Toll-like receptor-agonist-based therapies for respiratory viral diseases: thinking outside the cell

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Shareable abstract (@ERSpublications)
Respiratory virus infections are a significant disease burden and new treatment options are required. Treatments that stimulate innate immunity in the upper respiratory tract by targeting Toll-like receptors may provide rapid, pan-viral protection. https://bit.ly/3BNH2Em

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Abstract

Respiratory virus infections initiate in the upper respiratory tract (URT). Innate immunity is critical for initial control of infection at this site, particularly in the absence of mucosal virus-neutralising antibodies. If the innate immune response is inadequate, infection can spread to the lower respiratory tract (LRT) causing community-acquired pneumonia (as exemplified by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)/coronavirus disease 2019). Vaccines for respiratory viruses (influenza and SARS-CoV-2) leverage systemic adaptive immunity to protect from severe lung disease. However, the URT remains vulnerable to infection, enabling viral transmission and posing an ongoing risk of severe disease in populations that lack effective adaptive immunity.

Innate immunity is triggered by host cell recognition of viral pathogen-associated molecular patterns *via* molecular sensors such as Toll-like receptors (TLRs). Here we review the role of TLRs in respiratory viral infections and the potential of TLR-targeted treatments to enhance airway antiviral immunity to limit progression to severe LRT disease and reduce person-to-person viral transmission. By considering cellular localisation and antiviral mechanisms of action and treatment route/timing, we propose that cell surface TLR agonist therapies are a viable strategy for preventing respiratory viral diseases by providing immediate, durable pan-viral protection within the URT.

Introduction

Infection-induced severe respiratory diseases (even prior to the coronavirus disease 2019 (COVID-19) pandemic) are amongst the top contributors to death and disability in adults and children globally [1], causing an estimated 4 million deaths annually [2]. The World Health Organization states that community-acquired pneumonia and other infection-induced lower respiratory diseases were the fourth leading cause of death in 2019 worldwide [3]. Viral infection constitutes the most significant cause of infection-induced respiratory disease and can be attributed to a vast number of viral strains/subtypes from nine different families of respiratory viruses; namely, respiratory syncytial virus, influenza, parainfluenza, rhinovirus (RV), adenovirus, coronavirus, metapneumovirus and bocavirus [4]. Influenza infection alone causes an estimated 250 000–500 000 deaths globally and USD 71–167 billion in associated costs annually [5]. These data pre-date the ongoing and growing impact of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)/COVID-19 pandemic.

Innate immune-mediated protection from virus infections

Innate immunity is not constrained by the need to select antigen-specific lymphocytes, instead employing ubiquitously and constitutively expressed pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs). TLRs activate innate immunity by binding pathogen-associated molecular patterns

(PAMPs) [6]. TLRs on the cell surface (TLR1/2, TLR2/6, TLR4, TLR5 and TLR10) are constantly exposed to extracellular stimuli and regulate immune activation by maintaining quiescence during exposure to innocuous environmental molecules and commensal microbes [7]. The capacity for cell surface TLRs to differentiate 'background environmental and commensal microbial noise' from PAMPs is critical for homeostasis. Other TLRs (TLR3, TLR7/8 and TLR9) are localised intracellularly in endosomes and predominantly detect RNA- or DNA-based molecular structures associated with pathogens. Activation of intracellular TLRs typically induces a potent immune response mediated by type I interferons (IFNs) and inflammatory cytokines. When delayed and over-exuberant, this inflammatory response can contribute to severe respiratory viral illnesses such as COVID-19 [8] and avian influenza [9].

PRRs – TLRs

A key first step in immune activation is detection of pathogens *via* PRR binding of PAMPs, by both non-immune cells (*e.g.* epithelial cells) and immune cells (*e.g.* macrophages, neutrophils). Recognition triggers a "danger signal", activating a pro-inflammatory cascade that recruits and activates innate and adaptive immune cells [10, 11].

The first-described PRRs were the TLRs, with the discovery in 1998 that mammalian TLR4 binds the bacterial component lipopolysaccharide (LPS) [12]. It is now recognised that the human genome encodes 10 TLRs (TLR1–10), which recognise a range of PAMPs across all groups of pathogens. TLRs can be classified into two groups based on their cellular localisation (cell surface *versus* intracellular), which reflect the type of pathogen recognised and their role in regulating and triggering innate immunity. The current review focusses on TLRs, with a focus on the role of each TLR in viral infection and their suitability as therapeutic targets. A summary of TLR ligands, cellular distribution and effects of agonist stimulation is provided in table 1. While a range of additional novel treatment approaches are emerging (*e.g.* miRNAs, nanodrugs, *etc*.), these have been extensively reviewed elsewhere and are beyond the scope of the current review.

