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REVIEW

# Interplay of TLR4 and SARS-CoV-2: Unveiling the Complex Mechanisms of Inflammation and Severity in COVID-19 Infections

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**Abstract:** The late 2019 emergence of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, caused profound and unprecedented disruption to the global socio-economic structure, negatively affecting millions of lives worldwide. A typical hallmark of severe COVID-19 is hyper inflammation due to aberrant cytokine release (cytokine storm) by innate immune cells. Recent studies have revealed that SARS-CoV-2, through its spike (S) protein, can activate the body's innate immune cells via Toll-Like Receptors (TLRs), particularly TLR4. In silico studies have demonstrated that the S protein binds with high affinity to TLR4, triggering downstream signaling processes that result in pro-inflammatory cytokine release. Compared to other TLRs, such as TLR2, TLR4 plays a more significant role in initiating and sustaining the inflammatory response associated with severe COVID-19. Furthermore, interactions between the virus and target cells can enhance the cellular expression of TLR4, making cells more susceptible to viral interactions and subsequent inflammation. This increased expression of TLR4 upon viral entry creates a feedback loop, where heightened TLR4 levels lead to amplified inflammatory responses, contributing to the severity of the disease. Additionally, TLR4's potent activation of inflammatory pathways sets it apart from other TLRs, underscoring its pivotal role in the pathogenesis of COVID-19. In this review, we thoroughly explore the multitude of regulatory signaling pathways that SARS-CoV-2 employs to incite inflammation. We specifically focus on the critical impact of TLR4 activation compared to other TLRs, highlighting how TLR4's interactions with the viral S protein can exacerbate the severity of COVID-19. By delving into the mechanisms of TLR4-mediated inflammation, we aim to shed light on potential therapeutic targets that could mitigate the inflammatory damage caused by severe COVID-19. Understanding the unique role of TLR4 in the context of SARS-CoV-2 infection could pave the way for novel treatment strategies that specifically inhibit this receptor's activity, thereby reducing the overall disease burden and improving patient outcomes.

**Keywords:** SARS-CoV-2, cytokine storm, toll-like receptor 4, hyperinflammation, ACE2 receptors, innate immunity

## Introduction

The emergence of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic in late 2019 has contributed to an unprecedented global social and economic disruptive impact on humans. Infection with the SARS-CoV-2 virus causes coronavirus disease 2019 (COVID-19), a disease that has tragically claimed approximately 7 million lives worldwide.[1,2] The virus is a member of the betacoronavirus subgenus from the coronaviridae family. Compared to other coronaviruses in the same subgenus, SARS-CoV-2 exhibits a distinct ability to readily transmit from one host to another, even prior to the onset of noticeable symptoms. This might explain in part the initial rapid and extensive spread of the virus within populations, often going unnoticed. By infecting the cells of the upper respiratory tract, bronchial epithelium, and alveolar type II pneumocytes (ATII), SARS-CoV-2 can trigger the onset of variable clinical manifestations that can range from asymptomatic infection to acute lung injury, severe pneumonia, and death, especially in

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individuals with certain co-morbidities such as underlying lung infections, diabetes, obesity, etc.[1,3] Like other coronaviruses, SARS-CoV-2, has a large positive-sense RNA genome of roughly 30kb in size. This genome codes for and produces both non-structural and structural viral proteins including the characteristic envelope spike (S) glycoprotein. The spike (S) glycoprotein of SARS-CoV-2 interacts with receptors on the targeted cell membranes, specifically, it binds with the angiotensin-converting enzyme 2 (ACE2). ACE2 is recognized as the primary receptor that facilitates the infection and subsequent invasion of host cells by the virus. Indeed, research indicates a strong association between SARS-CoV-2 pathogenicity and two key processes. The attachment of the S protein to the ACE2 receptor, as well as the co-expression of TMPRSS2, a transmembrane serine protease 2 that facilitates the entry of the virus into target cells.[4] Infection by SARS-CoV-2 triggers multiple immune responses in the host. These include the production of virus-specific antibodies and T cells, which play a vital role in suppressing viral spread.[5] However, like most viruses, SARS-CoV-2 has adopted several strategies to evade the immune responses of its host, enabling it to replicate and spread effectively. Numerous studies have underscored the ability of SARS-CoV-2 to manipulate early antiviral interferon production.[6] Furthermore, SARS-CoV-2 has shown a remarkable capacity to evade antibody and T-cell recognition. This evasion is possible through a multitude of mutations that modify the virus's sites targeted by host antibodies, as well as specific HLA-1 restricted epitopes present on its spike protein.[5] Further, SARS-CoV-2 employs specific escape mechanisms that not only promote effective infection but also promote rapid replication within its host. These processes often culminate in detrimental lung injury, pulmonary thromboembolism, and multi-organ dysfunctions.[7] In relation to these consequences, the virus can provoke excessive inflammatory responses, often responsible for the heightened severity of COVID-19.

Following this, the initial stage of infection/entry is through the interaction of the virus' S protein and the ACE2 receptor, alongside the TMPRSS2 co-receptor, which are highly expressed on the ATII.[4] This further activates the ATII and triggers the release of pro-inflammatory cytokines, with subsequent recruitment of inflammatory cells to the alveolar space.[7] The virus can also trigger some cell death pathways such as pyroptosis[8] through the induction of the inflammasome, which is linked to additional destruction of the lung epithelium, disorientation of the alveolar architecture, and devastating inflammatory responses through the release of danger associated molecular patterns (DAMPs).[8]

Emerging evidence highlights the virus' ability to activate certain innate sensors, such as Toll-like receptors (TLRs), through its surface S protein or viral nucleic acid. TLRs are germline-encoded pattern-recognition receptors (PRRs), mostly expressed by innate immune cells. They are known to detect pathogens and self-derived molecules from damaged cells by recognizing pathogen-associated molecular patterns (PAMPs) and DAMPs, respectively.[9] Upon detecting these stimuli, TLRs initiate downstream signaling cascades, leading to the release of effector molecules such as inflammatory cytokines (IL6, TNF-α, IL-1β, CXCL10, CXCL8, etc.) (Table 1) and other mediators of inflammation.[9] Additionally, these sensors and their signaling pathways are vital for priming and initiating the adaptive immune response.[9] TLRs are classified by their cellular localization, distinguishing intracellular or endosomal TLRs from extracellular TLRs. Intracellular TLRs include TLR3, TLR7, TLR8, and TLR9. TLR3 detects double-stranded RNA, TLR7 and TLR8 recognize single-stranded RNA rich in guanosine and uridine, and TLR9, traditionally known for recognizing unmethylated CpG motifs in DNA, is also suggested to sense single-stranded RNA fragments from SARS-CoV-2.[10] In silico

