



## Review

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# Toll-like receptor 3 (TLR3) regulation mechanisms and roles in antiviral innate immune responses

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**Abstract:** Toll-like receptor 3 (TLR3) is a member of the TLR family, mediating the transcriptional induction of type I interferons (IFNs), proinflammatory cytokines, and chemokines, thereby collectively establishing an antiviral host response. Studies have shown that unlike other TLR family members, TLR3 is the only RNA sensor that is utterly dependent on the Toll-interleukin-1 receptor (TIR)-domain-containing adaptor-inducing IFN- $\beta$  (TRIF). However, the details of how the TLR3-TRIF signaling pathway works in an antiviral response and how it is regulated are unclear. In this review, we focus on recent advances in understanding the antiviral mechanism of the TRIF pathway and describe the essential characteristics of TLR3 and its antiviral effects. Advancing our understanding of TLR3 may contribute to disease diagnosis and could foster the development of novel treatments for viral diseases.

**Key words:** Toll-like receptor 3 (TLR3); Toll-interleukin-1 receptor (TIR)-domain-containing adaptor-inducing interferon- $\beta$  (TRIF); Innate immune; Antiviral response

## 1 Introduction

Antiviral innate immune and inflammatory responses are the body's front lines of defense against viral infection (Yang and Shu, 2020). Rapid and effective recognition of microbial infection or danger signals from within the cell by the innate immune system is a premise for eliciting host responses to repulse the threats (Liu and Gack, 2020). The innate immunity of animals depends on pattern-recognition receptors (PRRs) that specifically recognize pathogen-associated molecular patterns (PAMPs) and then activate a signaling cascade triggering the type I interferon (IFN)- and interleukin-1 (IL-1)-mediated proinflammatory responses (Rai, 2020). The virus-perceiving PRRs mainly include Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and DNA sensors, such as cyclic guanosine

mono-phosphate-adenosine monophosphate (GMP-AMP) synthase (cGAS), IFN- $\gamma$ -inducible protein-16 (IFI16), and the recently identified heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2B1) (Unterholzner et al., 2010; Sun et al., 2013; Wang L et al., 2019). TLRs are expressed mostly on the membranes of endosomes and lysosomes or on the surfaces of macrophages and dendritic cells (DCs), among other cell types, and are used to detect a wide range of microbial cell-wall-associated PAMPs and endosomal nucleic acids (Blasius and Beutler, 2010; Liu and Gack, 2020). RLRs are representative of cytoplasmic PRRs, which recognize both viral and host RNAs, even in sterile conditions via different mechanisms (Loo and Gale, 2011; Rehwinkel and Gack, 2020). Most RNA sensors reside in the endosome and cytoplasm, while PRR-mediated RNA sensing was recently shown also to occur in the nucleus and mitochondrion (Liu et al., 2018; Cao LL et al., 2019; Wang Y et al., 2019). In addition to PAMPs, PRRs can recognize tissue damage-associated molecular patterns (DAMPs) and play an essential role in promoting tissue repair and regeneration. However, they also cause numerous inflammatory diseases, such as metabolic disorders

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and autoimmune diseases (Mortaz et al., 2017; Gong et al., 2020).

TLRs were the first PRRs to be identified and are the best characterized (Kawasaki and Kawai, 2014). The TLR family consists of 10 members (TLR1–TLR10) in humans and 12 members (TLR1–TLR9, TLR11–TLR13) in mice (Takeda and Akira, 2015). Each TLR is composed of an ectodomain (ECD) that mediates PAMP recognition, a transmembrane domain, and a cytoplasmic Toll-IL-1 receptor (TIR) domain responsible for downstream signaling (Kang and Lee, 2011). The signaling pathway of TLRs mainly includes the myeloid differentiation primary response protein 88 (Myd88)-dependent TLR signaling pathway and the TIR-domain-containing adaptor-inducing IFN- $\beta$  (TRIF)-dependent TLR signaling pathway. The members of the TLR family selectively use adaptor proteins, including the TRIF (also called TIR-containing adapter molecule 1 (TICAM-1)), MyD88, transverse rectus abdominis musculocutaneous (TRAM) flap, and TIR-containing adaptor protein (TIRAP), to activate overlapping but distinct signaling pathways. These pathways initiate the transcriptional induction of mediators such as type I IFN and chemokines (Kawasaki and Kawai, 2014). TLR1, TLR2, and TLR5–TLR9 work via the Myd88-dependent TLR signaling pathway, TLR3 works via the TRIF-dependent TLR signaling pathway, and TLR4 triggers both pathways (Takeda and Akira, 2015).

