Antiviral Drugs Employing Toll-like Receptors in SARS-CoV-2

Subjects: Virology

Contributor: Abdul Manan, Rameez Hassan Pirzada, Muhammad Haseeb, Sangdun Choi

The innate immune system facilitates defense mechanisms against pathogen invasion and cell damage. Toll-like receptors (TLRs) assist in the activation of the innate immune system by binding to pathogenic ligands. This leads to the generation of intracellular signaling cascades including the biosynthesis of molecular mediators. TLRs on cell membranes are adept at recognizing viral components. Viruses can modulate the innate immune response with the help of proteins and RNAs that downregulate or upregulate the expression of various TLRs. In the case of COVID-19, molecular modulators such as type 1 interferons interfere with signaling pathways in the host cells, leading to an inflammatory response. Coronaviruses are responsible for an enhanced immune signature of inflammatory chemokines and cytokines. TLRs have been employed as therapeutic agents in viral infections as numerous antiviral Food and Drug Administration-approved drugs are TLR agonists.

Keywords: TLR ; immune system ; inflammation ; antiviral ; SARS-CoV-2

1. Introduction

Toll-like receptors (TLRs) are central mediators of the innate and adaptive immune responses. The immune system exhibits a defense mechanism for the host against pathogenic materials (exogenous and/or endogenous) at the cellular level [1]. Pattern recognition receptors (PRRs) including DNA sensors, RIG-1-like receptors, and TLRs are part of the innate immune system that protects against microbial infection. PRRs recognize conserved pathogen-associated molecular patterns (PAMPs) from microbes and endogenous danger-associated molecular patterns (DAMPs) produced by necrotic cells [2]. PAMPs are derived from viral, bacterial, parasitic, and fungal pathogens. The chemical nature of PAMPs recognized by TLRs varies greatly among organisms. In phylogenetics, TLRs are considered the most ancient class of PRRs. A large number of TLRs have been reported across a wide range of vertebrate and invertebrate species. The signaling pathways and adaptor proteins related to TLRs are evolutionary conserved, from Porifera to mammals. Moreover, similar domain patterns can be observed in most TLR homologs [3][4].

Viruses are responsible for initiating innate immunity through TLRs. Viruses, via a combination of small and unique proteins, not only escape the innate immune system but also destabilize the paybacks of the virus [5]. Similar to other pathogens, viruses are sensed by TLRs. Some viruses encode unique proteins that target TLR signaling. The hepatitis C virus encodes proteins that inhibit TLR-mediated signaling such as NS5A and protease NS3/4A [6][7], which inhibits MyD88 and cleaves TIR-domain-containing adapter-inducing interferon-β (TRIF), respectively. Moreover, the two vaccinia virus proteins have been reported as inhibitors of the TLR system; for example, A52R was observed to inhibit TLR-mediated NF-κB activation by targeting IRAK2 [8], whereas A46R exhibited a connection with TLR signaling downregulation by employing Toll-interleukin-1 receptor (TIR) domain-containing adaptors [9]. Intracellular TLRs not only sense viral and bacterial nucleic acids, but also identify self-nucleic acids in cellular abnormalities such as autoimmunity [10].

A novel single-stranded RNA (ssRNA)-containing virus causes coronavirus disease (COVID-19), also referred to as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which became a pandemic after the first case was identified in Wuhan, China in December 2019. With the spread of COVID-19, the pandemic poses a global challenge [11][12]. From a clinical point of view, the virus has various manifestations ranging from patients becoming critically ill with acute respiratory distress syndrome to asymptomatic infection. In the intensive care unit, multiorgan support therapy has been essential in almost every case of COVID-19 (**Figure 1**). The critical disease stage is typically observed at 7–10 days of clinical infection [11][13]. Hyperinflammatory outcomes (cytokine storm) are mainly associated with clinical impediments and mortality [14]. A possible treatment methodology in the form of vaccines is being employed for the prevention of SARS-CoV-2 infection, but there is no operative therapeutic treatment option available.

