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REVIEW

Role of Lipopolysaccharides in the Inflammation and Pyroptosis of Alveolar Epithelial Cells in Acute Lung Injury and Acute Respiratory Distress Syndrome

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Abstract: Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) represent a spectrum of common critical respiratory conditions characterized by damage and death of alveolar epithelial cells (AECs). Pyroptosis is a form of programmed cell death with inflammatory characteristics, and activation of pyroptosis markers has been observed in AECs of patients with ALI/ARDS. Lipopolysaccharides (LPS) possess strong pro-inflammatory effects and are a crucial pathological factor leading to ALI in patients and animals. In LPS-induced ALI models, AECs undergo pyroptosis. However, physiologically and pathologically relevant concentrations of LPS lead to minor effects on AEC cell viability and minimal induction of cytokine release in vitro and do not induce classical pyroptosis. Nevertheless, LPS can enter the cytoplasm directly and induce non-classical pyroptosis in AECs when assisted by extracellular vesicles from bacteria, HMGB1, and pathogens. In this review, we have explored the effects of LPS on AECs concerning inflammation, cell viability, and pyroptosis, analyzing key factors that influence LPS actions. Notably, we highlight the intricate response of AECs to LPS within the framework of ALI and ARDS, emphasizing the variable induction of pyroptosis. Despite the minimal effects of LPS on AEC viability and cytokine release in vitro, LPS can induce non-classical pyroptosis under specific conditions, presenting potential pathways for therapeutic intervention. Collectively, understanding these mechanisms is crucial for the development of targeted treatments that mitigate the inflammatory responses in ALI/ARDS, thereby enhancing patient outcomes in these severe respiratory conditions.

Keywords: alveolar cell death, inflammatory response, programmed cell death, pathological impact, cytokine activation

Introduction

Acute lung injury (ALI) is characterized by an uncontrolled inflammatory response that leads to acute noncardiogenic pulmonary edema and hypoxemia.[1] With further deterioration, ALI can progress to acute respiratory distress syndrome (ARDS), which is clinically characterized by progressive respiratory distress and refractory hypoxemia.[2] Currently, the primary drugs used in clinical ARDS treatment include steroids, monoclonal antibodies, and small-molecule inhibitors.[2] However, these drugs have significant side effects, high treatment costs, and cannot reverse the condition in all patients with ARDS, with mortality rates persisting at 27.5–53%.[3,4]

Damage and death of alveolar epithelial cells (AECs) are typical features of ALI, and the severity of lung epithelial damage serves as a crucial prognostic factor in patients with ARDS.[5–8] Various factors, including bacteria, viruses, acids, elevated oxygen levels, mechanical ventilation, and cytokines, can cause AEC injury.[1] In a healthy alveolar–capillary barrier, the pulmonary epithelial structure comprises a tight layer of type I and scattered type II AECs. Disruption of this barrier through type I AEC damage permits extensive fluid entry into alveolar spaces, resulting in pulmonary edema. After injury, reduced

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surfactant protein secretion by type II AECs leads to alveolar collapse.[9] AECs release pro-inflammatory cytokines (such as tumor necrosis factor alpha [TNF-α] and interleukin [IL]-1β) and damage-associated molecular patterns (such as nucleic acids and HMGB1), exacerbating pulmonary inflammation and leading to a detrimental cycle in the microenvironment.[10,11]

Lipopolysaccharides (LPS) are found in the cell walls of gram-negative bacteria and comprise three parts: O-specific side chains, core polysaccharides, and lipid A, with lipid A serving as the main toxic component. During gram-negative bacterial infections, the release of large amounts of LPS by these bacteria can lead to severe ALI and sepsis, positioning LPS as a pivotal pathological factor in sepsis and ALI/ARDS.[12] The LPS model is widely utilized in animal studies of ALI/ARDS, reflecting pathological characteristics similar to those of human conditions.[13] Both intraperitoneal and direct intratracheal LPS administrations induce ALI, albeit with distinct characteristics. Intratracheal administration substantially damages the alveolar epithelial structure, leading to significant death of type I and II AECs and subsequent hyaline membrane formation, whereas the vascular endothelial structure remains relatively intact.[14] In contrast, intraperitoneal injection mimics sepsis-related ALI, where LPS stimulates an influx of inflammatory factors into the circulation, resulting in indirect lung injury characterized by vascular endothelium damage, pulmonary interstitial edema, and a relatively preserved pulmonary epithelial structure.[14] Therefore, LPS is an important factor for inducing ALI in both clinical and experimental settings.

In this review, we explored the impact of LPS on AECs, focusing on inflammation, cellular activity, and pyroptosis, by reviewing prior studies. We have also summarized potential explanations for inconsistent findings regarding LPS effects on AECs. Collectively, our review highlights the necessity for further investigation into the immune role of AECs, which are among the first cells to encounter foreign stimuli.

Mechanism of LPS Signal Transduction

Overview of LPS Signal Transduction

Outside the cell membrane, the LPS-binding protein (LBP) binds to the lipid A portion of LPS, forming an LPS–LBP complex. LBP catalyzes the transfer of LPS via electrostatic interactions to cluster of differentiation 14 (CD14). Subsequently, CD14 transfers LPS to the toll-like receptor 4 (TLR4)/myeloid differentiation protein 2 (MD2) complex,[15] leading to dimerization of the extracellular domain of the TLR4/MD-2 complex (Figure 1).