**Table 1** Summary of the Mechanisms by Which TLR4 Agonists and Antagonists Function, and Their Regulatory Impact on Key Cytokines Responsible for Hyperinflammation During the COVID-19 Cytokine Storm

| TLR4 Agonist/Antagonist | Mechanism of Action | Impact on Key Cytokines Induction |
|---|---|---|
| Glucopyranosyl Lipid A (GLA) | Agonist | Promotes transient induction of MCP-1, TNFα, IFN-γ and CXCL10.[66] |
| Aptamer ST-6 | Antagonist | Dampens the release of TNF-α, IL-1 β, IL-6, and IFN- β.[67] |
| Qingwenzhike | Antagonist | Suppresses the expression of IL-6, TNF-α, MCP-1, IL-1β, IL-18, and IFN-γ.[58] |
| BZL-sRNA-20 | Antagonist | Reduce expression levels of IL-1 β, TNF-α and IL-6.[59] |
| Jinhua Qinggan granules | Antagonist | Promotes induction of TNF-α, IL-1 β, and IL-6.[68] |
| Lipid A analog CRX-527 | Agonist | Induce expression of TNF-α, IL-1 β, IL-6 and IFN-γ.[69] |

analyses have shown that TLR3, 7, 8, and 9 can effectively bind SARS-CoV-2 non-structural (NSP10) and structural protein mRNAs (E-protein), including those encoding the S protein.[10] On the other hand, extracellular TLRs such as TLR1, TLR2, TLR4, TLR5, and TLR6, primarily recognize microbial membrane components.[9] Specifically, TLR5 detects bacterial flagellin, TLR4 detects bacterial lipopolysaccharide, and TLR2, in combination with TLR1 or TLR6, detects various PAMPs like lipoproteins, peptidoglycans, and lipoteichoic acids.[9] Notably, it has been demonstrated that the SARS-CoV-2 S protein can bind to TLR2 and 4, with a particularly high affinity for TLR4,[11,12] which can trigger the activation of downstream signaling cascades in the target cells, followed by the release of inflammatory mediators. It is postulated that the virus–host interactions can indirectly implicate multiple cellular pathways to potentiate the upregulation of TLR4 on target cells, which can increase its accessibility to the S protein.[13] Advanced research aimed at exploring these pathways and their overall effect in the upregulation of TLR4 is needed to critically understand these interactions at the molecular level and develop therapeutic proposals. In this review, we provided a concise explanation on the various pathways explored by SARS-COV-2 to directly or indirectly induce inflammation and the implication of TLR4 in severe COVID-19. We equally identified research gaps that highlight the importance of understanding the molecular mechanisms involved in SARS-COV-2-spike protein-mediated activation of TLR4 for the development of effective anti-viral therapies.

Despite comprehensive investigations into the pathophysiology of SARS-CoV-2, numerous mysteries persist. The specific processes by which the virus instigates extreme inflammation remain undefined, as does the creation of cost-effective, curative antiviral treatments. This review article leverages an integrative approach and encapsulates vital advancements in our current understanding of SARS-CoV-2 pathophysiology, including the multifaceted cellular pathways involved in SARS-CoV-2 induced inflammation and the role of TLR4. This qualitative review of literature on the TLR4 pathway in the context of SARS-CoV-2 infection prioritizes articles from the past five years. To ensure a comprehensive understanding of TLR4's role and regulatory mechanisms, we also considered significant studies published before this window if they offered insights pertinent to understanding SARS-CoV-2. This approach allowed us to construct a nuanced overview of TLR4's involvement in the immune response to SARS-CoV-2, reflecting both recent findings and foundational knowledge.

## Current Understanding of How SARS-CoV-2'S S-Glycoprotein Modulates TLR4 Activation Inducing Inflammatory Responses

Similar to other TLRs, TLR4 is a type 1 transmembrane protein receptor consisting of three domains: the N-terminal extracellular domain that serves as a recognition site for several different PAMPs (pathogen-associated molecular patterns), a middle helical transmembrane domain, and the C-terminal intracellular domain that interacts with specific signal transduction adaptor molecules in the cytoplasm to initiate downstream signaling cascades through its homologous TIR (toll-IL1 receptor) domain. TLR4 is mainly expressed on the cell membrane of some sentinels innate immune cells such as macrophages, dendritic cells, and monocytes. They mainly recognize the LPS (lipopolysaccharide) endotoxin from gram-negative bacteria. Upon activation, several intracellular signaling events occur that drive the transcription of different inflammatory genes through the NF-kB (Nuclear factor-kB), AP-1 (activator protein-1), or IRF3 (interferon regulatory factor-3) pathways (see Figure 1).[14,15]

The processes by which LPS activates TLR4 go through a number of steps. The initial step involves the cleavage function played by the TLR4 coreceptor, CD14. Binding of LPS to LBP (LPS binding protein) initiates the transfer of LPS to CD14, which cleaves LPS aggregates to monomeric forms and localizes them into the hydrophobic pocket of MD2 (myeloid differentiation factor 2) that is part of the TLR4/MD-2 complex. The monomeric LPS triggers the heterodimerization and activation of the TLR4/MD-2 complex, thereby leading to the recruitment of the intracellular adaptor proteins, MyD88 (myeloid differentiation primary response 88) and/or TRIF (toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β), which in turn recruits other downstream signaling molecules required for the expression and secretion of pro-inflammatory mediators (see Figure 1).[15] The activation of the MyD88-dependent downstream signaling cascade results in an early activation of the NF-κB pathway with subsequent secretion of proinflammatory cytokines such as IL-6, TNFα, CXCL1, IL-1α, IL-1β, and IL-12. On the other hand, the activation of