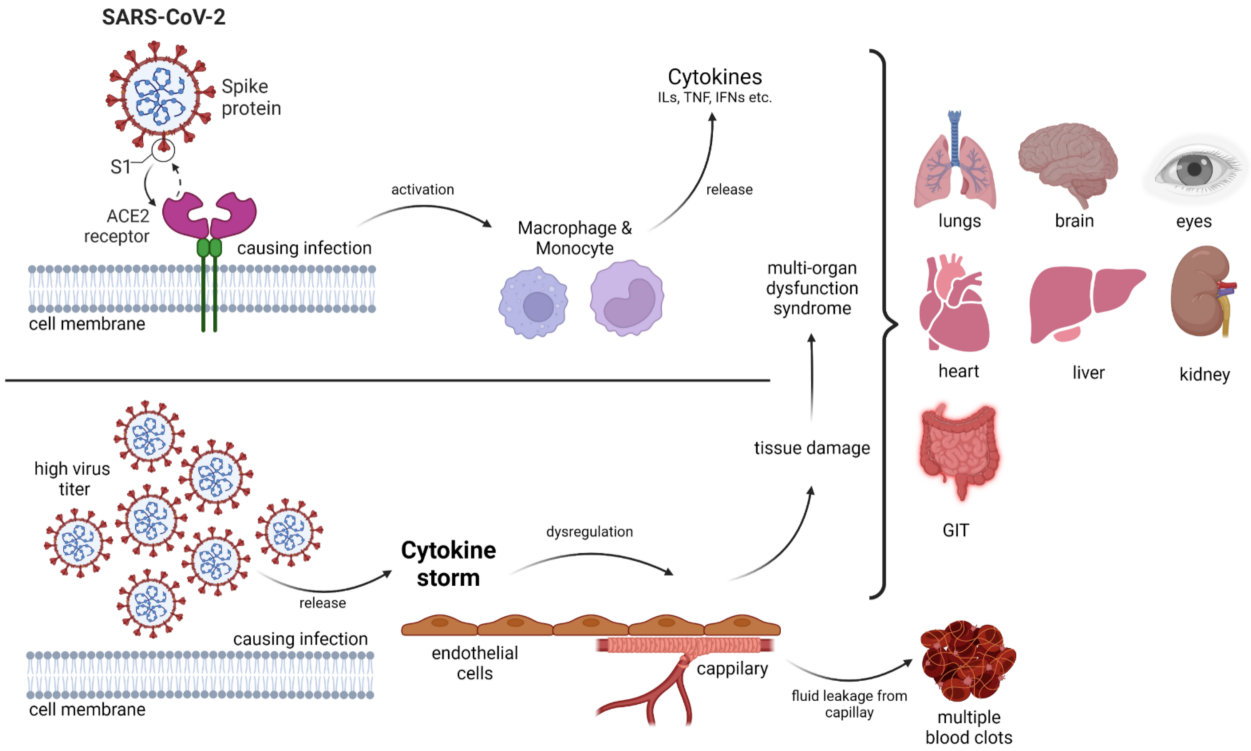

Figure 1. An overview of the SARS-CoV-2 infection pathway. During viral infection, immune cells are activated and release several cytokines as required for the biological system. A high virus titer is associated with a cytokine storm, and such dysregulation in the body of the patient may lead to multi-organ dysfunction syndrome. GIT—gastrointestinal tract.

Structurally, TLRs are type I transmembrane (TM) proteins with three distinct domains including an extracellular domain (ectodomain) that contains tandem copies of leucine-rich repeats, a single-pass TM as well as a cytoplasmic TIR downstream-signaling domain. TLRs experience either homodimerization or heterodimerization when encountering PAMPs and/or DAMPs, and adaptor proteins are employed; subsequently, a complex cellular event of downstream signal transduction is initiated, leading to the expression of inflammatory cytokines and interferons (IFN) that is observable at the molecular level [2]. The underlying TLR signaling cascades have been elucidated using structural, genetic, biochemical, and in silico methodologies [15].