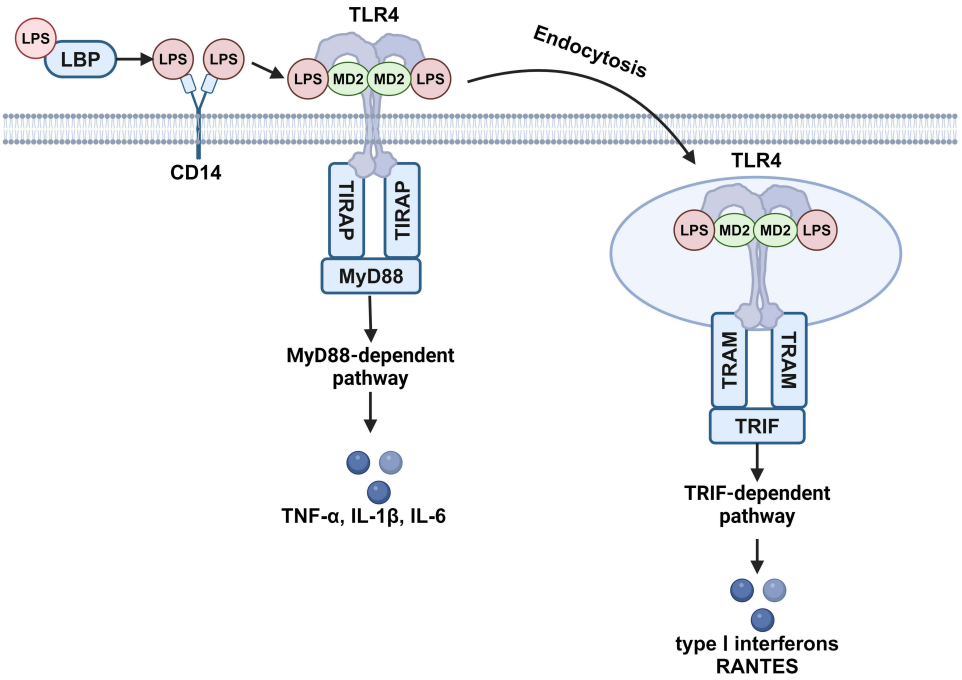

Figure 1 Schematic Diagram Depicting LPS/TLR4 Signaling. LBP binds to LPS and transfers it to CD14, which then transfers LPS to the TLR4/MD-2 complex. TLR4 activates the MyD88-dependent pathway, promoting the expression of TNF-α, IL-1β, and IL-6. Subsequently, the TLR4/MD-2/LPS complex undergoes endocytosis and activates the TRIF-dependent pathway, promoting the expression of type I interferons and RANTES. (Created with BioRender.com).

TLR4 belongs to a family of pattern recognition receptors responsible for identifying conserved pathogen-associated molecular patterns and initiating immune-inflammatory responses, with TLR4 serving as the primary receptor for LPS in mammals. TLR4 can bind to several adaptor proteins containing toll/interleukin-1 receptor domains (TIR domains), including myeloid differentiation primary response 88 (MyD88) and toll-like receptor adaptor molecule 1 (TRIF).[16] Consequently, downstream signaling from TLR4 is mainly divided into MyD88-dependent and TRIF-dependent pathways. Upon binding with MyD88, TLR4 rapidly activates downstream mitogen-activated protein kinase families and nuclear factor kappa beta, leading to the production of numerous cytokines.[16–18] Binding of TLR4 with TRIF activates the TRIF-dependent pathway, phosphorylating downstream interferon regulatory factor 3/7, thereby promoting the synthesis and release of type I interferons (IFN) (Figure 1).[16–18]

CD14 Plays a Key Role in LPS Signal Transduction

CD14 plays two major roles in LPS signaling. The first involves presenting LPS to the TLR4/MD-2 complex, enhancing the cellular response to low LPS concentrations. Upon stimulation with low concentrations of LPS (< 10 ng/mL), bone marrow-derived macrophages (BMDMs) from $CD14^{-/-}$ mice exhibit a significant reduction in TNF-α release, indicating that MyD88-mediated TNF-α release is CD14-dependent.[18] However, when stimulated with high concentrations of LPS (> 100 ng/mL), TNF-α release from $CD14^{-/-}$ BMDMs is even greater than that from wild-type BMDMs.[18] At low LPS concentrations, CD14 primarily facilitates the transfer of LPS to TLR4/MD2 complex. In contrast, at high LPS concentrations, the dependence of MyD88-dependent signaling on CD14 is overcome, potentially owing to the direct interaction between LPS and MD2.[19,20] The second role involves controlling the endocytosis of the LPS receptor complex, thereby activating the TRIF-related adaptor molecule (TRAM)–TRIF pathway and producing type I IFN.[17] TLR4 is the only receptor in the TLR family that activates both the MyD88-dependent and TRIF-dependent pathways.[21] Although LPS binds to the TLR4–MD2 complex on the cell membrane surface, the LPS–TLR4 complex must be endocytosed into the cell to exert its full biological effect. Upon binding of LPS, facilitated by CD14, the TLR4–MD2 complex is recruited to the lipid rafts.[22] Following this recruitment, TLR4 initiates the MyD88-dependent pathway, leading to an early and rapid inflammatory response.[23] Subsequently, the entire receptor complex is internalized by CD14 to form endosomes, thereby activating the TRAM–TRIF pathway and producing type I IFN.[17,18] The activation of the TRIF pathway and the production of type I IFN by LPS are highly dependent on CD14. In $CD14^{-/-}$ cells, even LPS concentrations of 1000 ng/mL fail to induce TRAM–TRIF-dependent regulated on activation, normal T cell expressed and secreted (RANTES) and type I IFN synthesis.[18]