Innate immunity to respiratory virus infection

Respiratory viruses typically enter the body *via* the nose and mouth in droplets ejected from the upper respiratory tract (URT) of an infected person. Following transmission, viral infection of airway epithelial cells (AECs) usually (but not always) triggers parallel signalling pathways to stimulate production of IFNs (type I and type III IFNs) and inflammatory cytokines *via* activation of IFN regulatory factors (IRFs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [13]. Timely expression of cytokines and IFNs, growth factors and chemokines rapidly mobilises a coordinated mucosal immune response that contains the infection to the URT and initiates adaptive immunity [13]. This immune response also causes symptoms generally referred to as the "common cold". Virus-induced growth factors and chemokines typically increase neutrophil numbers in blood and sputum [14]. However, virus infection, particularly RV, can also induce eosinophil recruitment in the context of allergic airways diseases such as asthma and lead to an exacerbation [15, 16]. Innate type I and type III IFNs have a range of complex antiviral effects, which are carefully coordinated in response to virus infection [17]. Of relevance to respiratory virus infection, type III IFN-λs are the first IFNs produced upon recognition of virus-associated PAMPs before viral entry [18] and have a key role in local suppression of viral replication by inducing AEC antiviral responses without stimulating systemic inflammation [19]. In contrast, type I IFNs are activated later during virus infection and enhance both antiviral and pro-inflammatory responses that can induce both local (airway) and systemic inflammation [19].

Recognition of PAMPs by TLRs activates IRF- and NF-κB-mediated intracellular signalling pathways [20]. Individual TLRs recruit adaptor molecules containing Toll-IL-1R (TIR) domains and stimulate downstream activation of signalling pathways (figure 1). The exact response induced depends on host factors such as the adaptor molecules recruited, the intensity of TLR activation and the TLR-expressing cell type [21]. The response is also shaped by viral immune evasion genes that interfere with all major components of antiviral innate immune activation pathways identified [22].

Sub-optimal antiviral immune responses characterised by both blunted/reduced [23, 24] and/or delayed [25] cytokine production occur in people with underlying inflammatory respiratory diseases (*e.g.* asthma and chronic obstructive pulmonary disease (COPD)), which limit virus clearance and contribute to exacerbated respiratory disease symptoms. For example, *in vitro* RV infection of AEC cultures from asthmatic donors produce lower levels of IFN-β and interleukin (IL)-15 with increased viral load, compared to epithelial cells from healthy donors [23, 24]. Further, induction of antiviral cytokine production was delayed in differentiated epithelial cell cultures from donors with asthma or COPD compared to healthy donors, in a physiologically relevant low multiplicity of infection model of RV

TABLE 1 Toll-like receptors (TLRs), known ligands, cellular localisation and effects of activation

Receptor	Cellular localisation	Ligand	Agonist	Evidence from agonist treatment of virus infection	Refs
TLR2 (as heterodimer with TLR1 or TLR6)	Cell surface	Pam_2Cys MALP-2 RV VP4 capsid SARS-CoV-2 envelope protein	Acylated lipopeptides Palmitic acid LP-1 $Pam_2C\text{-}SK_4$ PEG-diacylated lipopeptide Pam_2Cys	Type-I IFN responses to vaccinia Protects from lethal influenza infection with no effect on adaptive immune responses Intranasal treatment limits influenza spread to lower airways Induces innate immune priming, enhances early type-III IFN responses and reduces viral load after RV infection Reduces upper respiratory tract viral shedding in SARS-CoV-2 challenge model	[32, 36–40]
TLR3	Intra	dsRNA	Poly I:C PIKA Poly IC:LC	Protection from influenza yellow fever, Rift Valley fever, rabies Adjuvant protecting from HBV	[65, 71–76]
TLR4	Cell surface	LPS RSV F EBOV G VSV G DENV NS1	FimH MPLA	Protects from lethal influenza infection	[46–48]
TLR5	Cell surface	Flagellin		Vaccine adjuvant for influenza Protects from CMV and influenza infection	[51–55]
TLR7/TLR8	Intra	ssRNA	R-848 Imiquimod Loxoribine	Vaccine adjuvant for influenza Antiviral approach for HBV/HCV	[93, 125–130]
TLR9	Intra	Unmethylated DNA	CPG10101 CPG7909	Protects from HCV infection Vaccine adjuvant for HIV	[114–116]
TLR10	Cell surface	Unknown			

CMV: cytomegalovirus; DENV NS1: dengue virus non-structural protein 1; EBOV G: Ebola virus glycoprotein; FimH: fimbriae H protein; HBV: hepatitis B virus; HCV: hepatitis C virus; IFN: interferon; Intra: intracellular/endosomal; LPS: lipopolysaccharide; MALP: macrophage-activating lipopeptide; MPLA: monophosphoryl lipid A; PEG: pegylated; Poly I:C: polyinosinic:polycytidylic acid; Poly IC:LC: polyinosinic-polycytidylic acid stabilised with poly-L-lysine and carboxymethylcellulose; RSV F; respiratory syncytial virus *via* the fusion protein; RV: rhinovirus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; VSV G: vesicular stomatitis virus glycoprotein.

infection [25]. Viruses can also inhibit AEC innate immune activation. AECs infected with human β-coronaviruses OC43 and SARS-CoV-2, despite supporting sustained replication, exhibit deficient expression of type I/III IFNs and inflammatory cytokines which is likely to contribute to delayed onset of symptoms and facilitate transmission [26].