Downstream signaling is made possible by the presence of cytosolic TIR domain-adaptor proteins such as TRIF (also known as TICAM1), TRAM (TICAM2), MyD88, and MAL [5][16]. The involvement of TLRs with TIR adaptors leads to the activation of cytosolic signaling complexes including IRAK and TRAF proteins. These entities are responsible for the activation of transcription factors such as IRF and NF-κB. This executes the synthesis of type I IFNs and proinflammatory cytokines [16]. IRF7 is essential for IFN-α synthesis, NF-κB is necessary for TNF and IL-6 induction, and IRF3 and NF-κB are required for IFN-β production [17].

2. Structure of Coronavirus

SARS-CoV-2, a member of the β-coronavirus genus in the family Coronaviridae, has an envelope and positive-sense ssRNA genome of 29,891 nucleotides, encoding circular nucleocapsid proteins with 9860 amino acid residues [18]. The viral particle size ranges from 80 to 220 nm. Overall, 10 open reading frames (ORFs) have been identified in its genome to date (approximately 26–32 kb). The first ORF (almost 2/3 of the viral RNA) encodes polyproteins 1a (ORF1a) and 1b (ORF1b) [19]. Furthermore, these ORFs are cleaved by proteases into 16 nonstructural proteins (NSPs) that are responsible for genome replication and transcription [20]. Structural proteins (SPs) are encoded by the remaining ORFs [21] [22]. The main SPs and NSPs of SARS-CoV-2 are summarized in **Table 1** and **Table 2**, respectively. The name coronavirus is derived from the appearance under the electron microscope, in which the presence of crown-like spikes on the envelope resembles the corona of the sun [23]. SPs form the viral envelope that holds the RNA genome, while NSPs are expressed in host-infected cells but are not incorporated into virion infectious particles. These NSPs include various transcription factors and enzymes such as RNA-dependent RNA polymerase (RdRp) and hemagglutinin esterase (HE). Moreover, the virion employs enzymes such as RNA replicases and viral proteases to replicate itself [19][24][25][26].

Various SPs have been identified including the glycoprotein membrane (M), spike (S), small envelope (E), and nucleoprotein (N), and other accessory proteins. M-glycoprotein is the most abundant, spanning the membrane bilayer thrice [27]. S-glycoprotein (150 kDa) is a type-I TM protein on the outer surface of the virus and is responsible for the

binding of the virus to host cell receptors (ACE2). The S protein amino acid sequence of SARS-CoV-2 exhibits 86% similarity to that of SARS-CoV [28]. The S protein consists of oligosaccharides bound to serine amino acids through *o*-glycosides. The three major segments of S protein are the ectodomain, TM, and intracellular regions. The intracellular domain comprises the membrane fusion subunit S2 (trimeric stalk) as well as a short tail part known as the receptor-binding S1 domain (RBD; three S1 heads) [29][30]. Protein–protein interaction (PPI) between the human ACE2 and SARS-CoV-2 S protein facilitates viral attachment as well as the cellular entry of coronaviruses; thus, small-molecule blockage of these PPIs is a more inspiring therapeutic approach than inhibition via antibodies [31]. The S1 subunit of the S protein enables ACE2-mediated virus attachment, whereas the S2 subunit facilitates membrane fusion. Specifically, asparagine, glutamine, serine, phenylalanine, and leucine residues present in the S protein boost ACE2 binding [32].