Role of LPS in AECs

Pro-Inflammatory Effect of LPS on AECs

Owing to their roles in regeneration, immune regulation, and surfactant secretion, the role of alveolar type II (ATII) epithelial cells in inflammation has been well elucidated. Primary human ATII cells express functional TLR2 and TLR4 on their cell membranes. LPS stimulation induces the expression of TLR2/4, leading to increased expression on the cell membrane of ATII cells and secretion of cytokines such as IL-1β, TNF-α, and IL-6.[24,25] Compared with macrophages, ATII cells produce lower levels of TNF-α and IL-1β but higher levels of chemokines such as monocyte chemoattractant protein-1 (MCP-1) and IL-8, suggesting that ATII cells may be an important source of chemokines.[25,26] After LPS stimulation, ATI cells release more MCP-1 than IL-8, whereas ATII cells release significantly more IL-8 than MCP-1.[25]

Fluorescence-activated cell sorting (FACS) enables researchers to obtain high-purity primary cells. However, ATII cells sorted by FACS exhibit inflammatory characteristics that differ markedly from those reported in previous studies (Figure 2). Primary rat ATII cells treated with LPS produce only low levels of TNF-α, IL-6, and IL-1β. In contrast, ATI cells release much higher levels of these cytokines upon LPS stimulation.[27] Non-FACS-sorted ATII cells release hundreds of times more TNF-α and IL-6 upon LPS stimulation compared with that from FACS-sorted ATII cells, suggesting contamination by macrophages.[27] In addition, without 3D culture, the distinct lung epithelial phenotype of primary ATII cells disappears within 3–5 days of culture, which is evident from the loss of their cuboidal shape and decreased surfactant production.[28] Notably, single-cell transcriptomic results from human tissues show that TLR4

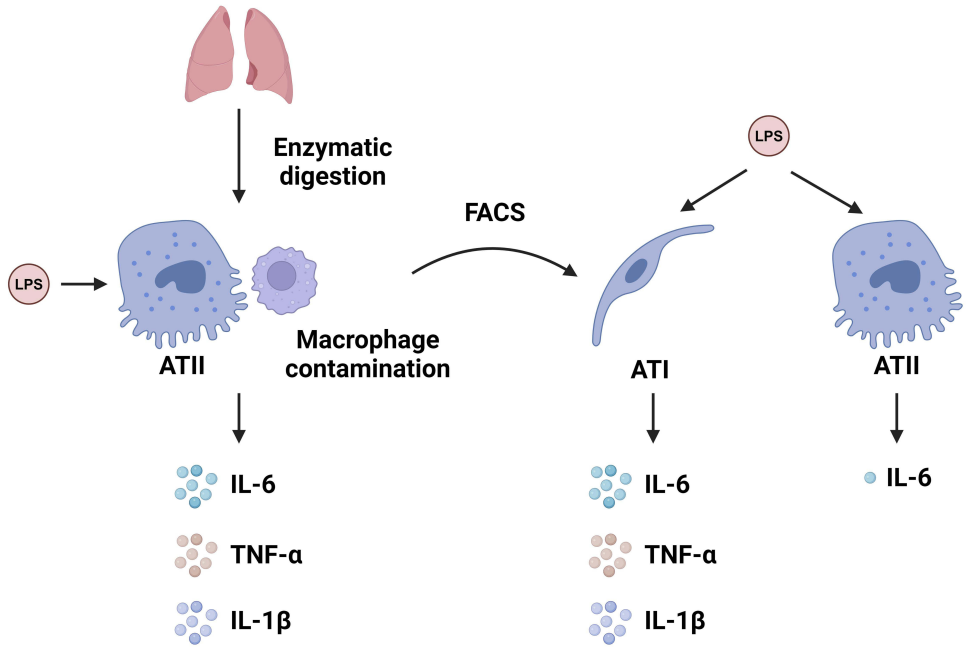

Figure 2 FACS More Accurately Differentiates the Cytokine Secretion Abilities of ATI and ATII Cells. In traditional methods of extracting primary AECs, the lung tissue is digested using enzymes, and ATII cells are obtained through differential adhesion. However, the ATII cells produced using this method are contaminated with macrophages. Consequently, early studies found that ATII cells could secrete large amounts of IL-6, TNF-α, and IL-1β upon LPS stimulation. However, after purifying ATI and ATII cells using FACS, researchers discovered that ATII cells secrete only small amounts of IL-6 after LPS stimulation, while ATI cells secrete large amounts of IL-6, TNF-α, and IL-1β upon LPS stimulation. (Created with BioRender.com).

expression levels are very low (< 1 nTPM) in both ATI and ATII cells.[29] Considering that even minimal immune cell contamination can significantly affect the assessment of immune responses in ATII cells, researchers must exercise caution when interpreting results from studies involving primary human ATII cells.

A549 cells, derived from patients with lung alveolar epithelial carcinoma, exhibit characteristics similar to that of ATII epithelial cells. Owing to challenges in isolating and purifying primary human ATII cells, often contaminated by immune cells, A549 cells frequently serve as substitutes.[30,31] Early studies in the 20th century demonstrated that A549 cells could secrete IL-6 and IL-8 in response to TNF-α, IL-1β, and other stimuli,[30,32] indicating their potential for cytokine release. Nevertheless, data on whether A549 cells functionally express TLR4 and release inflammatory cytokines upon LPS stimulation remain contradictory. A549 cells reportedly express TLR4,[33–38] MD-2,[34,38] and CD14;[38] the presence of TLR4 on the A549 cell membrane has been confirmed using flow cytometry and membrane protein isolation.[38,39] The human bronchial epithelial cell line BEAS-2B and primary human bronchial epithelial cells also express TLR4.[33,34] However, some studies suggest that although A549 and BEAS-2B cells express TLR4, this receptor may only reside in the cytoplasm, not on the cell membrane.[34,35,40] Even after LPS stimulation, TLR2/4 is absent from the surface of A549 cells.[40] Furthermore, multiple RNA-sequencing studies have indicated that A549 cells lack TLR4 mRNA under resting conditions.[41–43] Respiratory syncytial virus induces the expression of TLR4 in various AECs, including A549 cells, and promotes the translocation of TLR4 to the cell membrane. After respiratory syncytial viral infection, LPS binds to the cell membrane of AECs,[35] indicating that TLR4 activation in AECs may depend on pathological conditions, such as pathogen infection.