Agonist treatments for viral infection: targeting cell surface TLRs

TLRs localised on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) generally recognise bacterial components, are in constant contact with the surrounding extracellular microenvironment and are involved in maintaining homeostasis [27]. As such, their activation is tightly regulated, to limit unnecessary inflammation (particularly in the gastrointestinal tract [28]). TLR2, TLR4, TLR5 and TLR10 are discussed below. TLR1 and TLR6 function as co-receptors with TLR2, and as such have been included in the TLR2 section.

TLR2

TLR2 activation by virus infection

TLR2 was initially identified as a pathogen receptor for bacterial cell-wall components (*e.g.* peptidoglycan [29]), but is now recognised to be activated by virus components. TLR2 localises to the plasma membrane and forms heterodimers with TLR1 or TLR6, to recognise a broad repertoire of PAMPs (table 1) *via* a common signalling pathway regardless of the specific TLR2 heterodimer activated [30] and inducing similar gene expression signatures [31].

TLR2 has more recently been identified as involved in innate immune activation to a diverse range of viruses. Type-I IFN responses to the poxvirus vaccinia virus (large enveloped virus with double stranded DNA genome) are dependent on internalisation and activation of TLR2 on inflammatory monocytes [32].

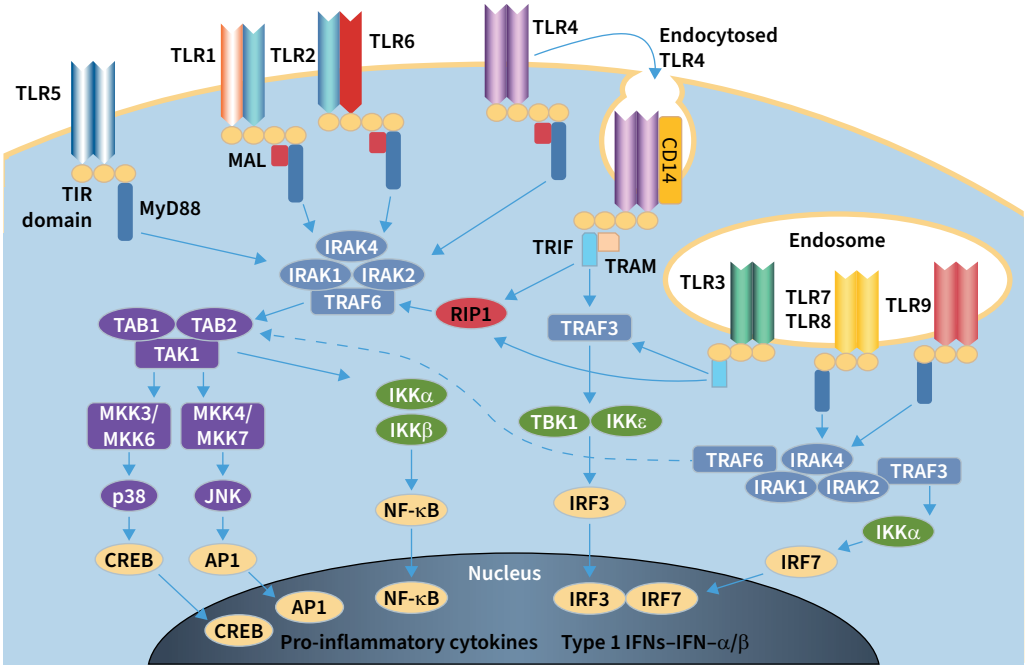

FIGURE 1 Overview of Toll-like receptor (TLR) signalling pathways. A schematic overview of TLR localisation, signalling molecules and downstream activation pathways. AP: activator protein; CREB: cAMP response element binding protein; IFN: interferon; IRAK: interleukin-1 receptor-associated kinase; IRF: IFN regulatory factor; IKKα: IkB kinase α; JNK: c-Jun N-terminal kinase; MKK: mitogen-associated protein kinase kinase; NF-κB: nuclear factor-κB; RIP: receptor-interacting protein; TBK: TANK-binding kinase 1; TIR: Toll-interleukin-1 receptor; TRAF: tumour necrosis factor receptor-associated factor; TRAM: TRIF-related adaptor molecule; TRIF: TIR-domain-containing adaptor-inducing IFN-β.

TLR2 is also implicated in the response to one of the smallest RNA viruses – RV. Myristoylated RV VP4 capsid protein interacts with TLR2 during cell entry and induces pro-inflammatory cytokine gene expression *in vitro* [33]. The envelope protein of β-coronaviruses, including SARS-CoV-2, is also sensed by TLR2. In this context, it has been suggested that TLR2-mediated inflammation contributes to cytokine storm-induced mortality in COVID-19 patients [34]. This might be true for the lower respiratory tract (LRT) and late-stage severe COVID-19 but the opposite appears to be the case for early URT responses to SARS-CoV-2, where innate immunity is significantly dampened and thought to underpin mild/asymptomatic illness and promote transmission [35]. With no pre-existing immunity, this inadequate innate immune response during the initial days following infection almost certainly enables SARS-CoV-2 transmission and contributed to the current pandemic disease.