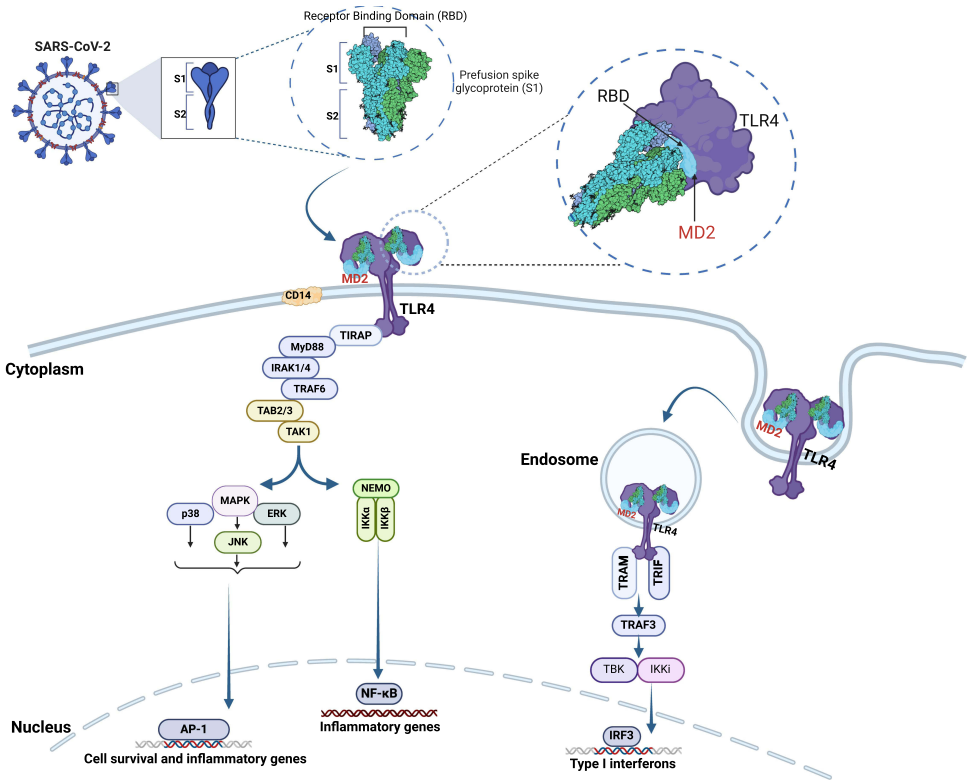


**Figure 1** Schematic overview of possible downstream signaling events triggered upon engagement of the SARS-CoV-2 spike protein with the TLR4/MD2 complex. The engagement of SARS-CoV-2's spike protein with the TLR4/MD2 complex on the surface of host cells triggers a critical immune reaction. This begins with a transformation in TLR4 that facilitates the attraction of MyD88 and TRIF proteins, initiating the NF-κB pathway which results in inflammation and an immune reaction through the activation of the IRAK4-IRAK1/IRAK2 complex and TRAF6. This process leads to the activation of TAK1, which in turn causes the degradation of IκBα, permitting NF-κB to enter the nucleus and initiate the transcription of genes for immune activation. In parallel, TAK1 stimulates the MAPK pathways (ERK, JNK, p38 MAPK), culminating in the activation of AP1, which is crucial for cell survival and the proliferation of immune cells. The endosomal pathway dependent on TRIF leads to the activation of TRAF3 and TRAF6, which then activates TBK1 and IKKε. These enzymes phosphorylate IRF3, and the phosphorylated IRF3 moves into the nucleus to induce the transcription of genes for type I interferon, essential for the antiviral response and for halting the spread of the virus. This concise schematic underscores the pivotal roles played by AP1, NF-κB, and IRF3 in the body's defense mechanisms against viral infections, illustrating a complex yet systematic response that encompasses inflammation, cell proliferation, and antiviral activities. Created with BioRender.com.

the TRIF-dependent downstream signaling results in the activation of the IRF3 pathway, which induces the production of type I IFNs and chemokines such as CXCL10.[14]

Emerging evidence suggests that several different viruses can engage and activate the TLR4 through the surface glycoprotein.[15] Although the precise mechanisms by which these viruses activate the TLR4 through their surface glycoprotein remain elusive, several studies have emphasized the potential role of glycosylation or hydrophobic interactions as the crucial mechanisms at play. Moreover, recent studies have shown that the membrane-bound glycoproteins of some viruses such as the RSV (respiratory syncytial virus), VSV G (vesicular stomatitis virus glycoprotein), and EBOV (Ebola virus) can bind to the hydrophobic pocket of MD-2 through hydrophobic interactions and mediate the activation of the MD-2/TLR4 complex. In connection to this, Rallabhandi et al, clearly demonstrated the indispensable role of MD-2 in RSV F-protein mediated-TLR4 activation. Their results showed a significant upregulation of IL-1β mRNA in primary peritoneal macrophages from wild-type C57BL/6J mice, after activation with the RSV F-protein. However, this effect was not seen in MD-2−/− mice derived macrophages, thereby providing evidence of hydrophobic interactions mediated by the RSV F-protein and the hydrophobic pocket of MD-2 to initiate the activation of cellular mechanisms downstream of the TLR4 pathway.[16] Results from other studies involving the VSV G[17] and EBOV sGP (secreted glycoprotein)[18] revealed similar activation dynamics between the viral surface protein and TLR4-coreceptor complex. Altogether, these observations provide key evidence on the activation of the TLR4 by several highly glycosylated viral glycoproteins and the potential role of hydrophobic interactions and glycosylation in mediating receptor-ligand binding and protein stability, respectively.

Several preliminary studies[11,12] have revealed evidence of the ability of SARS-CoV-2 to induce TLR4 activation through the envelope spike glycoprotein. Perhaps, with the cytokine storm inducing multi-organ dysfunction seen in most severe COVID-19 cases, it is intriguing to relate these effects to the possibility of the virus to aberrantly activate the TLRs to induce hyperinflammation. It has been suggested that the SARS-CoV-2 may bind to TLR4 to facilitate ACE2 expression, viral entry and hyperinflammation in affected organs.[19] Moreover, recent reports have shown that the S1 subunit of the SARS-CoV-2 spike protein can activate murine primary macrophages and human THP1 cell derived-macrophages in a TLR4-dependent manner. This activation led to the transcription of several proinflammatory genes and the release of inflammatory mediators such as TNF-α, IL-6, nitric oxide and IL-1β through the NF-kB and JNK signaling pathways (Figure 1). On the other hand, these effects were remarkably suppressed following the addition of specific antagonists targeting the TLR4/NF-kB/JNK axis. Thus, highlighting the direct impact of the SAR-CoV-2 spike protein in mediating inflammatory responses through the TLR4 pathway.[20] Other in vivo studies with murine models have revealed the possibility of the spike protein in inducing neuroinflammation and memory dysfunction through this receptor pathway.[21] It should be noted that patients suffering from long COVID-19 display some extrapulmonary defects such as cognitive impairment,[22] thereby highlighting the possibility of the virus or its structural components in displaying neurotropism. While Fontes-Dantas et al demonstrated that the spike protein introduced into the brain could traverse the blood–brain barrier in mice and trigger neuroinflammation, it is yet to be investigated whether the virus or its structural elements produce comparable effects in human Long COVID-19 patients experiencing cognitive dysfunction.

There is no doubt that the TLR4 signaling pathway is very crucial in inducing antiviral immune responses. However, understanding the exact threshold of activation needed for protection can guide researchers in determining the extent to which viruses, through their surface glycoproteins, activate the receptor beyond this threshold to promote the cytokine storm seen in most severe viral infections. Although some studies have proposed the use of an omicron peptide-based chimeric vaccine construct as an ideal therapeutic candidate against SARS-CoV-2, the utilization of high-dimensional immunoinformatic techniques has demonstrated TLRs engagement including TLR4.[23] Therefore, it is crucial to pinpoint the precise optimal threshold at which innate immune cells may become activated by the vaccine through this receptor axis in ideal experimental settings.[23] This can provide valuable information needed to enhance the safety and effectiveness of the vaccine without triggering abnormal inflammatory reactions following the activation of the immune system.[23]

The complexity of the pathophysiology of SARS-CoV-2 poses a major setback for researchers to accurately understand the multiple host factors contributing to the severity of the clinical manifestations of COVID-19. The dysregulation of cells of the innate immune system as a result of this viral infection has contributed to a cytokine storm seen in most severe COVID-19 cases.[24] Therefore, understanding the various mechanisms involved in SARS-CoV-2-mediated upregulation/hyperactivation of TLRs including TLR4, known to bind viral S protein with a higher affinity, could pave a gateway for the development of cost-effective antivirals that can prevent cytokine storm.

In this review, we have proposed certain hypotheses based on the current understanding of the pathomechanisms of SARS-CoV-2 and described the various cellular pathways that could be hijacked by SARS-CoV-2 to increase the upregulation of TLR4 and ultimately promote hyperinflammation.

## Decrease in Type II Pneumocytes Surfactant Production During SARS-COV-2 Infection Upregulates the Expression of TLR4

The human pulmonary alveolar cells (pneumocytes) play a crucial role in gas exchange and protection from inhaled harmful substances. They consist of mainly two cell types: type I and type II alveolar cells. These two cell types differ in their shapes, size, and secretory capabilities. Type I alveolar cells represent about 85% of alveolar cells,[25] consisting of large squamous epithelial cells with non-secretory functions and occupying most of the alveolar surface area. Type II alveolar cells, on the other hand, make up about 15% of alveolar cells, are comprised of small cuboidal-shaped epithelial cells, and are known for surfactant secretion. Surfactants consist of a mixture of both lipids and proteins, with lipids constituting a greater percentage. The protein component of surfactant consists of specific proteins SP-A, SP-B, SP-C and SP-D which are involved in immune responses within the alveoli. Surfactants are important in reducing the surface tension of the alveoli and preventing lung collapse and edema during gas exchange.[26] However, since the respiratory

system is constantly exposed to the external environment, threats from pathogens can thus interfere with the functionality of pneumocytes, leading to deleterious downstream events in the host.