Moreover, N protein bound to nucleic acids is an important structural component of the virus, which is responsible for viral replication and cellular response to infection in the host cellular machinery [28] (**Table 1**). The N protein comprises a serine-rich linker region sandwiched between the N-terminal domain (NTD) and the C-terminal domain (CTD). These termini are crucial for viral entry and processing in host cells. The CTD regulates nucleocapsid formation and the NTD adheres to the viral genome in the form of orthorhombic crystals. Phosphorylation sites are also present in the linker region, which control its function [32]. In the case of SARS-CoV, the N protein enhances the activation of cyclooxygenase-2 (COX-2), resulting in the inflammation of pulmonary cells [33]. Moreover, the N protein interacts with the p42 proteasome subunit, which degrades the virion [34]. This also disables type-I IFN, which is responsible for suppressing the host immune responses produced by biological systems against viral infections [35]. The interaction of the N protein with heterogeneous nuclear ribonucleoproteins leads to increased viral RNA synthesis [36]. The N protein sequence of SARS-CoV-2 shows a 94.3% similarity to that of the SARS-CoV [28].

The smallest TM structural protein in coronaviruses is the E protein (**Table 1**), which comprises two different domains: the NTD (1–9 residues) as well as a hydrophobic domain (10–37 residues), with a chain at the terminal (38–76 residues) [37][38][39]. The E protein plays a crucial biological role, not only in the structural integrity of the virus, but also in host virulence [40]. The E protein sequence of SARS-CoV-2 shows a 96.1% similarity to that of SARS-CoV [28].

The M protein plays a crucial role in maintaining the shape of the viral envelope (**Table 1**). This function can be achieved by interacting with other viral proteins that exhibit PPIs [41]. The M protein is also known as the central organization of coronavirus proteins. The binding of E to M produces the virus envelope, and this interaction is sufficient for the synthesis and release of viruses [42][43]. The binding of M with S is an important event for the retention of the S protein in the endoplasmic reticulum–Golgi complex as well as its integration into new viruses [43][44]. Moreover, the interaction of N with M stabilizes the nucleocapsid (RNA–N protein complex) and the internal core of viruses, resulting in the completion of viral assembly [44][45]. The M protein amino acid sequence of SARS-CoV-2 exhibits a 96.4% similarity with that of SARS-CoV [28].

Table 1. The structural proteins (SPs) of coronaviruses and their physiological significance.

Sr. No.	SPs	PDB ID	Residues	Physiological Significance	Reference
1	E	7K3G	76–109	Virus assembly, morphogenesis, viral–host interaction, membrane permeability	[46]
2	M	8CTK	220–260	Virus assembly, protein interactions (M–M, M–S, M–N)	[47]
3	N	6VY0, 6YUN	422	Abundant RNA-binding protein, virion genome packaging	[48]
4	S	6VYB	1273	Main antigen component, triggers the host immune response	[49]

Table 2. The non-structural proteins (NSPs) of coronaviruses and their physiological significance.

Sr. No.	NSPs	PDB ID	Residues	Physiological Significance	Reference
1	NSP1	7K3N	180	Protein synthesis, prevents antiviral activity of host cells, degrades host mRNA	[50][51][52]
2	NSP2	7MSW	638	Genome replication, disruption of intracellular host signaling	[53][54][55]
3	NSP3 (Papain-like protease, PL_{pro})	7KAG, 6WEY, 6WUU, 7LG0	1945	Integral to viral replication, post-translational processing of the two polyproteins, suppresses host protein synthesis	[19][55][56]
4	NSP4	3GZF	500	Protects new replicated virions, replication and assembly of viral structures in host cell	[57][58]
5	NSP5 (3C-like protease, $3CL_{pro}$)	6LU7	306	Protein cleavage capacity (conserved feature)	[59][60]
6	NSP6	-	290	Induction of autophagosomes, inhibition of viral components to reach host lysosomes	[61][62][63]
7	NSP7	7JLT	83	Primase complex (NSP7-NSP8), hetero-oligomeric complex (NSP7-NSP8-RdRp), viral replication	[64][65][66]
8	NSP8	7JLT	198	Primase complex (NSP7-NSP8), hetero-oligomeric complex (NSP7-NSP8-RdRp), viral replication	[64][65][66]
9	NSP9	6WXD	113	RNA synthesis, carries viral RNA to the host cell, responsible for proliferation	[67][68][69]
10	NSP10	6ZPE	139	Cofactor activation for replicative enzymes, complex NSP10-NSP14, viral RNA proofreading	[70][71][72]
11	NSP11	-	13	Cleavage product of PP1a by $3CL_{pro}/M^{Pro}$	[18][73]
12	NSP12 (RNA polymerase, RdRp)	6YYT	932	RNA polymerase activity	[26][74][75][76][77]
13	NSP13	6JYT	601	Helicase activity	[26][78]
14	NSP14	7R2V	527	Viral RNA methylation, viral RNA proofreading, methyltransferase activity	[70][79][80][81]
15	NSP15	6WXC	346	Endoribonuclease activity	[78][82]