Although LPS stimulation induces the secretion of IL-8 and other cytokines in A549 and BEAS-2B cells,[34,44–46] several studies have reported that LPS does not induce cytokine secretion in A549 cells.[25,30,35,39,47] The low responsiveness of A549 cells to LPS may also be due to the lack of surface CD14,[25] and they only secrete IL-8 under combined stimulation with LPS and soluble CD14.[33,39] However, other studies have reported the presence of CD14 on the surface of A549 cell membranes and shown that biotinylated LPS can bind to CD14 on the cell membrane of A549 cells.[40] Even in the absence of TLR4 or CD14 on the cell membrane, LPS can still enter cells through scavenger receptors with the help of LBP, and then bind to TLR4 in the cytoplasm, thereby exerting its pro-inflammatory effects.[48,49]

Despite being extensively studied in lung epithelial cell lines, A549 and BEAS-2B cells exhibit significant differences from primary cells in their physiological characteristics. Although A549 cells are often used as a substitute for ATII cells, they only exhibit ATII characteristics under specific culture conditions.[50,51] Moreover, BEAS-2B cells cultured under serum-free and serum-containing conditions exhibit different phenotypes, with BEAS-2B cells cultured in serum lacking epithelial cell characteristics but exhibiting interstitial cells.[52] These cells also exhibit an abnormal karyotype, which may render them unsuitable for use as "normal" cells.[53]

Physiological Concentration of LPS Does Not Inhibit AEC Cell Viability

Regulated cell death (RCD) is a crucial mechanism for eliminating damaged cells and maintaining internal homeostasis, including apoptosis, necroptosis, and pyroptosis.[54] LPS induces various forms of RCD, including apoptosis,[55–59] autophagy,[55] pyroptosis,[60] ferroptosis,[61,62] and PANoptosis,[63] in AECs. Cell viability indicates alterations in cell proliferation and death. Despite the ease of detection, studies report contradictory effects of LPS on AEC viability.

Under physiological conditions, the concentration of LPS in human serum ranges from 0–1.0 ng/mL; however, in pathological conditions, this concentration can increase to 10 ng/mL.[64] In inflammation-related studies involving AECs, LPS is often used at concentrations exceeding 1 μg/mL, hundreds of times higher than those found clinically. Some studies have determined that stimulation with 1 μg/mL LPS for 24 h is sufficient to reduce A549 cell viability by 50%.[65,66] However, other studies indicate that when stimulated for 24 h, the LPS concentration must exceed 15 μg/mL to achieve a 50% reduction in A549 cell viability.[67,68] Researchers have also reported that at concentrations of 0–15 μg/mL, LPS had no effect on cell viability after 72 h of treatment.[69] After stimulation with a high LPS concentration (500 μg/mL) for 24 h, A549 cell viability decreased only to 67% (Table 1).[50] Interestingly, A549 cell viability increased with prolonged LPS stimulation, potentially owing to LPS tolerance and cell fusion.[50]

Table 1 Effects of Different Concentrations of LPS on A549 Cell Viability

Concentration	Time	Detection Reagents/Methods	Result Type	Rate of Change	Ref
0.1μg/mL	24h	MTT assay	Cell viability	ns	[65]
1μg/mL	12h	MTT assay	Cell viability	ns	[65]
1μg/mL	24h	MTT assay	Cell viability	49%↓	[65]
1μg/mL	48h	MTT assay	Cell viability	73%↓	[65]
8μg/mL	24h	CCK8 assay	Cell viability	ns	[67]
10μg/mL	24h	MTT assay	Cell viability	74%↓	[65]
10μg/mL	24h	MTT assay	Cell viability	18%↓	[50]
0–15μg/mL	12h-72h	MTT assay	Cell viability	ns	[69]
0.1–25μg/mL	16h	CCK8 assay	Cell viability	ns	[68]
10–200μg/mL	48h	MTT assay	Cell viability	<10%↓	[50]
10–200μg/mL	72h	MTT assay	Cell viability	<10%↓	[50]
50–200μg/mL	24h	MTT assay	Cell viability	25%↓	[50]
500μg/mL	24h	MTT assay	Cell viability	33%↓	[50]
500μg/mL	48h	MTT assay	Cell viability	21%↓	[50]
500μg/mL	72h	MTT assay	Cell viability	15%↓	[50]

In summary, pathologically significant LPS concentrations are unlikely to suppress AEC cell viability. Moreover, although high concentrations of LPS can reduce AEC cell viability and induce various forms of RCD,[60–62,70–72] the pathological significance of RCD is unclear.

Effect of LPS on AEC Pyroptosis

Classical Pyroptosis and ALI

Pyroptosis is a form of programmed cell death characterized by cell swelling, membrane pore formation, and the release of inflammatory contents, presenting a "fried eggs" morphology upon rupture.[73] In classical pyroptosis, various pattern recognition receptors (such as NLRP3 and AIM2) form inflammasomes by binding to scaffold proteins ASC and pro-caspase-1. Upon assembly of the inflammasome, pro-caspase-1 undergoes self-cleavage to form the active p33/p10 complex, which cleaves pro-IL-1β and pro-IL-18 to generate mature IL-1β and IL-18.[74] Additionally, the p33/p10 complex can cleave gasdermin D (GSDMD) to form the GSDMD N-terminal domain, which binds to lipids on the cell membrane, creating pore structures and releasing intracellular contents (Figure 3).[74–76]

Pyroptosis is a crucial mechanism of the immune system for combating infections, wherein immune cells release a large number of cytokines, amplifying the inflammatory response against pathogenic infections.[77] However, during