Therapeutic targeting of TLR2

Administration of the TLR2 agonist Pam_2Cys to the lung protects against lethal influenza infection [36], but had no effect on subsequent development of adaptive immune responses [37]. Further, TLR2 agonist treatment *via* intranasal delivery specifically prevented the spread of influenza infection to the lower airways [38]. We recently reported that prophylactic intranasal administration of a pegylated Pam_2Cys analogue reduced RV lung viral load in mice *in vivo* and in air–liquid interface-cultured primary human bronchial epithelial cell cultures *in vitro* [39]. Prophylactic treatment was effective when administered 7 days before infection and was associated with innate immune activation, increased IFN expression and inhibition of neutrophilic inflammation *in vivo* [39]. Similarly, treatment *in vitro* primed innate immunity defined by upregulated IFN-λ, chemokine and anti-microbial gene expression and an accelerated response to infection, enriched for NF-κB-regulated anti-microbial genes. This boosted response resolved rapidly, coincident with reduced viral load [39]. Of note, protection also occurred in epithelial cell cultures derived from donors with asthma [39], which have delayed responses to RV infection [25]. Further, we recently reported that prophylactic intranasal administration of the related TLR2/6 agonist (INNA-051) reduced levels of SARS-CoV-2 shedding in a ferret infection model [40].

These data highlight that TLR2 promotes innate immune responses to multiple respiratory viruses (influenza, RV and SARS-CoV-2) and that prophylactic TLR2 activation can prime airway immunity for an accelerated response to infection that is primarily associated with early induction of IFN-λ (rather than increased IFN-β expression). A schematic overview of the proposed mechanism of action of TLR2-mediated antiviral responses is included in figure 2.

TLR4

TLR4 activation by virus infection

Prior to ligand engagement, TLR4 localises to the cell surface and is best known for binding to the bacterial cell-wall component LPS, a ligand predominantly associated with Gram-negative (and some Gram-positive) bacteria [12]. TLR4 is unique in that it can be internalised and signal from endosomes where it can sense viral glycoproteins and activates multiple intracellular signalling pathways [41]. CD14-mediated binding of LPS and subsequent interaction with TLR4 induces TLR4 dimerisation and downstream Toll-IL 1 receptor domain containing adaptor protein (TIRAP)/MyD88 activation [42]. TLR4 also migrates to endosomal membranes (in association with CD14), where ligand binding results in TRIF-related adaptor molecule/TIR-domain-containing adaptor-inducing interferon-beta (TRAM/TRIF), IRF-3 signalling and IFN-β expression [43]. Thus, TLR4 intracellular endosomal signalling downstream of TLR4 activation stimulates the production of type I IFNs *via* NF-κB and IRF-3-mediated processes [44]. Like TLR2, TLR4 activation was not previously thought to occur during virus infection. However, a growing list of viruses is now recognised to induce inflammatory responses *via* TLR4 including respiratory syncytial virus (RSV) *via* the fusion protein, Ebola virus glycoprotein, vesicular stomatitis virus