Sr. No.	NSPs	PDB ID	Residues	Physiological Significance	Reference
16	NSP16	6WVN	298	Viral replication, immune response evasion Viral RNA methylation, methyltransferase activity	[81][83][84]

3. Overview of TLR Signaling

Invading pathogens stimulate the release of proinflammatory mediators in response to infection (**Figure 1** and **Figure 2**). Signaling networks are necessary for the protection of the host against invading microorganisms. TLR signaling dysregulation plays a central role in the development and progression of infection. Inflammatory secretory molecules including chemokines, ILs, IFNs, and tumor necrosis factor-alpha (TNF-α) are part and parcel of TLR signaling, resulting in the modulation of cellular characteristics such as apoptosis, immune response, and proliferation [85][86][87]. Mitogen-activated protein kinases (MAPKs) and NF-κB are activated by TLRs. TLR3 and TLR4 are involved in the stimulation of IRF3. In contrast, IRF7 is triggered by TLR7–9 [88]. TLRs are stimulated by interactions with ligands to initiate an intracellular downstream signaling cascade, leading to activation of the host defense system [89].

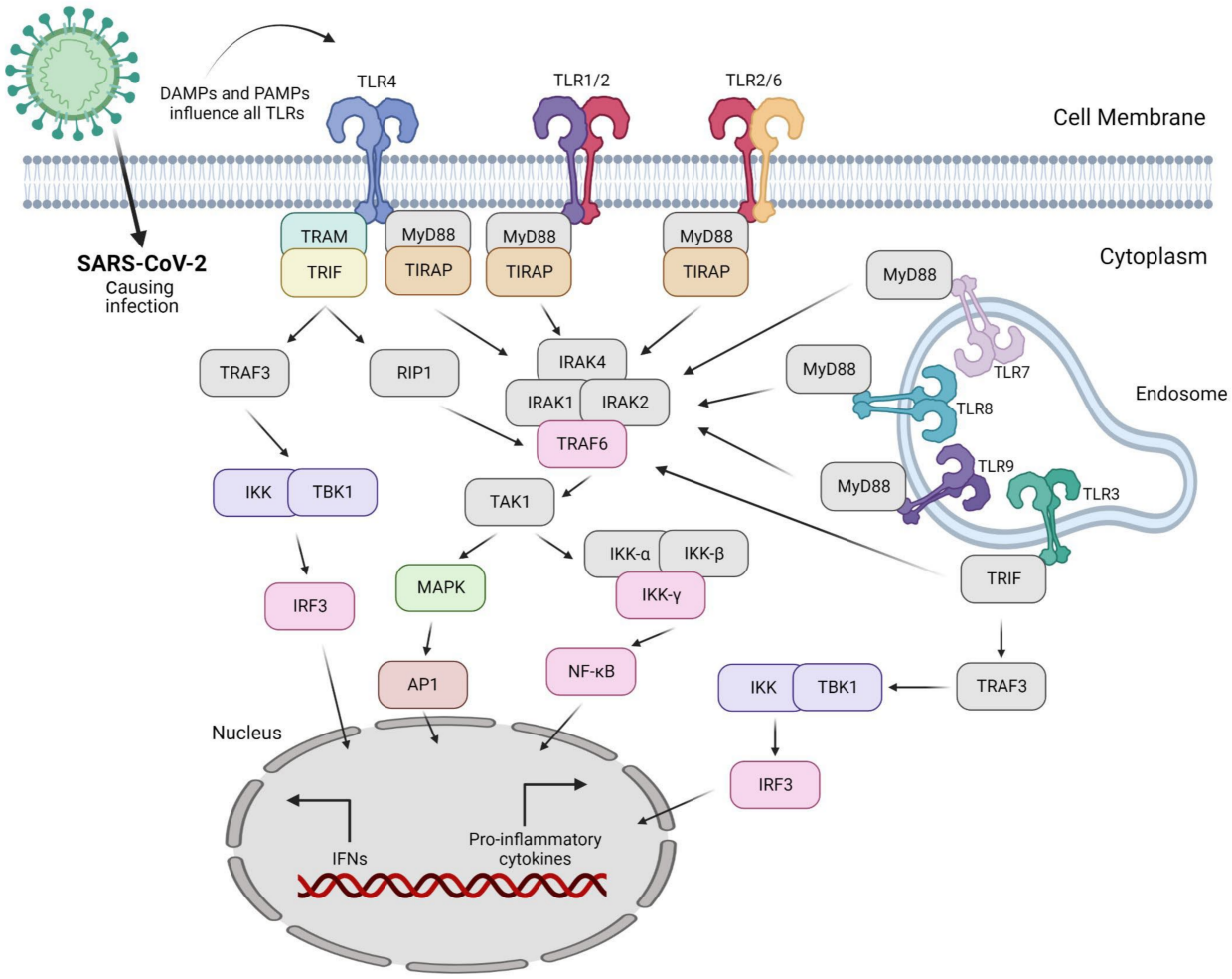