Although the alveolar type II cells represent a smaller percentage of the alveolar cells compared to type I cells, they are strong targets for SARS-CoV-2 infection due to their high co-expression of the canonical receptors ACE2 and TMPRSS2 required for viral cell entry.[27,28] Infections of the ATII cells lead to suppressed surfactant production, thereby predisposing the alveoli to harmful effects due to their inability to carry out vital functions as mentioned earlier. One such consequence is the fact that decreased surfactant production enables viral escape due to the lack of the opsonization action of SP-A and SP-D.[29] Furthermore, since SP-A and SP-D are also involved in maintaining lung homeostasis and innate immune responses, depletion of surfactant by SARS-CoV-2 leads to infiltration of immune cells into the alveoli, creating a proinflammatory milieu (see Figure 2). In addition, it has also been recently reported that patients with severe SARS-CoV-2 infection had increased serum concentrations of SP-A and SP-D which further corroborated with the increase in proinflammatory cytokines.[29] However, apart from the immunological consequences of suppressed surfactant production, its detergent-like function capable of interacting with the lipid and protein components of the SARS-CoV-2 envelope will be missing, thus fostering viral survival and replication.[30,31] Interestingly, surfactants are known to block TLR4 in the lungs to avoid untoward proinflammatory effects and its depletion, therefore, leading to TLR4 upregulation (see Figure 2).[13] TLR4 has a low expression profile in both type I and II alveoli cells. However, the in silico study by Choudhury and Mukherjee et al purported that TLR4 interaction with SARS-CoV-2 spike protein is stronger than other TLRs. They also showed that the same spike protein interaction with TLR4 is stronger compared to the interaction between spike protein and ACE2.[12] Furthermore, another unique feature of TLR4 binding to the SARS-CoV-2 spike protein is that this process is independent of the proteolytic cleavage of TMPRSS2 for viral cell entry.

As previously mentioned, TLR4 is present on APCs (antigen-presenting cells) such as monocytes, macrophages and dendritic cells. It can also be found in a membrane-bound form expressed on ATI (alveolar type I pneumocytes) and ATII with a TIR domain cytoplasmic tail.[32] TLR4 in concert with MD2 interacts with LPS and components of viral proteins.

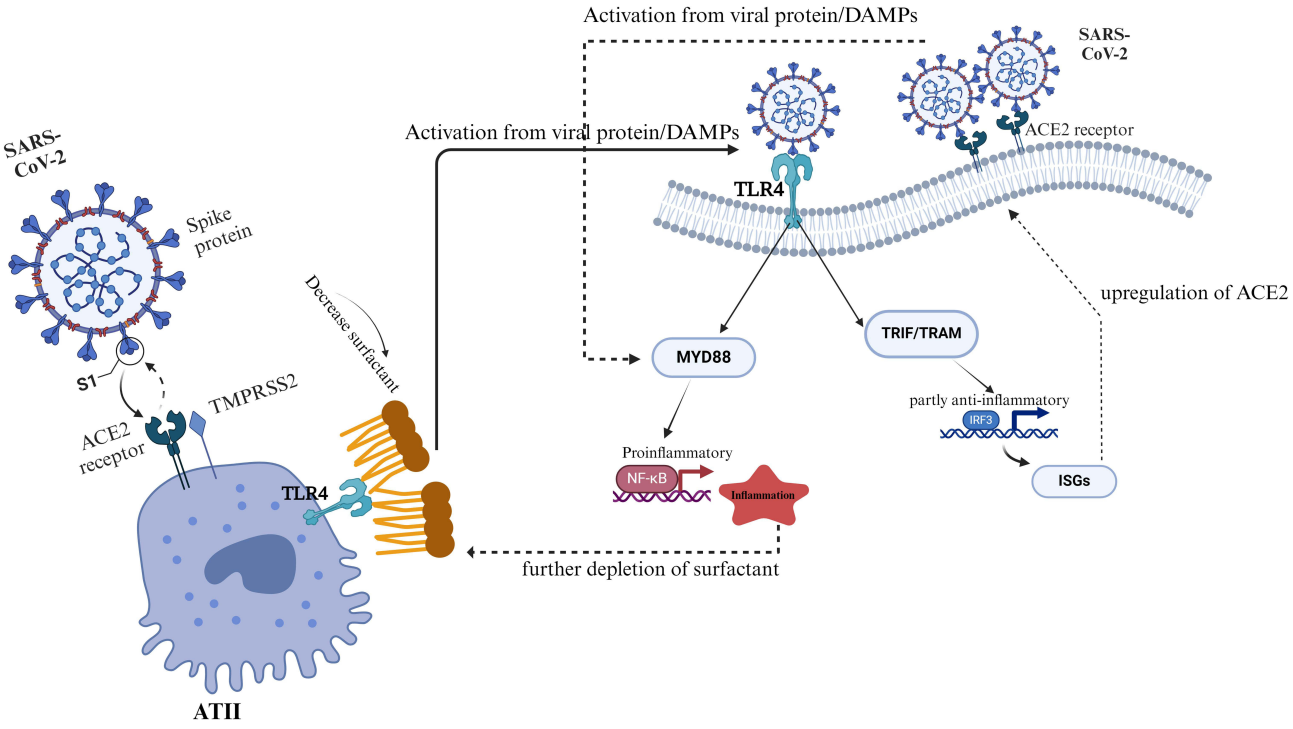


**Figure 2** Decrease in surfactant production due to SARS-CoV-2 infection of ATII cells leads to TLR4 upregulation. The exposition of TLR4 engenders its interaction with SARS-CoV-2 viral proteins and DAMPs released from dying cells leading the TLR4 activation and signaling pathways. TLR4 signaling via MyD88 leads to inflammation via NF-kB proinflammatory pathway while signaling via TRIF/TRAM leads to a partial anti-inflammatory pathway via IRF3. The IRF3 pathway fosters the upregulation of ISGs, including ACE2, which further binds to SARS-CoV-2, enhancing viral infection and replication. The increased viral replication then indirectly exacerbates the proinflammatory cascade, depleting surfactant more, and causing a hyperinflammatory state leading to potential cytokine storm. Created with BioRender.com.

The signaling pathway of TLR4 can be regarded as a "two-edged sword" involving both pro-inflammatory and anti-inflammatory outcomes (see Figure 2). The canonical pathway (MyD88 dependent) fosters the activation of NF-kB leading to a proinflammatory downstream effect while the non-canonical pathway (MyD88 independent) promotes the activation of IRF3 which creates an anti-inflammatory downstream effect.[33] Interestingly, the non-canonical pathway has been shown to upregulate ISGs (interferon-stimulated genes) including ACE2 in the lungs. This results in high ACE2 expression and thus more infection of the alveoli cells. This process then reinforces the canonical pathway through the presence of more viral DAMPs, creating a hyper-inflammatory state in the lungs.

Previous studies from viruses such as VSV and RSV whose membrane-bound glycoprotein also interacted with TLR4 were shown to lead to the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-18.[15] This was also observed with SARS-CoV-2 S protein interaction with TLR4 leading to the action of the transcription factor NF-kB.[34] The presence of these cytokines, together with viral DAMPs from lytic cells, sensitizes innate immune cells in the lungs to further upregulate TLR4 and worsens the inflammatory response by intensifying the canonical signaling MyD88-dependent pathway. These increased inflammatory responses may then culminate in "cytokine storms" as reported in patients with severe SARS-CoV-2 infection or ARDS (acute respiratory distress syndrome) due to the incessant depletion of surfactants in the alveoli.