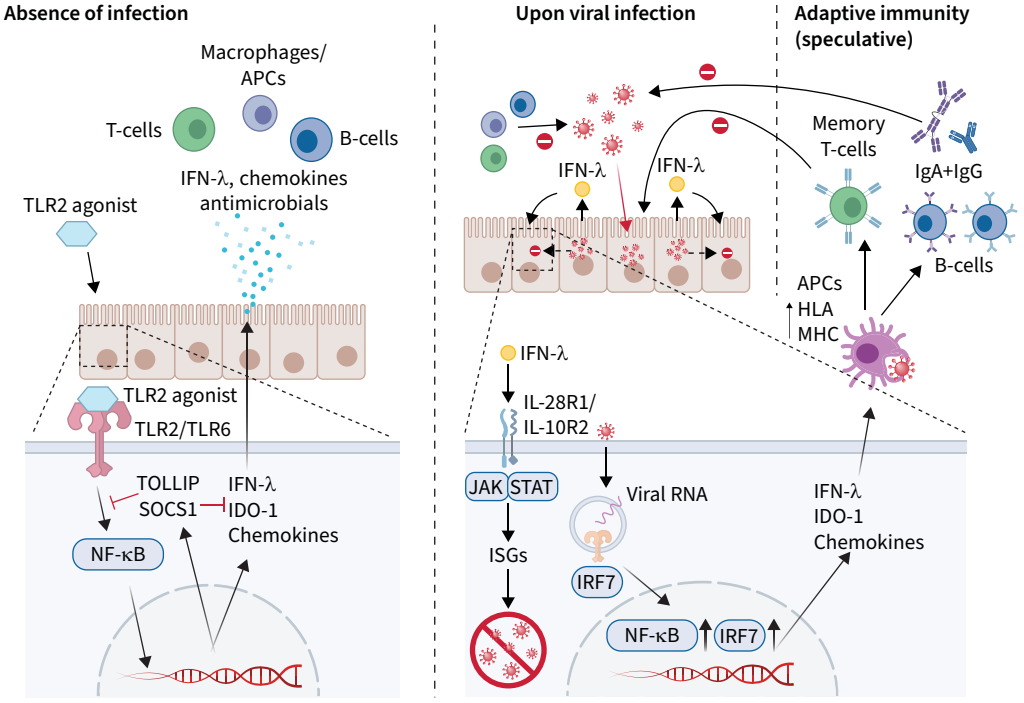

FIGURE 2 Proposed Toll-like receptor 2 (TLR2) agonist treatment mechanism of action. TLR agonists engage the TLR2 heterodimer receptor inducing expression of interferon (IFN)-λ, antimicrobial proteins (*e.g.* indoleamine 2,3-dioxygenase 1 (IDO1)) and chemokine expression through nuclear factor-κB (NF-κB) signalling. Chemokines release recruited lymphocytes, macrophages and antigen-presenting cells (APCs) to the respiratory mucosa to establish innate immune priming. Virus infection after TLR2 agonist treatment increases NF-κB and (IFN regulatory factor 7 (IRF7) signalling, resulting in early expression of IFN-λ following recognition of viral RNA by endosomal TLR3 (double-stranded intermediate) and/or TLR7/8 (single-stranded RNA). Synergy between IFN-λ-mediated IFN-stimulated genes (ISGs), antimicrobial factors, and mucosal lymphocyte activation reduce viral load. Transcriptome data indicate enhanced antigen presentation leading to humoral and cell mediated adaptive immunity. JAK/STAT: Janus-associated kinase/signal transducer and activator of transcription; HLA: human leukocyte antigen; Ig: immunoglobulin; IL: interleukin; MHC: major histocompatibility complex; SOCS1: suppressor of cytokine signalling; TOLLIP: Toll-interacting protein. Image generated using BioRender.

glycoprotein and the dengue virus non-structural protein 1 [45]. Excessive and/or chronic TLR4 activation can contribute to severe inflammatory disease, including acute respiratory distress syndrome (ARDS) and systemic organ failure.

Therapeutic targeting of TLR4

Prophylactic stimulation of TLR4 *via* fimbriae H protein administration protected against lethal influenza A virus infection in a mouse model [46]. Protection was associated with local innate immune activation, increased neutrophil infiltration to the airway lumen and increased levels of inflammatory cytokines tumour necrosis factor-α (TNF-α), RANTES and IL-12 in bronchoalveolar lavage fluid [46]. Administration of monophosphoryl lipid A (MPLA; another TLR4 agonist) also enhanced both mucosal and systemic immune responses to vaccine components, and MPLA treatment prior to infection protected against lethal influenza infection [47, 48]. Whilst there is some encouraging data to support development of TLR4 agonists as a protective treatment for respiratory virus infection, more research has focussed on TLR4 blockade treatment to suppress viral inflammation-induced acute lung injury and ARDS [49]. TLR4's ability to signal from both the plasma membrane and endosomes and reliably controlling immune activation (production of inflammatory cytokines and IFN-β) represent challenges for TLR4-agonist based therapies as an approach to prevent and/or treat respiratory virus infection. TLR4-targeted treatment will require careful consideration of therapeutic windows/timing to provide protection from disease while limiting unintended severe inflammatory disease.

TLR5

TLR5 activation by infection

TLR5 localises to the cell surface and recognises flagellin, a component of bacterial flagella, though there is also clear evidence that TLR5 is involved in the immune response to cytomegalovirus (CMV) [50].

Therapeutic targeting of TLR5

Flagellin is highly immunogenic and has been used as a vaccine adjuvant to stimulate antiviral immunity, *via* fusion to influenza A and influenza B antigens [51–53]. Flagellin administration reduced influenza A virus replication, independently of type I IFN responses and IL-22 signalling, and increased the efficacy of Oseltamivir treatment [54]. TLR5 ligands have also been assessed as standalone prophylactic treatments for CMV infection in mouse models, where administration reduced viral load in the liver, increased cytotoxic natural killer (NK) cell activity and increased numbers of IFN-γ-, granzyme B- and CD107a-producing NK cells [55]. TLR5 ligand administration is a potent adjuvant when administered to the airways [56]. However, TLR5 is quickly degraded in the lung and signalling through TLR5 in the lung is regionalised [56]. Thus, TLR5 agonists may promote antiviral responses in certain settings, but may have limited utility for the treatment of respiratory virus infections.

TLR10

TLR10 activation by virus infection

TLR10 is an orphan receptor lacking a known distinct agonist and is only present as a pseudogene in mice, which has made it difficult to characterise the function of TLR10. TLR10 gene variant overexpression and/or treatment with anti-TLR10 antibodies had no effect on NF-κB activity in the absence of immunological stimulation [57]. However, TLR10 may be anti-inflammatory upon dimerisation with TLR2 [58]. TLR10 antibody treatment in conjunction with Pam_3Cys stimulation of TLR1/2 enhanced production of inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α by peripheral blood mononuclear cells (PBMCs) [58]. Further, silencing TLR10 in monocyte-derived macrophages stimulated greater production of IL-6 [58]. Expression of human TLR10 in mice reduced inflammatory responses (IL-6 and C-X-C motif ligand 1 (CXCL1)/ keratinocytes-derived chemokine (KC)/murine IL-8) to systemic Pam_3Cys administration. In B-cells, antibody-mediated engagement of TLR10 inhibited B-cell proliferation, cytokine production and signal transduction, and TLR10 transgenic mice demonstrated diminished antibody responses to T-cell-dependent and -independent antigens [59]. Dendritic cell (DC) maturation markers and capacity to activate T-cells were reduced upon TLR10 antibody treatment of human monocytes cultures and B-cells after TLR4 or TLR8 stimulation [60]. In addition, stable TLR10 knockdown in a human monocyte cell line reduced cytokine production in response to agonist stimulation of TLR2/6 heterodimers and TLR5 [61].