Figure 2. SARS-CoV-2 causes infection in the lungs mainly via DAMPs and PAMPs produced as a result of the action of nearly all Toll-like receptors (TLRs). Only TLRs involved in virus sensing and/or signaling are displayed here.

The nature of the ligand and downstream adaptor molecules directs the TLR signaling cascade (**Table 3**). Two distinct pathways play critical roles in TLR signaling: MyD88-dependent and -independent pathways [90] (**Figure 2**). The former pathway employs all TLRs (except for TLR3), resulting in the biosynthesis of inflammatory cytokines [91]. In contrast, the latter pathway (also referred to as the TRIF-dependent pathway) involves TLR3 and TLR4, resulting in the expression of IFN-I [92]. In other words, the interaction of PAMP and PRR leads to the biosynthesis of proinflammatory cytokines as well as IFN-1, which is a cellular indication of the immune response [93]. Several negative regulators that enhance the activation of the innate immune response are involved in TLR-dependent signaling cascades. Hence, the overactivation of

TLRs can lead to the interruption of immune cell homeostasis, resulting in the risk of inflammatory disorders [94]. Consequently, inhibitors (antagonists) targeting these receptors and/or cascades can serve as novel therapeutics to treat such disorders [95].

Table 3. Toll-like receptors (TLRs) and their physiological significance.