SARS-CoV-2 infection of ATII cells through ACE2 receptors leads to viral replication and destruction of these cells. Since ATII cells are the sole producers of surfactants, their destruction leads to decreased surfactant and a compromise in the inhibition of TLR4 in the lungs.[19] Upregulation of TLR4 might engender its interaction with the S protein of SARS-CoV-2, which could lead to a proinflammatory signaling pathway either via the MyD88 or TRIF/TRAM pathway enhancing ISGs and ACE2 expression.[19] Therefore, it is tempting to postulate that increased expression of ACE2 and TLR4 could favor viral infection, replication, and infiltration of proinflammatory innate immune cells with a bias towards the canonical signaling pathway leading to hyperinflammation within the alveoli.

## SARS-CoV-2 Infected Platelets May Trigger the Upregulation of TLR4

Platelets are the smallest of the human blood cells produced from megakaryocytes from the bone marrow. They play a central role in several physiologic and pathologic processes of hemostasis, inflammation, and host defense.[35] Activation of platelets is important for their function and involves a complex interplay of various adhesion and signaling molecules.[35] In addition to playing a key role in regulating tissue homeostasis and clot formation after injury, evidence emerging from numerous reports suggests the involvement of platelets in the pathology of several diseases, including SARS-CoV-2 infection.[36] It should be noted that one of the clinical hallmarks seen in severe COVID-19 patients is abnormal clot formation, which in some cases results in multiple thrombi and disseminated intravascular coagulation.[37] This provides a clear indication of platelets' involvement and contribution to the severity of SARS-CoV-2 pathology, including the heightened inflammatory reactions seen in most COVID-19 patients.[36] Recent studies from Zhang et al involving COVID-19 patients and mice transfused with human ACE2 transgenic platelets revealed an ACE2-MAP kinase-dependent pro-thrombotic and inflammatory response of activated platelets following SARS-CoV-2 infection.[36] The study provided evidence of the expression of the canonical ACE2 and TMPRSS2 receptors of spike protein on platelets, which serve as a key mediator for the spike protein-platelet interactions and subsequent activation of platelets (see Figure 3). The release of inflammatory mediators such as IL-1β, TNF-α and IL-8 was observed in the activated platelets. The authors also showed that the spike protein of SARS-CoV-2 could potentiate thrombus formation following mesenteric arteriole injury in mice transfused with the hACE2 transgenic platelets. In addition, their results revealed a significant expression of the P-selectin adhesion molecule on platelets that mediates the interaction between platelets and PMN leukocytes through the PSGL-1 (P-selectin glycoprotein ligand 1). Although not investigated by Zhang et al, several studies have provided evidence of a possible transfer of the thrombogenic TF (tissue factor) from leukocytes to platelets,[38] with others revealing a direct release of the TF from activated platelets without any prior interactions with monocytes or PMN leukocytes.[39] It should be noted that TF is a key molecule of the extrinsic coagulation pathway and plays an essential role in the production of thrombin (crucial for clot retraction through the production of insoluble fibrin threads from soluble fibrinogen) from the zymogenic prothrombin. Aside from being an important molecule in clot formation, thrombin is also known to be a potent platelet activator and a mediator in TLR4 surface trafficking in activated platelets.[40] There is no existing data on whether the spike protein-ACE2 downstream signalosome in activated

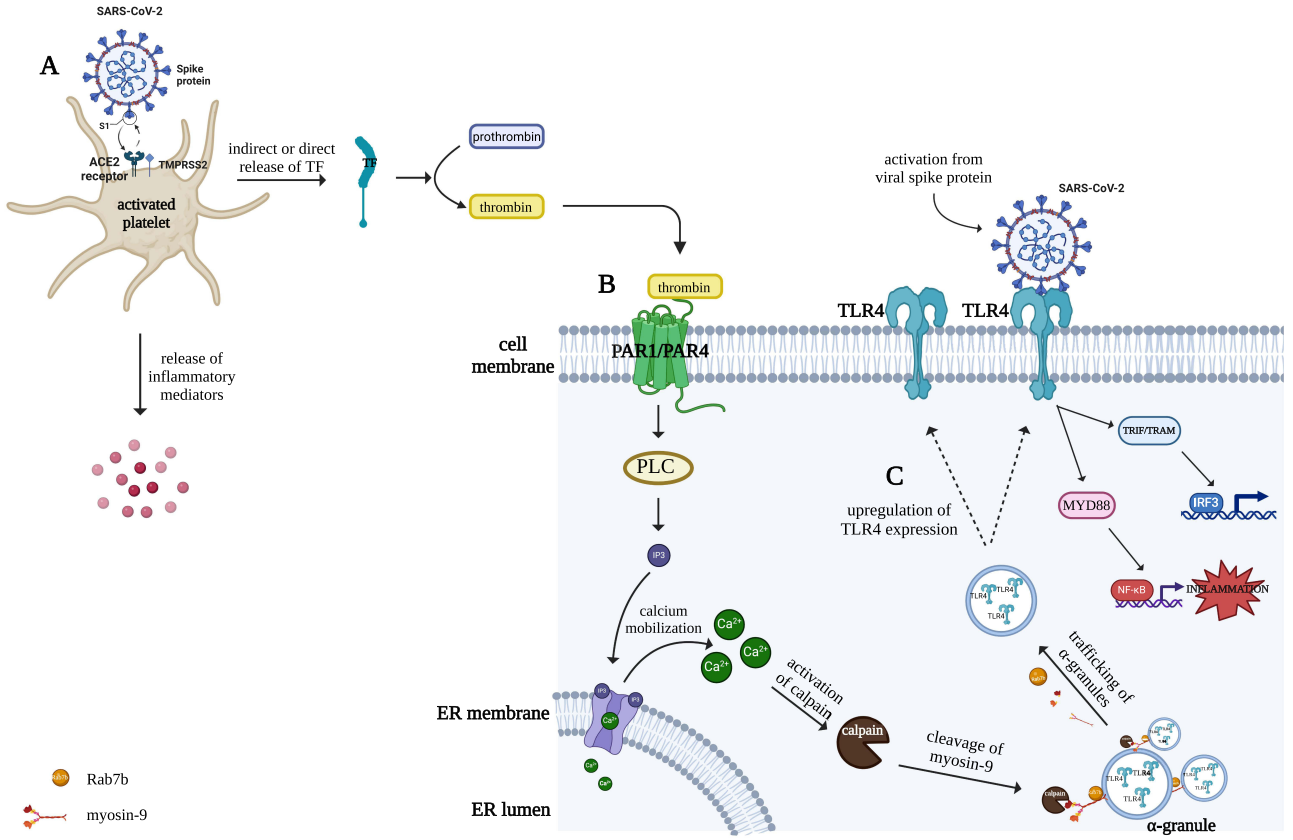


**Figure 3** The figure summarizes the downstream effect of activation of platelets by the SARS-CoV-2 spike protein via the ACE2 receptor. (**A**) Binding of SARS-CoV-2 virus to the ACE2 receptor activates downstream signaling cascades leading to the release of inflammatory mediators and TF which will go on to aid in converting prothrombin to thrombin. The release of TF can be indirect if the platelet previously interacted with monocytes or PMN leukocytes which transferred TF to the platelet for release. Alternatively, it is considered direct when the platelet releases TF upon activation without prior interactions with these cell types. (**B**) Binding of thrombin to the PAR1 or PAR4 receptor leads to intracellular calcium mobilization through the PLC pathway, the calcium-dependent protease calpain is activated and cleaves myosin-9 on the surface of α-granules containing TLR4. This disrupts myosin-9 interaction with Rab7b and the two no longer coordinate to negatively regulate the trafficking of these α-granules. (**C**) TLR4 expression is upregulated due to the intracellular events and the receptor is then accessible for further binding by additional SARS-CoV-2 viruses within the site of infection, which may lead to further inflammation. Created with BioRender.com.