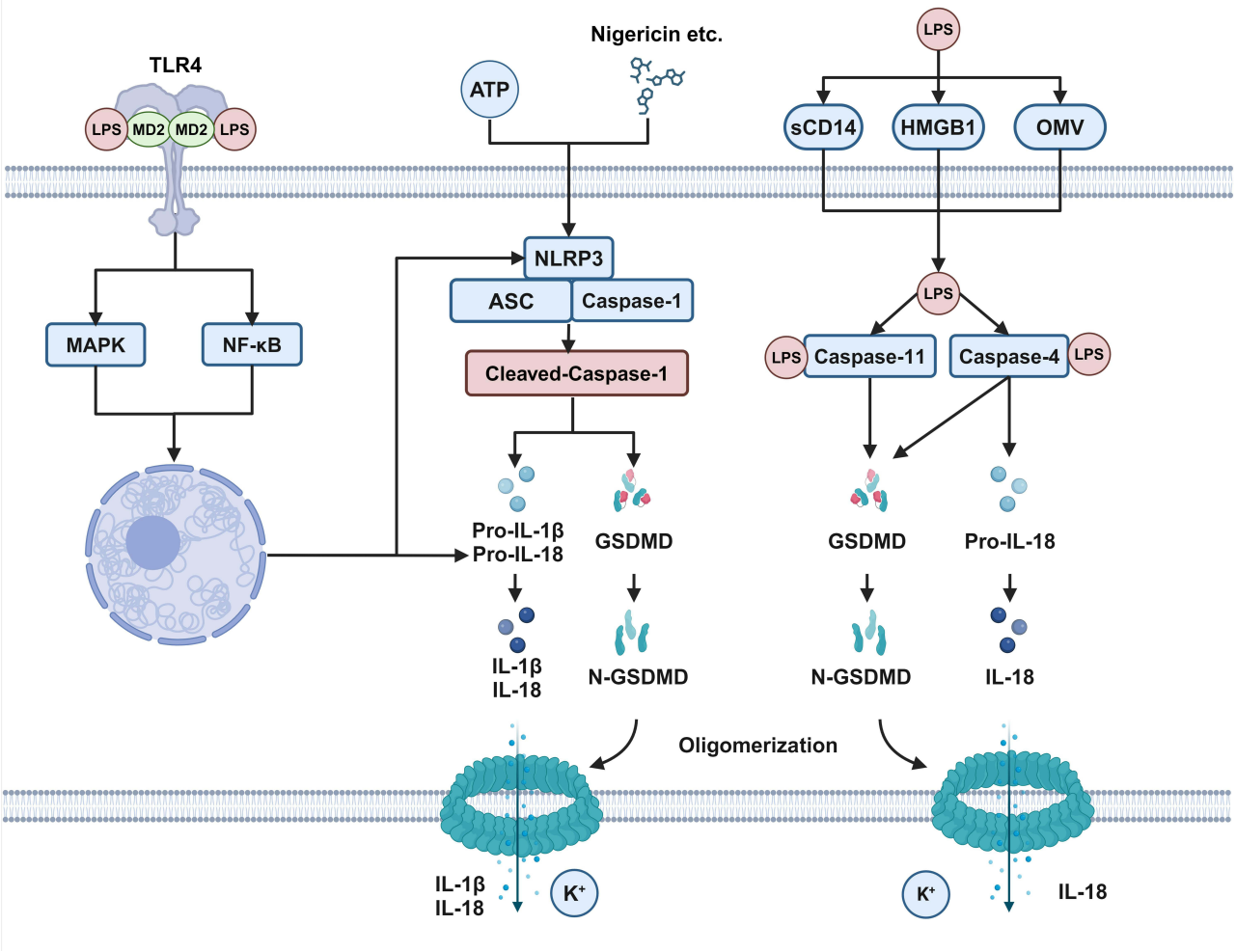

Figure 3 Schematic Diagram of LPS-induced Classical and Non-classical Pyroptosis in AECs. After LPS stimulation, the expression of NLRP3 and pro-IL-1β increases. When the cells are subsequently stimulated with ATP or Nigericin, the NLRP3-ASC-Caspase-1 complex forms in the cytoplasm. Within the complex, Caspase-1 undergoes autolysis and then cleaves pro-IL-1β, pro-IL-18, and GSDMD. Cleaved N-GSDMD oligomerizes and forms pores in the cell membrane, resulting in the release of active IL-1β, IL-18, and potassium ions. With the assistance of sCD14, HMGB1, OMV, and others, LPS can directly enter the cytoplasm and bind to cytoplasmic caspase-4/5 (human) or caspase-11 (mouse). Activated caspase-4/5/11 can cleave GSDMD, leading to cell membrane perforation. Additionally, caspase-4 can cleave IL-18, resulting in the release of active IL-18. (Created with BioRender.com).

pathogen infections, inflammation acts as a double-edged sword—excessive inflammation leads to tissue damage, whereas weak inflammation impedes pathogen clearance. For instance, in *Yersinia* infection, GSDMD$^{-/-}$ results in an increased bacterial load, exacerbating the risk of mortality in mice.[78] In SARS-CoV-2 infection, GSDMD$^{-/-}$ does not alleviate infection-induced weight loss and lung damage.[79] In *Leishmania* infection, NLRP3$^{-/-}$, GSDMD$^{-/-}$, and caspase-1/11$^{-/-}$ mice display significantly larger ear lesions.[80] However, in H1N1 influenza infection, GSDMD$^{-/-}$ can reduce mortality rates and alleviate lung damage in mice.[81,82] In sepsis caused by *Candida albicans*, GSDMD$^{-/-}$ mice exhibit reduced mortality rates, whereas caspase-1/11$^{-/-}$ mice experience increased mortality rates.[83]