In the context of virus infection, TLR10 expression was linked to the production of cytokine and IFN responses *in vitro* [62]. TLR10 expression was induced by influenza infection (H1N1 and H5N1) in primary human macrophages and THP-1 cells (human monocyte cell line) [62]. Virus replication (*de novo* protein synthesis) and soluble factors induced by virus infection both induced TLR10 expression, and experimental reduction of TLR10 expression resulted in suppressed IFN and cytokine responses to influenza A virus [62].

Therapeutic targeting of TLR10

Due to the lack of a specific ligand for TLR10, the absence of clear-cut signalling pathway and conflicting reported roles for TLR10 function, TLR10 is not currently a likely target for treatment approaches.

Agonist treatments for viral infection: targeting intracellular-endosomal TLRs

TLRs located on endosomal membranes within intracellular organelles, including TLR3, TLR7, TLR8 and TLR9, directly recognise motifs within viral nucleic acids [63]. Recognition of viral-associated ligands (or intracellular exposure to endogenous ligands as a result of infection) leads to rapid and potent activation, which initiates innate antiviral immunity through IFN production [64]. Viruses with an ssRNA genome that enter cells *via* receptor-mediated endocytosis and replicate in the cytoplasm (*e.g.* RV, paramyxoviruses such as RSV, coronaviruses) generate both single-stranded and double-stranded RNA molecules during their replication cycle. SsRNA directly activates intracellular TLR7/8, while dsRNA is recognised by TLR3. Influenza is somewhat different, being the only RNA-based respiratory virus that replicates in the nucleus. Influenza ssRNA and dsRNA intermediates are sensed by intracellular TLR7 and TLR3, respectively (as well as by additional cytoplasmic PRRs, such as RIG-I and MDA-5) [65, 66].

Activation of intracellular TLRs requires appropriate localisation of the agonist. Drug delivery therefore must be considered. Further, stimulation of intracellular TLRs is more likely to trigger intense pro-inflammatory responses [67–69], which may contribute to unintended pathology – particularly in clinical scenarios where excessive inflammation is the primary driver of disease such as advanced, severe LRT viral illness and viral exacerbation of asthma or COPD.

TLR3

TLR3 activation by virus infection

TLR3 localises to the endosomal membrane and recognises dsRNA, a feature of many virus replication cycles [70]. TLR3 activation stimulates NF-kB activation and downstream antiviral type I IFN production [70]. TLR3 signalling is also activated by exposure to the synthetic molecule Poly I:C (polyinosinic: polycytidylic acid), a chemically stabilised dsRNA analogue.

Therapeutic targeting of TLR3

Numerous studies have demonstrated effects of TLR3 stimulation on virus infection outcomes. Treatment with PIKA, a stabilised derivative of Poly I:C, protected mice from influenza A virus infection (including the 2009 pandemic H1N1 virus) [71]. Protection was associated with increased TNF-α, IFN-γ, CXCL1 (mouse IL-8/KC), IFN-β and recruitment of interstitial macrophages, neutrophils and plasmacytoid dendritic cells (pDCs) [71]. Administration of PIKA or Poly I:C as adjuvants protected against hepatitis B virus (HBV), associated with enhanced cellular and humoral immune responses [72, 73]. TLR3 stimulation *via* polyinosinic-polycytidylic acid stabilised with poly-L-lysine and carboxymethylcellulose (Poly IC:LC) administration protected mice and rhesus monkeys from yellow fever, Rift Valley fever and rabies virus infections and also provided protection against multiple, lethal influenza strains [65, 74]. These studies demonstrate potential for the application of TLR3 agonists for protection against viral infection, however they also identified potential safety concerns.

Toxic effects of Poly IC:LC administration have been observed in animal models (*e.g.* hypothermia and weight loss) which could limit their clinical utility. These effects were mitigated by formulating Poly IC: LC within liposomes, which also enhanced protection from lethal influenza (PR8) infection in mice [75, 76]. Thus, TLR3 agonists have a strong potential to enhance antiviral responses, although careful consideration will be required to determine dosage and treatment regimens to provide protection, while limiting toxic side-effects and excessive inflammation. These considerations will be particularly important when considering TLR3 agonist treatment for patients with pre-existing inflammatory airways diseases.

TLR7/8

TLR7/8 activation by virus infection

TLR7 and TLR8 localise to the endosomal membrane and detect guanosine and uridine-rich ssRNA in the cytosol of infected cells during infection by a range of viruses including human immunodeficiency virus (HIV), influenza virus and RV [77, 78]. TLR7 is activated in immune phagocytes, when viral nucleic acids colocalise with TLR7 following endo-lysosomal fusion [79]. Upon activation, TLR7 signals through MyD88-dependent pathways to induce IFN production [80] *via* the transcription factor, IRF-7 [81–83], a master regulator of type I IFNs [84]. Although TLR7 is expressed in multiple cell types, pDCs are the predominant source of TLR7-induced IFN [85].