TLRs	Ligand Recognition	Form	Localization	Adaptor Molecules	Negative Adaptors	Response	Reference
TLR1	Triacyl lipopeptides, soluble factors	Heterodimer	Cell surface	MyD88, Mal	-	NF-κB activation and proinflammatory cytokines	[96][97]
TLR2	Hsp70, lipopeptide, HCV, Nonstructural protein 3	Heterodimer	Cell surface	MyD88, Mal	-	NF-κB activation and proinflammatory cytokines	[98][99]
TLR3	dsRNA	Homodimers	Endosomal membrane	TRIF	SARM negatively regulates TRIF	IRF activation, production of type 1 IFNs and proinflammatory cytokines	[100][101]
TLR4	Lipopolysaccharide, Taxol, S protein of SARS-CoV-2	Homodimers	Cell surface	MyD88, Mal, TRIF, TRAM	SARM negatively regulates TRIF and TRAM to consequently reduce inflammation	Activation of NF-κB, pro-inflammatory cytokines, and IFN-inducible genes	[102][103]
TLR5	Flagellin	Homodimers	Cell surface	MyD88	-	Activation of NF-κB and proinflammatory cytokines	[104][105]
TLR6	Diacyl lipopeptides, lipoteichoic acid, fungal zymosan	Heterodimer	Cell surface	MyD88, Mal/TIRAP	-	Activation of NF-κB and proinflammatory cytokines	[106][107]
TLR7	SARS-CoV-2 ssRNA, imadozoquinoline	Homodimers	Endosomal membrane	MyD88	-	IRF activation, production of Type 1 IFNs and proinflammatory cytokines	[108][109]

TLRs	Ligand Recognition	Form	Localization	Adaptor Molecules	Negative Adaptors	Response	Reference
TLR8	SARS-CoV-2 ssRNA		Endosomal membrane	MyD88	-	IRF activation, production of type 1 IFNs and proinflammatory cytokines	[110][111]
TLR9	Unmethylated CPG-containing ssDNA, hemozoin from the malaria parasite	Homodimers	Endosomal membrane	MyD88	-	IRF activation, production of type 1 IFNs and proinflammatory cytokines	[112][113]

4. Role of Antiviral Drugs Employing TLRs

When a pathogen such as a virus invades, an antiviral immune response is evident in the host cells. Various conserved molecular patterns of PAMPs have been identified. As discussed above, TLRs are the key constituents of the innate immune system, and multiple TLRs (TLR1–4, TLR6–9) identify viral ligands [17][114][115][116]. With respect to their functional importance, TLRs might be potentially employed to treat not only inflammatory disorders but also viral diseases. This can be explained by a deep insight into the positive and negative mediators of TLRs [94][117]. TLR agonists lack accessory molecules but can mimic natural ligands; hence, they exhibit a low molecular weight and have potential for expanded pharmacokinetics and pharmacodynamics in comparison with the parent molecule. Moreover, TLR antagonists help to deal with autoimmune and inflammatory disorders by defeating unnecessary inflammation, resulting in an antibody- or cell-mediated response that suppresses disease progression [94][118][119].

Different approaches are employed by viruses in which they weaken their recognition by masking and/or increasing the dysregulation of mediators. Viruses disturb TLR signaling through their own mechanisms. Thus, TLRs are largely involved in the molecular interaction between viruses and host cells [5]. Various PRRs are engaged in the response to viral infection, which is also the case for TLRs. A thorough understanding of this interaction has facilitated the development of various strategies to limit viral infection including antiviral immunity as well as therapeutics [5]. Moreover, viral infection activates TLRs to increase cytokine levels, resulting in an antiviral innate immune response. The interaction between viruses and TLRs at every step of the signaling pathway plays an important role in developing effective antiviral therapies as well as in identifying novel molecular targets for the advancement in antiviral drugs [120]. The regulation of invasion, replication, and immune responses is a significant factor in viral pathogenesis [114]. Viral glycoproteins and NSPs released in the extracellular region are responsible for the stimulation of TLR2 and TLR4 due to their presence on the cellular surface [114][121][122]. In contrast, TLR3, TLR7/8, and TLR9, which are present in the endosomal compartment, contain viral double-stranded RNA (dsRNA) [123], ssRNA [111], and CpG DNA (unmethylated) [113], respectively.