In direct lung injury induced by LPS and sepsis-related lung injury, lacking interference from pathogens, the situation appears to be more "pure". In LPS-induced sepsis models, GSDMD$^{-/-}$ improves mouse survival rates, whereas caspase-1$^{-/-}$ does not improve survival rates.[84,85] Similarly, caspase-1$^{-/-}$ mice show more severe neutrophil infiltration in lung tissue following intratracheal LPS administration.[86] However, caspase-1$^{-/-}$ can alleviate lung injury caused by high tidal volume mechanical ventilation.[87] In cecal ligation and puncture-induced sepsis, GSDMD$^{-/-}$ improves mouse survival rates and reduces lung injury.[88] In the LPS intratracheal administration model, neutrophil-specific GSDMD$^{-/-}$ mitigates lung injury by reducing neutrophil extracellular traps.[89] Additionally, GSDMD$^{-/-}$ alleviates acute pancreatitis-related lung injury.[90] Overall, GSDMD$^{-/-}$ can alleviate ALI caused by various etiologies, but caspase-1 may lack a strong role in mitigating this condition, possibly owing to the existence of the Caspase-8/GSDMD axis.[78,91]

NLRP3-mediated classical pyroptosis has been extensively studied in clinical and animal models of ALI. Targeted inhibition of NLRP3 using small molecule inhibitors showed good efficacy in ALI animal models.[92–94] However, the activity of NLRP3 inflammasomes in AECs remains controversial. Clinically, elevated markers of pyroptosis have been detected in ALI/ARDS caused by various factors and are associated with poor prognosis in patients.[95–97] Postmortem examinations of deceased patients with SARS-CoV-2 further confirm pyroptosis as a significant cause of pulmonary epithelial cell death.[98] In animal models of ALI, pulmonary epithelial cells undergo NLRP3 inflammasome-mediated pyroptosis.[99] Co-localization of caspase-1 p20 and an ATII marker (SPC) is observed in the lung tissues of ALI mice.[99]

A549 cells are commonly used as AECs in pyroptosis research. Numerous studies have shown that they express NLRP3, ASC, and Caspase-1, providing the molecular basis for classical pyroptosis, and can undergo classical pyroptosis when stimulated by agents such as LPS and SARS-CoV-2.[100–104] SARS-CoV-2 activates NLRP3 inflammasome activity in A549 cells leading to pyroptosis.[104–106] Notably, although A549 cells release IL-1β upon SARS-CoV-2 infection, the quantity is significantly less than that released by macrophages.[106] Therefore, further elucidation of inflammasome activity in lung epithelial cells is required.

However, many studies have questioned the ability of AECs to undergo pyroptosis. Single-cell sequencing and transcriptomic studies suggest that primary human AEC and A549 cells either do not express NLRP3 and caspase-1 or express them at substantially low levels.[29,107] Studies have found that primary human AECs and A549 cells do not show NLRP3 protein via Western blotting.[108,109] Other studies have reported that caspase-1 is undetectable in lung epithelial cell lines (16-HBE, HBEC, and BEAS-2B cells) using Western blotting.[110] Similarly, mouse lung epithelial cells (TC-1 cells) also lack the expression of NLRP3, ASC, and caspase-1.[111] These studies provide contrasting evidence, suggesting that AECs may lack NLRP3 and caspase-1, and therefore cannot undergo classical pyroptosis.

Cytoplasmic LPS Induces Non-Classical Pyroptosis in AECs

After binding to TLR4, LPS enters the cells via lipid raft transport and is subsequently degraded in lysosomes without leaking into the cytoplasm. In contrast to the "gentle" extracellular action of LPS, entry into the cytoplasm can lead to non-classical pyroptosis.[112] In human macrophages, epithelial cells, and endothelial cells, cytoplasmic LPS triggers caspase-4-dependent pyroptosis.[85,113] Once inside the cytoplasm, LPS can directly bind to caspase-4/5 (human) or caspase-11 (mouse), leading to their self-cleavage and oligomerization.[113,114] Activated caspase-4/5/11 cleaves GSDMD into NT-GSDMD, resulting in pyroptosis.[115] Unlike the activation of caspase-1 in classical pyroptosis, caspase-4/5/11 lacks the activity to cleave pro-IL-1β.[116,117] Notably, caspase-4 can cleave IL-18;[114] moreover, activated caspase-11 can cleave pannexin-1, leading to cytoplasmic ATP release, which induces P2X purinoceptor 7-mediated classical pyroptosis.[118] Furthermore, both GSDMD- and pannexin-1-dependent pores cause K+ efflux, which can activate NLRP3 inflammasomes, leading to the cleavage of pro-IL-1β and pro-IL-18 by caspase-1.[85,119] However, the exact

mechanism underlying the entry of LPS into the cytoplasm remains unclear. LPS may enter the cytoplasm by binding to HMGB1[120–122] or CD14[123] or through bacterially secreted outer membrane vesicles[124,125] and host-derived extracellular vesicles,[126] thereby inducing pyroptosis (Figure 3).

Currently, research on the relationship between non-classical pyroptosis and ALI is limited. Reportedly, caspase-11 knockout and endothelial-specific caspase-11 knockout can reduce mortality from endotoxic shock and alleviate lung damage secondary to endotoxic shock.[84,85] Activation of the caspase-11/gasdermin D pathway induces mitochondrial DNA release, which hinders endothelial cell proliferation and impairs lung vascular repair.[127] This implies that in ALI, caspase-11-mediated pyroptosis is a crucial mechanism leading to endothelial cell injury and inhibition of repair. *Burkholderia pseudomallei, Shigella flexneri*, and LPS electroporation can induce caspase-4-dependent pyroptosis in A549 cells;[128,129] LPS transfection does not cause significant cell death in A549 cells.[129] This is because A549 cells lack guanylate-binding proteins (GBPs), which directly bind and aggregate "free" LPS. IFN-γ can induce the expression of GBPs in A549 cells; therefore, IFN-γ-primed A549 cells can undergo pyroptosis after LPS transfection.[129] Overall, the mechanism of non-classical pyroptosis in AECs needs to be further studied in primary AECs, and in vivo studies are needed to demonstrate the physiological significance of non-classical pyroptosis in AECs.