Of relevance to respiratory virus-induced exacerbations of lung disease, several studies have reported impaired TLR7 signalling associated with deficient IFN production in people with asthma during RV infection. RV infection of bronchoalveolar lavage or bronchial epithelial cells isolated from people with asthma results in deficient IFN production, compared to infection of cells from healthy controls [23, 86]. Further, IFN production in response to TLR7 stimulation is reduced in PBMCs isolated from people with asthma, compared to cells isolated from healthy controls [87]. Interestingly, people with well-controlled asthma are less likely to exhibit IFN-deficiency [88]. Impaired TLR7 function may also contribute to more severe RV infections in the context of asthma, based on data obtained from mouse models. Eosinophilic inflammation induced by IL-5, suppressed TLR7 expression in a mouse model of allergic airways disease, resulting in less IFN production in response to RV-A1 infection [89]. TLR7 knockout mice ($TLR7^{-/-}$) also had decreased IFN responses, exaggerated eosinophilic inflammation and increased airway hyperresponsiveness to methacholine, which was restored by adoptive transfer of TLR7-competent wild-type pDCs [89]. In line with these observations, TLR-agonists can be used to potentiate allergic inflammation or even induce tolerogenic profiles in response to allergens and although this falls outside the scope of this review, this concept has been reviewed elsewhere [90].

Therapeutic targeting of TLR7/8

TLR7 and TLR8 activation and downstream IFN production can be induced by exposure to a range of synthetic agonists, including R-848, imiquimod or loxoribine (a synthetic nucleoside) [91, 92]. Because of the pivotal role of TLR7 in immune responses against viruses, TLR7 agonists are currently used as vaccine adjuvants for multiple strains of influenza [93] and being assessed as an antiviral treatment approach for HBV and hepatitis C virus (HCV) infection [94–99]. For example, TLR7 agonist treatment suppressed HBV replication in a HepG2.2.15 cell line [94], provided long-term suppression in HBV-infected chimpanzees [98] and had efficacy in a mouse HCV infection model [99]. TLR7 agonist treatment was relatively well-tolerated in patients with ongoing HBV [97] or HCV infection [96], although treatment did not significantly alter virus levels. In other studies, TLR7 agonists such as PF-4878691 have exhibited a narrow therapeutic range, with severe adverse effects observed with the dose levels required to generate antiviral efficacy mediated by IFN-α induction, leading to early termination of clinical trials [100].

Thus, TLR7 activation is relevant in the context of respiratory disease (*i.e.* asthma) and respiratory virus infection. TLR7 agonist administration has been assessed for vaccine development and potential antiviral therapy, but not as a therapeutic approach for the prevention of respiratory virus infection in the context of underlying inflammatory disease. TLR7 agonist treatment is also not well suited for treatment of acute, severe respiratory viral lung disease with potential contribution to acute virus-induced immunopathology exemplified by a study that reported blockade of TLR7 reduced mortality in a mouse model of severe influenza. Rather than reducing the innate immune response in AECs, the TLR7 antagonist reduced excessive type I IFN and inflammatory cytokine/chemokine production by pDC and monocytes and this reduced mortality [101]. This also highlights the safety concerns related to bystander immune cell activation by topical (*e.g.* inhaled) exposure to TLR7 agonists which may be addressed using drug delivery platforms such as nanoparticles that shield the agonist from immune cells and facilitate delivery to virus-infected AECs [102].

TLR9

TLR9 activation by virus infection

TLR9 localises to the endosomal membrane and recognises microbial DNA containing unmethylated CpG dideoxynucleotides [103, 104]. TLR9 is expressed by a diverse range of immune cells [105, 106] and is activated in response to infection by DNA viruses, including poxviruses [107], herpesviruses [108] and adenoviruses [109]. Overlap between TLR9-expressing cell types and downstream pathology have prompted speculation that TLR9 may mediate the severe symptoms of SARS-CoV-2/COVID-19 infection [110].

Therapeutic targeting of TLR9

Synthetic CpG oligodeoxynucleotides have primarily been assessed as vaccine adjuvants [111, 112] and standalone therapies for the treatment of chronic infections (*e.g.* HCV). Numerous clinical trials assessing TLR9 agonist administration have provided valuable insight into the feasibility and safety of TLR agonists as a treatment approach. Subcutaneous administration of CPG7909 (a synthetic TLR9 agonist) to healthy volunteers promoted systemic Th1 responses, characterised by increased IL-6, IL-12p40, IFN-α, and IFN-inducible chemokines (including CXCL10) [113]. Administration of the TLR9 agonist CPG10101 prior to HCV infection stimulated cytokine production (CXCL10), antiviral responses (IFN-α, oligoadenylate synthetase) and decreased HCV RNA [114]. Treatment was well-tolerated, and the mild adverse events observed were similar to those observed following recombinant IFN-α treatment [115]. Vaccines containing CPG7909 reduced HIV pro-viral load and increase numbers of HIV-specific $CD8^{+}$

T-cells [116]. While some clinical trials assessing TLR9 agonists have reported adverse events [117], the majority of the events have been mild (*e.g.* injection site reactions) and TLR9 agonist treatment has generally been well tolerated. Severe adverse effects (*e.g.* high-grade neutropaenia and electrolyte disturbances) were reported when TLR9 agonists were paired with chemotherapy for the treatment of advanced non-small-cell lung cancer [118, 119], although these effects have not been observed in subsequent trials [120]. Whether TLR9 agonist administration or the combination therapies applied cause these effects remain unclear.