TLR agonists have a positive effect on antiviral immunity and exhibit significant resistance against experimental infections [124][125][126]. The TLR–virus interaction involves a complex mechanism that is associated with the type of TLR as well as the type of virus. Moreover, multiple PRRs are required to initiate an immune response to various viral infections. Moreover, significant differences in TLR signaling have been reported between mice and humans. Therefore, therapeutic manipulation of TLRs requires an understanding of human cellular immunity [127]. Some examples are presented below.

TLR2 activation enhances the innate immune response to viral infections and can be used to treat viral respiratory diseases. Using the shock-and-kill strategy, immune cell recognition is enhanced and latently infected cells are eliminated [109][128]. TLRs can be used to reverse HIV-1 latency and trigger innate immune responses. In an evaluation of the effectiveness of SMU-Z1 (a novel TLR1/2 agonist), in addition to enhancing latent HIV-1 transcription (ex vivo), the NF-κB and MAPK pathways were also targeted in cells [128]. Latency-reversing agents have been employed for HIV reactivation, resulting in enhanced immune activation [109]. Dual TLR2/7 agonists were synthesized and characterized based on their latency-reversing ability, which were found to effectively reactivate the latency. TLR2 components reactivate HIV by NF-κB stimulation and the secretion of IL-22 (thereby enhancing the antiviral state and inhibiting HIV infection), whereas TLR7 components induce the secretion of TNF-α [109]. The activation of TLR2 in vivo has been assessed against rhinovirus

infection [129]. Airway epithelial cells promote an extended immune response characterized by IFN-λ expression, NF-κB activation, and lymphocyte recruitment, resulting in a reduction in viral-induced inflammation and continued antiviral innate immunity [129].

TLR3 (the first identified antiviral TLR) in humans confers protective immunity against vaccinia virus (VACV) infection. In contrast, TLR3 is responsible for the detrimental effects of VACV infection in mice and TLR4 has the same effect in humans [130][131]. The recognition of dsRNA by TLR3 is further evidence of the role of TLRs in the antiviral response [116][123][132]. TLR3 signaling can be activated by a synthetic dsRNA agonist (a potent immune stimulant), resulting in protective immunity against multiple viruses including coronaviruses [133][134][135][136]. Viral-origin ssRNA sequences (rich in GU- and AU-) are detected by TLR7 and TLR8, which are functionally similar and only differ with respect to their expression patterns [110][127]. TLR7/8 expression is evident in dendritic cells, monocytes, and macrophages [137]. Additional examples are listed in **Table 4**.

Table 4. Reported antiviral agonists employing Toll-like receptors (TLRs).

Drugs	TLRs	Viruses	Significance	References
Pam_2CSK_4	TLR2	Parainfluenza	Reduced virus replication	[138]
INNA-051	TLR2	SARS-CoV-2	Reduces viral RNA load	[139]
PIKA	TLR3	Influenza A	Reduces virus load	[140]
Poly ICLC	TLR3	HIV	Release of IFN-α/β/γ	[141]
NA6	TLR4	Norovirus	Induction of IFN-β	[142]
MPL	TLR4	VZV	Stimulate cytokines	[143]
Flagellin	TLR5	Influenza A	Reduces virus replication	[144]
CBLB502	TLR5	ConA	Activation of NF-κB	[145]
Pam_2CSK_4	TLR6	Parainfluenza	Reduces virus replication	[138]
INNA-051	TLR6	SARS-CoV-2	Reduces viral RNA load	[139]
GS-9620	TLR7	HIV	Reactivates latency	[109]
Vesatolimod	TLR7	HIV	Modest delay in viral rebound	[146]
R848	TLR7/8	Zika	Activation of NF-κB	[147]
GS-9688	TLR8	HBV	Activation of dendritic and natural killer cells	[148]
ODN2395	TLR9	Parainfluenza	Reduces viral replication	[138]

CBLB502—Entolimod; ConA—Concanavalin A; GS-9688—Selgantolimod; R848—Resiquimod; NA6—neoagarohexaose; VZV—Varicella-Zoster virus.

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