Factors Influencing the Effect of LPS on AECs

Effects of Extraction Methods and Bacterial Strain Sources on LPS Effects

The potency of LPS is influenced by the extraction method and bacterial strain. Common LPS is extracted using the phenol method and contains other bacterial components, such as lipoproteins, activating both TLR4 and TLR2. However, ultrapure LPS extracted through repeated enzymatic hydrolysis, followed by purification using the phenol–TEA–DOC method, only activates TLR4.[130] Both TLR2 and TLR4 can activate the MyD88-dependent pathway, and TLR2 can also form a complex with RAC1, activating downstream nuclear factor kappa beta through the PI3K/AKT pathway.[131] Common LPS additionally activates TLR2, making the signaling pathway exceptionally complex and, thus, unsuitable for studying downstream TLR signaling. However, LPS has been widely used in numerous studies, and many early studies did not effectively distinguish between LPS sources and purity.[132]

LPS from different bacterial strains also produces markedly different pro-inflammatory effects. LPS comprises lipid A, a core polysaccharide, and an O-polysaccharide. O-polysaccharides are polysaccharide chains composed of multiple oligosaccharide repeat units exhibiting high variability.[133] Bacteria with long O-polysaccharide chains form smooth colonies and produce smooth LPS (sLPS), whereas those lacking long O-polysaccharide chains or with short O-polysaccharide chains form rough colonies and produce rough LPS (rLPS).[133] At low LPS concentrations, neither sLPS nor rLPS activate TLR4 in the absence of CD14.[134] However, at concentrations of ≥ 100 ng/mL, regardless of CD14 presence, rLPS can activate both the MyD88-dependent and TRIF-dependent pathways, whereas sLPS can only activate the MyD88-dependent pathway (Figure 4).[134] It is worth mentioning that this study was conducted in RAW264 and J774A.1 cell lines, and the LPS concentration reached the μg/mL level, which may explain the difference from the results obtained by Zanoni et al.[18] Thus, highlighting the extraction method and bacterial strain source of LPS is crucial to ensure the purity of LPS bioactivity and provide assurance of reproducibility by other researchers.

Effect of Serum Concentration on LPS Effects

The effect of serum on LPS cannot be overlooked because the concentration, manufacturer, and batch of serum can influence the biological activity of LPS. Fetal bovine serum (FBS) in the culture medium significantly increases LPS-induced release of TNF-α, MCP-1, and IL-8 in human primary lung macrophages and AECs.[25] Moreover, at the same LPS concentration, higher concentrations of normal human serum (NHS) in the culture medium result in greater IL-8 and IL-6 release from AECs.[33] This effect may be due to the presence of soluble CD14 in NHS. Under serum-free conditions, 200 ng/mL of CD14, combined with physiological concentrations of LPS (10 ng/mL), induces significant cytokine release from A549 and BEAS-2B cells.[33] The amount of IL-8 produced by A549 cells stimulated with LPS+CD14 is no different from that produced by cells stimulated with 10% NHS + LPS; however, the amount of IL-8 produced by BEAS-2B cells stimulated with LPS+CD14 is only approximately one-tenth of that stimulated with 10% NHS + LPS,[33]