platelets could lead to a thrombin-mediated platelet activation with elevated TLR4 surface expression. However, through data from Jui-Chi et al, where researchers used healthy washed human platelets to determine the expression of TLR4 in thrombin stimulated platelets, it was revealed that there was an increased expression of TLR4 on the surface of thrombin activated platelets. The authors showed that thrombin can either signal through the PAR1 or PAR4 receptors expressed on platelets to induce a PLC (phospholipase C)-dependent intracellular calcium mobilization, which eventually activates calpain and favors intracellular α-granules-containing TLR4 trafficking towards the surface of platelets (see Figure 3). Therefore, the increased expression of TLR4 by thrombin activated platelets was mediated through the intracellular PLC-calcium-calpain axis.[40] It is tempting to speculate that SARS-CoV-2 activation of platelets, as demonstrated by Zhang et al, may also promote intracellular α-granules-containing TLR4 trafficking to the surface of platelets, thereby increasing the expression of TLR4 on the surface of platelets. Whether or not this happens remains to be elucidated. Perhaps, the formation of multiple thrombi seen in severe COVID-19 patients indicates the involvement/presence of thrombin in circulation, which could trigger similar effects seen in the study of Jui-Chi et al. Therefore, further research is needed to explore whether SARS-CoV-2 mediated activation of platelets could culminate in an increased expression of TLR4 on the surface of platelets and if the virus can hijack this receptor to propagate the release of inflammatory mediators. Moreover, it was recently reported that the spike protein of SARS-CoV-2 can colocalize with TLR4 expressed on platelets isolated from COVID-19 patients to drive inflammatory responses and to favor platelet-dependent thrombus growth.[41] This observation was further validated in different experimental settings involving the in vitro stimulation of collagen or thrombin activated platelets (isolated from healthy human donors) with SARS-CoV-2 spike protein. Similar

proinflammatory events were detected followed by increased platelet aggregation and thrombus growth.[41] However, these biological effects were blunted after the cells were treated with inhibitors of the TLR4 signaling pathway, thereby, highlighting the direct impact of the spike protein on TLR4 in driving inflammation and thrombosis.

## The Role of the Renin-Angiotensin System (RAS) Pathway in SARS-COV-2 Mediated Inflammation

The RAS (renin–angiotensin system) is a key component of the cardiovascular system involved in the regulation of blood pressure and blood volume.[42] Renin is produced by the juxtaglomerular cells of the afferent arteriole of the kidney in response to a decline in blood pressure and volume. It is involved in the cleavage of angiotensinogen, which is primarily produced by the liver, to AngI (angiotensin I), which is then converted to AngII (angiotensin II) by angiotensin-converting enzyme produced by the vascular endothelial cells of the pulmonary circulation. AngII binds to either AT1-R (Angiotensin II Type 1 Receptor), or AT2-R (Angiotensin II Type 2 Receptor) to exert its physiological functions.[43] It stimulates vasoconstriction of the arterioles under normal physiological conditions by acting on AT1-R.[43] Although, the predominant functions of AngII are mediated via the AT1-R, there is evidence of vasodilatory effects via interaction with AT2-R. In pathological conditions, AngII-AT1-R interaction is associated with inflammation, oxidative stress, and fibrosis.[44] Its effector function is regulated by ACE2 which cleaves AngII to AngI and has an anti-inflammatory effect.[44] Thus, ACE2 is paramount in the regulation of the physiological and pathological effects of AngII. As earlier explained, ACE2 is the main receptor essential to SARS-COV-2 pathogenesis. Conditions associated with high expression of ACE2 like diabetes, cardiovascular diseases, cigarette smoking, and drugs like statins aggravate the severity of SARS-COV-2 infection by augmenting the expression of receptor-binding sites of the virus.[42,45]

Dysregulation of the RAS pathway is strongly associated with severe COVID 19.[42] This is due to an imbalance in the AngII-ACE2-Ang axis, which results in hyper-activity of AngII. It is well established that the ACE2 receptor is the main binding site of the S1 RBD (receptor-binding domain) of SARS-COV-2 virus. Binding of SARS-COV-2 virus to the ACE2 receptor is associated with downregulation of ACE2 receptors via different mechanisms such as clathrin-dependent and caveolae-dependent endocytosis, and saturation of the binding site by the virus (see Figure 4).[46] The downregulation of the ACE2 receptor favors increased accumulation and effector functions of AngII which contributes to severe COVID-19 disease. Hyper-activation of the AngII-AT1-R axis triggers excessive vasoconstriction, hypoxemia, increased endothelial injury, and tissue necrosis which contribute markedly to increased inflammation and thromboembolic effects characterizing ARDS.[47–49] Furthermore, increased accumulation of danger signals following hypoperfusion-mediated tissue injury contributes to hyper-activation and release of inflammatory mediators by innate immune cells. Other possible mechanisms associated with the induction of AngII mediated hyper-inflammation include the possibility of increased catecholamine activation of macrophages via β-adrenergic receptors expressed in macrophages and other immune cells. Although this mechanism needs further investigation, some studies have shown that β2-AR blockade can reduce inflammatory cytokines in the patient's serum contributing to rebalancing of the immune system in COVID-19.[50]

Several downstream signaling pathways have been linked to the AngII-AT1-R axis, especially in cardiovascular diseases. This includes MAP kinases (ERK 1/2, JNK, p38MAPK), receptor tyrosine kinases (PDGF, EGFR, insulin receptor), NF-κB, and nonreceptor tyrosine kinases (Src, JAK/STAT, FAK (focal adhesion kinase)), and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase pathways (see Figure 4).[51]

The downstream inflammatory signaling pathways associated with activation of the AngII-AT1-R axis in COVID-19 disease are still unclear. Nevertheless, there is increasing evidence of the activation of the NF-KB pathway following Ang II-ATI-R interaction.[52] It has been proposed that this interaction leads to activation of the CBM signalosome and IκB kinase complex resulting in the phosphorylation of IκB and release of NF-κB that stimulates the transcription of various inflammatory mediators.[52] Interestingly, these results have been supported by few animal studies which have elucidated an upsurge in TLR4/NF-κB signaling and M1 cytokine production in macrophages following AngII-AT1-R interaction.[53] Nonetheless, the contributions of these models on hyperinflammation in COVID-19 disease and the underpinning mechanisms still need further investigation. Furthermore, more studies are required to investigate whether the above signaling pathways occur both in vascular endothelial cells and immune cells.

