limited information on the immunological function of TLR9 compared to that of TLR2 and TLR4, it might not be the right point to make conclusion on the role of TLR9 in regulating pneumococcal infections.

Pneumococci are known to invade cells via Platelet Activating Factor (PAF)-receptor and polymeric IgR (plgR) of alveolar epithelial cells. As many intracellular microbes actively interact with cytoplasmic receptors, pneumococci inside epithelial cells are also sensed by cytoplasmic nucleotide-binding oligomerization domain protein 2 (NOD2) via PGN of pneumococcal cell wall (52, 81–84). Deficiency of NOD2 in mice results in reduced pneumococcal clearance along with enhanced sepsis and sepsis-associated neuronal damage (SAND) (82, 85), which suggests that NOD2 also participates in regulating pathophysiology of pneumococcal infections by sensing of pneumococcal PAMP. It is interesting to know that NOD1, a meso-DAP receptor, is critically involved in regulating pathogenesis of pneumococcal infections. NOD1 KO mice are highly susceptible to early pneumococcal sepsis, while activation of NOD1 by its agonist KF565 inhibits pneumococcal disruption of epithelial barrier (86, 87). Since NOD1 is not thought as a direct sensor for pneumococcus, these findings also support the idea that TLR4 responds to pneumococcus indirectly by detecting host-driven TLR4 ligands.

Cytoplasmic pneumococcal DNA was also found to be sensed by DNA-dependent activator of IFN-regulatory factors (DAI) and stimulator of IFN genes (STING), which are cytoplasmic nucleic acid sensing receptors, in alveolar epithelial cells (88). Since the activation of these cytoplasmic DNA receptors by pneumococcus upregulates interferon-
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regulatory factor 3 (IRF3)-dependent type 1 IFN expressions, these findings somehow can be used for explaining the reason why pre-disposition of pneumococcus is protective for infections with influenza A virus or other respiratory viral infections.

Alveolar epithelial scavenger receptors for pneumococcus were also found, including scavenger receptor A1 (SR-A1), mannose receptor, and macrophage receptor with collagenous structure (MARCO, also known as SR-A2), and CD36 (73, 89, 90). These non-opsonic scavenger receptors are expressed in lung and known to detect respiratory pneumococcus (73). Studies in KO mice found significant defects in pneumococcal clearance in MARCO KO and CD36 KO mice following pneumococcal infections, but no

Figure 1. Schematic diagram of epithelial sensors for pneumococcus. Abbrs: AP-1 activator protein 1; DAI, DNA-dependent activation of IRF; IRF, Interferon regulatory factor; LTA, lipoteichoic acid; NFκB, nuclear factor kappaB; NOD, Nucleotide-binding oligomerization domain 2; PGN, peptidoglycan; plgR, polymeric immunoglobulin receptor; PLY, pneumolysin; TLR, Toll-like receptor.
significant impact of loss of SR-A1 or mannose receptor on regulating pneumococcal infections has been found (73, 89). In addition, polymorphism of mannose binding lectin (MBL) in human was found to be associated with higher colonization of pneumococcus in the nasopharynx (65). Since scavenger receptors were originally found as opsonic receptors of immune cells, their non-opsonic functions in alveolar epithelial cells, especially as epithelial sensors for pneumococcus, have not been fully investigated yet. Further investigations on these topics are required.

As shown in Fig. 1, among many known microbial PRRs, three TLRs, TLR2, TR4, and TLR9, cytoplasmic receptors NOD2, DAI, and STING, and cell surface scavenger receptors SR-A1, MARCO, mannose receptor, and CD36 have been found to recognize pneumococcal PAMPs in alveolar epithelial cells. TLR2 is likely to be a major sensing receptor for pneumococcus in these cells. Although it is clear that sensing of pneumococcal PAMPs by these PRRs activates specific receptor-dependent intracellular signaling pathways in vitro, the roles of sensing pneumococcal PAMPs by these receptors seem not to be simply conclusive in vivo as live pathogens, such as pneumococcus, not only contain PAMPs for multiple PRRs but also contains many other pathogenic factors which are not exclusively recognized by PRRs but play critical roles for the pathogenesis of pneumococcal infections (34).

**Epithelial anti-pneumococcal responses**

Sensing of microbial pathogens by PRRs triggers down-
stream signaling pathways of these receptors, which are engaged in a diversity of immunophysiological cellular responses. Intracellular signaling pathways of PRRs are largely classified into two groups based on their primary intracellular adaptors, which form cytoplasmic signaling complex, a MyD88-denepdent pathway and a MyD88-independent, TRIF-dependent pathway. These two signaling pathways activate a various signaling molecules, but finally they are converged into three transcription factor families, nuclear factor-κB (NF-κB), activating protein-1 (AP-1), and IRF3 (32, 33, 91). Pneumococcal sensing by these PRRs and subsequent activation of these signaling pathways regulate various epithelial innate immune responses (Fig. 2): 1) expression of pro- and anti-inflammatory cytokines and chemokines, such as IL-1β, TNF-α, IL-6, IL-8, IL-10, TGF-β, type 1 IFNs, and so on (71, 79, 81, 83, 87, 90, 92), 2) expression of epithelial anti-microbial peptides, such as defensins and plasminogen activator inhibitor-1 (PAI-1) (77, 93, 94), 3) expression of mucin (75, 95), 4) regulation of epithelial barrier function (71, 77, 87), 5) expression of bacterial and viral PRRs, such as TLR2, TLR7, NOD1, and NOD2 (39, 50, 59, 76, 84, 96), 6) expression of negative regulator of innate immune signaling pathways, such as MAPK phosphatase-1 (MKP-1) and cylindromatosis (CYLD) (75, 77, 92), and 7) regulation of cellular apoptosis (46, 72, 97). Since pneumococcus regulates such a diversity of innate immune responses, we are likely to observe the outcomes of complex interactions between these signals when the roles of PRRs are studied in vivo. Therefore, it is also not surprising even if we do not observe a significant impact of deficiency in a single PRR on regulating pneumococcal infections in vivo, and their immunological roles should not be underestimated.

CONCLUSION

Despite extensive studies on pneumococcus, we still did not find the right way to deal with this old friend of man, and it is still a major cause of death worldwide. Since the first discovery of TLR4 as a receptor for microbial endotoxin LPS (98), the tremendous progress has been made in understanding the molecular mechanisms by which microbial pathogens are sensed by host cells and intracellular signaling pathway are activated by these pathogens. The roles of PRRs, including TLRs, NLRs, and scavenger receptors, in pneumococcal infections also have been extensively studied, but the in vivo roles of these receptors are not conclusive yet.

Because pneumococcus resides in the nasopharynx of healthy individuals, respiratory epithelium is the primary entry site of the pneumococcus. However, its role in regulating anti-pneumococcal defense has long been underestimated compared to the extensive studies in immune cells. As we discussed above, alveolar epithelial cells showed distinctive expression regulation of PRRs from immune cells and also from intestinal epithelial cells, which suggests a possible existence of alveolar epithelial specific regulation and role of PRRs.

Future studies in these aspects may open new insight in understanding the roles of PRRs, and also bring novel therapeutic strategies, which will be effective in both immunocompetent and immunocompromised individuals.

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