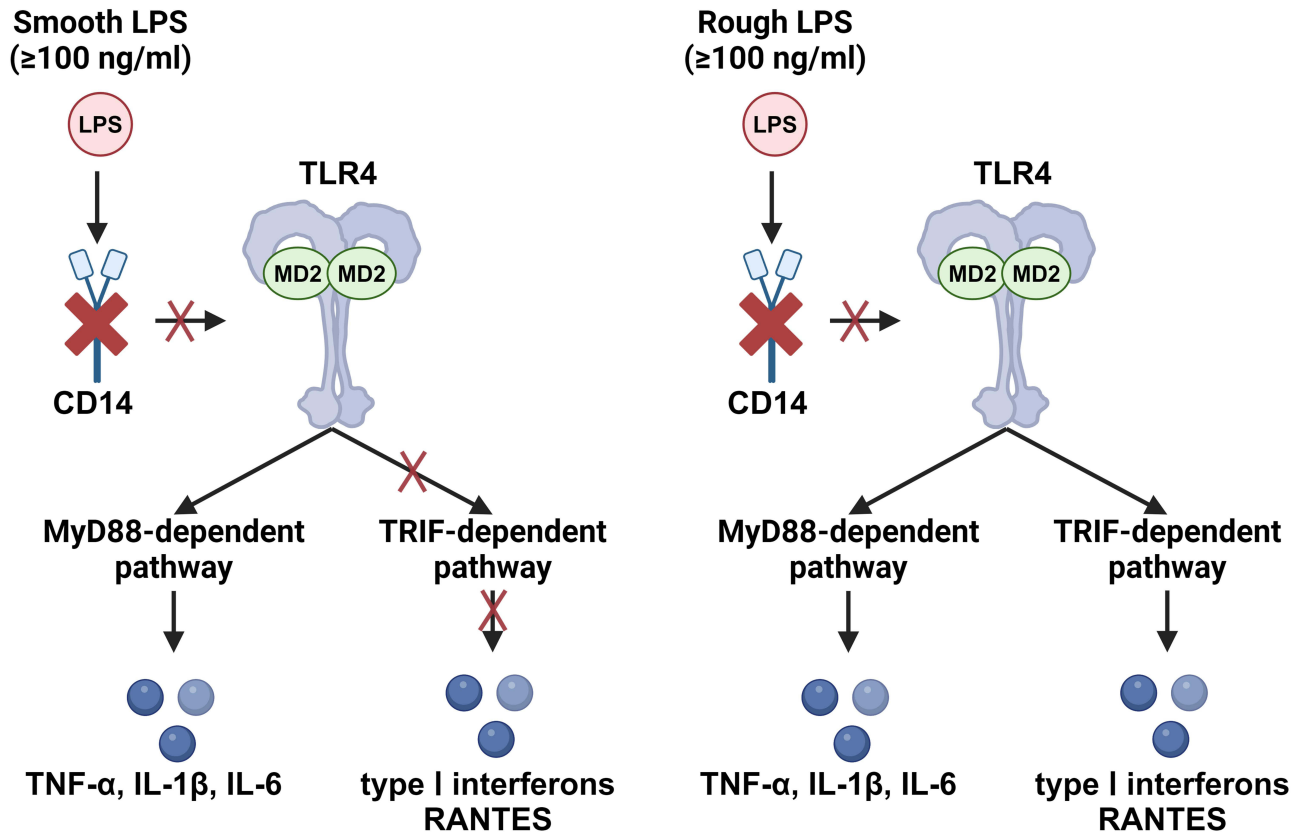

Figure 4 Smooth LPS and Rough LPS Exhibit Different Pro-inflammatory Activities in the Absence of CD14. In CD14-deficient cells or environments lacking CD14 (such as those with monoclonal antibodies that deplete CD14), smooth LPS at concentrations ≥100 ng/mL can activate the MyD88-dependent pathway but cannot activate the TRIF-dependent pathway. Under the same conditions, rough LPS can activate both the MyD88-dependent pathway and the TRIF-dependent pathway. (Created with BioRender.com).

indicating that other substances in NHS may promote the LPS-induced cytokine secretion from BEAS-2B cells. Serum enhances not only the pro-inflammatory effects of LPS but also the inhibition of cell activity by LPS (Table 2).[50,135,136] Tang et al[136] observed that a FBS concentration of 5% was required for LPS to induce A549 cell death. Nova et al[50] found that, compared with A549 cells cultured with 10% FBS, the inhibitory effect of LPS on cell activity was reduced by approximately 50% in cells cultured with 4% FBS.

Serum from different manufacturers and different batches may lead to markedly different inflammatory responses.[135,137] Serum from the same manufacturer, such as Gibco, but of different origins, can increase IL-8 release induced by LPS by thousands of times.[135] Serum from different manufacturers or batches can induce different levels of IL-8 secretion in HCT-8 and HT-29 cells through extracellular signal-regulated kinase phosphorylation, which may be related to the presence of 1-palmitoyl-sn-glycero-3-phosphocholine in the serum.[138]

Table 2 Effects of Different Concentrations of FBS on A549 Cell Viability

LPS Concentration	FBS (%)	Time	Cell Viability	Ref
10 μg/mL	4%	24h	92%	[50]
10 μg/mL	10%	24h	82%	
500 μg/mL	4%	24h	85%	
500 μg/mL	10%	24h	67%	

(*Continued*)

Table 2 (Continued).

LPS Concentration	FBS (%)	Time	Cell Viability	Ref
10 μg/mL	0%	48h	ns	[136]
	0.10%	48h	ns	
	1%	48h	ns	
	5%	48h	↓	
	10%	48h	↓	
100 μg/mL	0%	48h	ns	
	0.10%	48h	ns	
	1%	48h	ns	
	5%	48h	↓	
	10%	48h	↓	

Owing to the potent effect of serum, which can mask multiple subtle changes, many studies have used serum starvation to eliminate serum interference.[135,137] However, serum starvation lacks standardized criteria and can lead to the inhibition of proliferation, cell death, and phenotypic changes in AECs.[51,139–141] The effect of serum starvation on cells is also time-dependent;[139] thus, the duration of serum starvation may directly affect the responsiveness of AECs to LPS.

Conclusions and Future Perspectives

LPS is one of the most common triggers of ALI and ARDS in both humans and animals. AECs, as the most important cell group in the lungs, still hold many unknowns regarding the immune response to LPS. AECs can clearly produce a weak response to direct LPS stimulation. The intensity of this response largely depends on serum concentration, CD14, and LPS itself. Therefore, whether the weak response of AEC to LPS stimulation plays a significant role in disease requires further investigation. Clinical studies of ALI/ARDS have revealed that AECs undergo classical pyroptosis, characterized by caspase-1 and IL-1β cleavage. However, in vitro studies on primary AEC and lung epithelial cell lines have shown a lack of NLRP3 and caspase-1. Although SARS-CoV-2 can induce weak IL-1β release from AECs, the significance of this change in ALI/ARDS requires further investigation. Apart from its pro-inflammatory role, LPS also plays a vital role in inducing non-classical pyroptosis, which is gaining increasing attention in research aiming to improve our understanding of sepsis and ALI/ARDS. AECs can undergo non-classical pyroptosis under conditions such as bacterial infection or electroporation. However, the significance of AEC non-classical pyroptosis in ALI/ARDS requires confirmation through AEC-specific gene-knockout mice. Collectively, our review stresses the need for meticulous methodological consistency and further investigation into the immune functions of AECs, which play a critical role in the host defense against pathogenic threats. This enhanced understanding is crucial for developing targeted therapies that can effectively manage and mitigate conditions such as ALI and ARDS.

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Disclosure

The authors declare that this study was conducted without any commercial or financial relationships that could be construed as conflicts of interest.

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