Treatment delivery route

It is important to also consider the route / method of treatment delivery and downstream effects. As outlined above, respiratory virus infections begin upon exposure to virus in URT, resulting in "common cold" symptoms [121]. Severe symptoms leading to community acquired pneumonia are typically associated with subsequent spread of the virus into the lower respiratory tract. Emerging evidence suggests that severe disease may also be triggered by direct droplet aspiration of SARS-CoV-2 [122], although the relative contribution of this route of entry remains uncertain. Treatments which are delivered systemically (*e.g.* oral delivery and injected vaccines) often fail to establish effective adaptive immune responses in the URT, which has also been reported in primate studies of SARS-CoV-2 vaccines [123, 124]. Targeting the virus at the URT involves directly administering to the nasal mucosa using a nasal spray. Depending on the drug, this has the potential added benefit of limiting systemic exposure and associated adverse events. In particular, chronic fatigue associated with systemic TLR-driven inflammation can be avoided with localised administration, restricted to the URT mucosa [125, 126]. We propose that immune-modulatory treatments which can be delivered and/or targeted directly to the respiratory tract will safely limit virus replication in the early stages of infection, which may reduce peak viral load and reduce virus spread, even where effective vaccines are available. Children are less susceptible to SARS-CoV-2-induced severe illness, likely due (in part) to enhanced innate immune control of viral infection in the URT, with differences in cell populations and IFN-mediated responses observed, in addition to heightened expression of PRRs [127, 128] leading to reduced markers of systemic IFN responses, indicative of better control of the virus in respiratory tract [129]. URT delivery (*e.g.* nasal spray) has the potential to establish early local immunity, limit infection initiation and reduce progression to LRT disease and person-to-person transmission.

Prevention of respiratory viral disease

The phenomenal success of vaccines at preventing human viral diseases (recently exemplified by COVID-19 vaccines) cannot be overstated and is irrefutable evidence that immunity can be harnessed to prevent infectious diseases. Of the 10+ vaccine-preventable viral diseases, only two target respiratory viruses (influenza and COVID-19), though a vaccine to respiratory syncytial virus is on the horizon [130]. This will likely increase in coming years, expedited by the emergence of new vaccine platforms (*e.g.* mRNA vaccines) and enhanced recognition of the burden of respiratory virus infections. However, several factors limit the availability and efficacy of vaccine approaches for respiratory viral infection.

RV serves as a useful case study for the challenges confronting preventative treatment/vaccine development. RV is the most common human respiratory viral pathogen and causes up to 60% of annual respiratory illnesses globally [131, 132]. Failure to adequately control RV infection to the URT can lead to LRT infection and severe disease, including bronchiolitis and community acquired pneumonia [121]. There are no preventive treatments or vaccines for RV infection and treatment is limited to supportive, non-specific options (*e.g.* non-steroidal anti-inflammatory drugs [133]).

Vaccine limitations

Effective vaccines for respiratory viruses are difficult to develop, particularly for viruses with numerous subtypes and/or genetic/antigenic diversity. Decades of research since the discovery of RV [134] have not yielded an effective vaccine. A major challenge is the large number (>160) of genetically distinct subtypes [135]. While early RV vaccines effectively induced humoral immune responses against specific RV strains [136], they failed to induce broad cross-reactive immunity [137, 138]. While multivalent vaccines effectively generated antibody responses against 25 and 50 RV strains in mice or rhesus macaques, respectively [139], it remains unclear how many subtypes would have to be included to yield broad clinical benefit. Influenza virus can also reshuffle its segmented genome, requiring updated influenza vaccines that protect against new virus strains [140]. Further, influenza A viruses (like β-coronaviruses) reside in animal reservoirs, provide ongoing potential for spill over and emergence of novel human viruses [141]. As vaccine development requires a detailed knowledge of virus genome sequence, structure and replication cycle, vaccine availability lags for new human viruses. Even where vaccines are available,

non-vaccine preventative treatments are important for population groups that are not well protected by vaccination. A broadly effective preventative treatment that leverages innate immunity could address this unmet need.

Conclusions

TLRs are promising therapeutic targets for the prevention of respiratory virus infection. Standalone TLR agonist treatments have demonstrated antiviral effects in a range of infections. We note that careful consideration should be given to the effects of TLR agonist treatment on inflammatory mediators and type I IFN production. Cell surface TLRs that are not classically associated with antiviral virus immunity, particularly TLR2/6, have recently shown promise in pre-clinical studies against multiple different respiratory viruses *via* innate immune priming of the respiratory mucosa. COVID-19 has reinforced the understanding that innate immunity in the URT plays a key role in disease caused by respiratory viruses and is therefore a viable preventative treatment option, particularly for those at high risk of infection and severe disease.

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