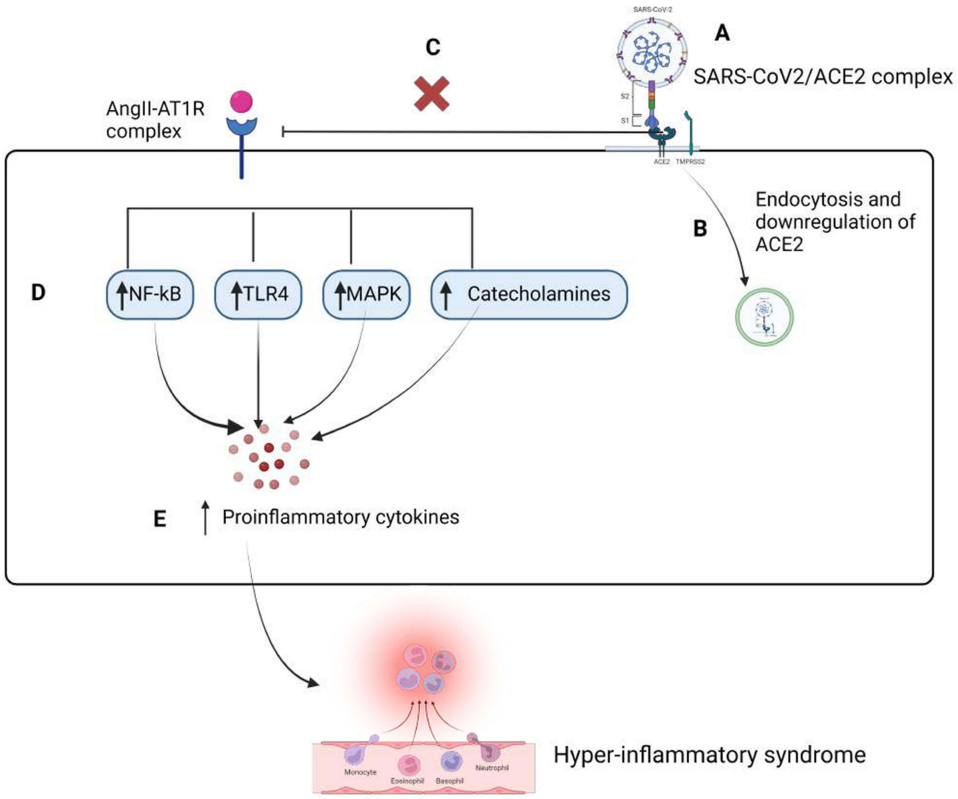


**Figure 4** The Effect of SARS-CoV-2 on the AngII/AT1-R Pathway and the Decrease of ACE2. This illustration presents the complex pathophysiological processes initiated by the abnormal activation of the Angiotensin II (AngII)/Angiotensin II Type 1 Receptor (AT1-R) pathway due to SARS-CoV-2 infection. The initial steps where the virus binds to and reduces ACE2 receptors, which are crucial for managing cardiovascular and inflammatory reactions. This reduction disturbs the equilibrium, enhancing the AngII/AT1-R interaction because of the decreased ACE2-mediated control (**A** and **B**).The effects of this imbalance highlight the increase in signaling pathways that cause inflammation and damage to tissues. The uncontrolled stimulation of the AngII/AT1-R pathway increases pro-inflammatory reactions, leading to a hyperinflammatory state characterized by significant cytokine production, termed a "cytokine storm". This event is a key element in the progression of serious COVID-19 complications, such as acute respiratory distress syndrome (ARDS) and systemic multi-organ failure (**C**–**E**).This figure not only offers a graphical overview but also portrays a detailed summary of the vital interactions between SARS-CoV-2 and the AngII/AT1-R pathway, which are at the core of the severe symptoms of COVID-19 through the reduction of ACE2 and activation of subsequent signaling pathways. It highlights the potential profound effects of viral infection on the regulation of the cardiovascular and immune systems, enhancing our comprehension of the disease's pathology and identifying potential areas for therapeutic intervention. Created with BioRender.com.

## Targeting TLR4 as a Therapeutic Strategy for COVID-19

In response to the COVID-19 pandemic, various existing drugs have been repurposed and used as therapeutic options. However, the most effective strategy against the pandemic has remained vaccination, particularly the mRNA-based vaccines (Pfizer and Moderna) and DNA-based vaccines (AstraZeneca and Johnson & Johnson).[54,55] The high emergence of new strains of the SARS-CoV-2 virus has posed a significant challenge for researchers, as these variants can often evade the protection provided by some vaccines.[54] Given the major role that TLR4 plays in both the immune response and disease pathogenesis, exploring therapeutics targeting TLR4 presents a promising avenue. This approach could potentially offer a broader protective effect that is less influenced by specific viral strains.

One such example of this approach is the AbhiSCoVac vaccine, in which the constructed vaccine peptide is designed to stably engage major immune sensors like TLR2, TLR4, major histocompatibility complex (MHC) class I, and MHC class II.[55] Recognizing the spike protein's interaction with TLR4 as common among coronaviridae family viruses, researchers are focused on identifying specific amino acid sequences responsible for this interaction and screening immunogenic epitopes within these sequences to develop a multi-epitope multi-target chimeric vaccine effective against SARS-CoV-2 and related coronaviruses.[55]

Moreover, understanding TLR4's role in COVID-19 immunopathology suggests that TLR4 antagonists could alleviate inflammation in severe COVID-19 cases, while TLR4 agonists might enhance immunity in high-risk individuals.[54] Accordingly, several TLR4 agonists and antagonists have reached various phases of clinical trials,

including peptides (EC-18), chemical compounds like imiquimod, hydroxychloroquine, and artesunate, as well as small molecules such as PUL-042.[54] Repurposed drugs like alogliptin, sitagliptin, and linag, dipeptidyl peptidase IV-antagonists, have also been investigated for their effectiveness against TLR4-induced inflammation in COVID-19 patients with diabetes comorbidity.[54]

Alternative strategies targeting TLR4 include monoclonal antibodies designed to inhibit its activity. For instance, EB05 (paridiprubart), currently in clinical trials, prevents TLR4 dimer formation, thereby blocking the response to TLR4 agonists such as the SARS-CoV-2 S protein. In a study conducted by Zhou et al, the impact of the TLR4/MyD88 signaling pathway on sepsis-associated ARDS in rats was investigated using an anti-TLR4 monoclonal antibody.[56] The findings indicated that pretreatment with this antibody resulted in reduced lung injury, inflammation, and accumulation of lung fluid, along with improved arterial oxygen levels.[56] Additionally, the levels of TNF-α and IL-1β in both lung fluid and blood were lower, accompanied by decreased expression of TLR4, TLR9, MyD88, and NF-κB in macrophages.[56] While this study focused on sepsis-associated ARDS, the development of monoclonal antibodies targeting TLR4, such as EB05, holds potential for yielding similar beneficial effects in mitigating the exaggerated inflammatory response observed in COVID-19-induced ARDS. This suggests that targeting TLR4 could be a promising therapeutic approach for managing severe respiratory complications in COVID-19 patients by dampening the inflammatory cascade and reducing tissue damage.

Extending beyond traditional antibodies, aptamers are being explored as an innovative approach in targeting TLR4 for COVID-19. Aptamers are short, single-stranded DNA or RNA molecules that can fold into unique 3D structures and specifically bind to target molecules with high affinity and selectivity.[2] Due to their small size, aptamers can generally offer less steric hindrance and better access to binding sites compared to antibodies, and combined with benefits like easy synthesis, lower immunogenicity, and existing uses in detection and therapy, it is evident that aptamers could offer enhanced therapeutic outcomes in the context of COVID-19.[2] Strides have already been made to interfere with the S protein–ACE2 interaction by using aptamers blocking the S1 RBD or the ACE2 receptor. Specifically targeting TLR4, APToll is a notable aptamer currently being developed and is in clinical trials, demonstrating the potential of aptamer-based therapies in addressing the challenges posed by COVID-19.

While vaccination remains the cornerstone of defense against COVID-19, the pivotal role of TLR4 in its immuno-pathology highlights the potential significance of TLR4-targeted therapies. These therapies could not only serve to mitigate inflammation but also to enhance immunity in COVID-19 patients. Additionally, they could offer a broad protective effect that is less influenced by the emergence of new viral strains. From innovative vaccines like AbhiSCoVac to promising treatments such as TLR4 antagonists and novel aptamer-based approaches, ongoing research is poised to advance therapeutic options. Despite these advancements, further exploration and regulatory approval of additional TLR4-targeted therapies are crucial to expand the arsenal of effective treatments against COVID-19.

## Conclusion and Future Perspectives

Since the moment COVID-19 became a global concern, it was evident that we were facing an unprecedented challenge. The rise of different variants of concern not only tested the strength of public health systems but also exposed weaknesses in our existing vaccination approaches, leading to a decrease in vaccine effectiveness and a variety of clinical outcomes. This situation highlights the intricate relationship between the SARS-CoV-2 virus and the human immune system, presenting a complex puzzle for scientists and medical professionals to solve to devise accurate and cost-effective treatments.

The interaction between SARS-CoV-2 and the human immune system involves both the innate and adaptive immune responses, with the virus exploiting certain entry points to enhance its replication and cause severe illness. A significant part of this interaction includes the virus's ability to activate innate immune sensors, particularly Toll-like receptors (TLRs), with TLR4 playing a pivotal role in initiating the inflammatory response observed in severe COVID-19 cases. This response, while a part of the body's defense mechanism, often exacerbates the condition, complicating treatment efforts.

The limitations of current medications, such as non-steroidal anti-inflammatory drugs (NSAIDs), are becoming clearer—they mainly provide symptomatic relief without addressing the virus's replication. This underscores the critical need for antiviral treatments that directly target SARS-CoV-2, offering a more direct approach to combating the

pandemic. Investigating the TLR4 signaling pathway as a therapeutic target could be key in mitigating the intense inflammation seen in many cases.

Despite the extensive global vaccination efforts, they have not entirely halted SARS-CoV-2 transmission. Nevertheless, vaccines have been effective in lessening the severity of the disease in breakthrough infections, underscoring the importance of developing new antiviral drugs that are effective, accessible, and affordable to ensure broad protection against the virus.

At this crucial point in the pandemic, innovative solutions are more necessary than ever. Our review focuses on targeting the TLR4 signaling pathway as a promising strategy, potentially offering the dual benefits of viral suppression and inflammation reduction. This approach represents the comprehensive strategy needed to fight SARS-CoV-2, combining specific therapeutic interventions, continued vaccination campaigns, and adaptable public health measures to defend against current and future threats.

The fight against COVID-19 is far from over, requiring ongoing collaboration among the global scientific community, policymakers, and healthcare providers. Through cutting-edge research and a holistic approach to disease management and prevention, we have a better chance of overcoming the complexities of this pandemic and emerging stronger. Therefore, there is a need for the development of cost-effective drugs against the virus. It has been hypothesized that targeting the TLR4 signaling pathway could help mitigate the hyperinflammation seen in most COVID-19 patients.[57] Recently, several drug molecules targeting the TLR4 pathway[58–62] have shown promising outcomes in alleviating the typical inflammatory reactions induced during SARS-CoV-2 infection. These results support the role of TLR4 signaling pathway in mediating severe COVID-19 associated complications. Therefore, exploring the role of TLR4 signaling in COVID-19 associated lung disease could open therapeutic strategies to improve patient clinical outcome. Undoubtedly, the virus possesses the ability to elicit significant inflammatory responses, either directly or indirectly, by intricately manipulating the host's immune system. Yet, our comprehension of the full array of mechanisms through which this virus prompts inflammation or induces a cytokine storm remains limited. Consequently, there is a pressing need for more in-depth research to unravel the molecular underpinnings of SARS-CoV-2 pathogenesis. This includes a detailed exploration of how the virus interacts with and activates various cell types within the host's body. By gaining a clearer understanding of these processes, we can pave the way for the development of more effective preventive and therapeutic interventions, ultimately advancing our quest to find the ultimate solution to this pandemic.

While this review focuses on the TLR4 pathway and its significant role in the severe inflammation seen with SARS-CoV-2, it does not fully encompass the disease's complex mechanisms. It is crucial to recognize that the immune response to SARS-CoV-2 is far more intricate, involving a wide network of immune sensors and pathways. Specifically, this analysis overlooks the contributions of other innate sensors like Nod-like receptors (NLRs) and C-type lectin receptors (CLRs), which also play pivotal roles in the immune system's response to SARS-CoV-2, potentially influencing the severity of the hyperinflammatory state induced by the virus. Furthermore, recent research has expanded our understanding of SARS-CoV-2's pathophysiology, revealing a diverse immune response that implicates additional toll-like receptors such as TLR7 and TLR3, among others, in the severity of COVID-19. This broader perspective underscores the complexity and dynamic nature of the immunological challenges posed by SARS-CoV-2, highlighting the ongoing discovery of how various innate immune pathways contribute to the disease's progression and severity.

Further, long COVID-19 and post-acute sequelae of SARS-CoV-2 infection (PASC) introduces a significant dimension to the therapeutic strategy against COVID-19. Long COVID-19 and PASC represent a range of symptoms that persist for weeks or months after the acute phase of the infection has resolved, affecting multiple organ systems and significantly impacting the quality of life of post-infection individuals.[63] Persistent inflammation and immune dysregulation are thought to play a central role in these prolonged symptoms. TLR4's involvement in initiating and sustaining inflammatory responses makes it a prime target for therapeutic intervention.[64] By modulating TLR4 activity, we can potentially reduce the chronic inflammation that contributes to ongoing symptoms in long COVID-19 patients. Furthermore, pharmacologic agents targeting TLR4 could help in rebalancing the immune system, reducing the likelihood of autoimmune-like conditions that have been observed in PASC patients.[65] Thus, TLR4 inhibitors not only offer a means to mitigate the acute inflammatory response during the initial infection but also provide a promising strategy to address the long-term sequelae of COVID-19, improving recovery and quality of life for millions of patients worldwide.

## Abbreviations

ACE2, Angiotensin-converting enzyme 2; AP-1, activator protein-1; ARDS, acute respiratory distress syndrome; ATI, alveolar type I pneumocytes; ATII, alveolar type II pneumocytes; AT1-R, Angiotensin II Type 1 Receptor; AT2-R, Angiotensin II Type 2 Receptor; DAMPs, danger associated molecular patterns; FAK, focal adhesion kinase; IRF3, interferon regulatory factor-3; LBP, LPS binding protein; MD2, myeloid differentiation factor 2; MyD88, myeloid differentiation primary response 88; NADPH, nicotinamide adenine dinucleotide phosphate; NF-kB, Nuclear factor-kB; NSAIDs, Non-steroidal anti-inflammatory drugs; PAMPs, pathogen associated molecular patterns; PLC, phospholipase C; PSGL-1, P-selectin glycoprotein ligand 1; RAS, renin-angiotensin system; RBD, receptor-binding domain; sGP, secreted glycoprotein; TF, tissue factor; TIR, toll-IL1 receptor; TLR, Toll-like receptor; TMPRSS2, transmembrane serine protease 2; TRIF, toll/interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β.

## Data Sharing Statement

All data is included within the article.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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The authors declare that they have no competing interests in this work.

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