TLR3 recognizes double-stranded RNA (dsRNA), which is a viral replication intermediate, and initiates downstream signal transduction, thereby up-regulating the expression of IFN- $\alpha/\beta$  and inducing antiviral protein (AVP) synthesis activity (Matsumoto et al., 2011). DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 (DDX1), DDX21, and DHX36, the members of the DExD/H-box helicase cytosolic sensors of dsRNA, share with TLR3, the TRIF for downstream type I IFN signaling (Zhang et al., 2011). TLR3 is the only receptor in the TLR family that depends entirely on the TRIF to induce IFN- $\beta$  production (Yang and Shu, 2020). According to the different downstream products activated by TRIF, the TLR3-mediated signaling pathway can be divided into the TRIF-dependent nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B) pathway and TRIF-dependent IFN-regulatory factor 3/7 (IRF3/7) pathway (Matsumoto et al., 2011). Once activated on the

plasmalemma by exogenous dsRNA, TRIF interacts with tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) and TRAF6 to trigger a series of cascade reactions. The mechanisms and negative regulation of TRIF are currently areas of active research that we emphasize in this review.

## 2 Characteristics and recognition of TLR3

### 2.1 Basic characteristics of TLR3

TLR3 is widely expressed in neurocytes, immune cells, fibroblasts, and various epithelial cells (Kulka et al., 2004; Town et al., 2006; Fang et al., 2013; Bugge et al., 2017; Chen et al., 2019). Among immune cells, only myeloid DCs, macrophages, and mast cells (MCs) express TLR3. TLR3 localizes both at the cell surface and in endosomes in MCs and macrophages, but only in endosomes in myeloid DCs (Matsumoto et al., 2003, 2011; Agier et al., 2016). DCs are one of the most important immune cells that produce IFNs, including type I IFNs ( $\alpha$  and  $\beta$ ), which are associated with viral clearance (Matsumoto et al., 2003). TLR3 works in antigen-presenting DCs to induce lymphocyte-mediated antigen-specific immune responses (Matsumoto et al., 2020; Soto et al., 2020). MCs have been reported to act as sentinel cells of innate immunity, extensively engaged in infection control and clearance (Piliponsky and Romani, 2018; Marshall et al., 2019). MCs express TLR3 molecules and other proteins associated with the cellular antiviral response, like IRF3, types I and II IFN receptors, and major histocompatibility complex (MHC) I (Witczak et al., 2020).

TLR3 consists of an ECD, a cytoplasmic TIR domain, a transmembrane domain, and two flanking regions, known as the leucine-rich repeat (LRR) C-terminal (LRR-CT) and N-terminal (LRR-NT) regions (Choe et al., 2005). The TLR3-transmembrane domain and ECD structures have been resolved through X-ray crystallography; nevertheless, the structure of a membrane-solvated full-length receptor remains inaccessible (Liu et al., 2008; Mineev et al., 2014). Molecular dynamics simulation has demonstrated that the stabilization of the TLR3-TIR dimerization interface relies on reciprocal contact between the  $\alpha$ C and  $\alpha$ D helices of one subunit and the  $\alpha$ C helix and BB loop of the other (Patra et al., 2020). As a

highly conserved region in the TIR domain, the BB loop is essential for mediating interactions among TIR domain-containing proteins (Singh et al., 2014). In the TLR3 A795P homodimer, the individual subunits are tilted slightly toward each other, which influences the orientation of the BB loops on the homodimer and, in turn, the binding of the TIR domain of TRIF to the homodimer (Mahita and Sowdhamini, 2018).

## 2.2 Recognition of dsRNA by TLR3

### 2.2.1 Activation of TLR3

The common ligands of TLR3 exist on poly(I:C), dsRNA viruses (e.g., rotavirus (RV), respiratory syncytial virus (RSV), murine cytomegalovirus (MCMV)), and single-stranded RNA (ssRNA) virus (e.g., West Nile virus (WNV)) (Alexopoulou et al., 2001; Topping and Kelly, 2019; Uehata and Takeuchi, 2020). A recent report showed that endothelial TLR3 could also detect extracellular dsRNA that is secreted from highly metastatic tumors (Tavora et al., 2020). TLR3 exists as a monomer and a membrane receptor in resting cells, and dimerizes only when bound to ligands via its ECD (Botos et al., 2009). The TLR3 ECD is formed by 23 LRRs, and the crystal structure resembles a sizeable horseshoe-shaped solenoid (Botos et al., 2009). Binding of the ECD domain of TLR3 to dsRNA requires an acidic environment (Leonard et al., 2008). As TLR3 functions mainly in the endosomes, several mechanisms have been suggested to account for delivery of extracellular TLR3-activatory dsRNA molecules into endosomes (Tabeta et al., 2006; Barton and Kagan, 2009; Pelka et al., 2018). Mainstream theories include the uptake of apoptotic bodies from infected cells (Salio and Cerundolo, 2005), clathrin-dependent endocytosis (Itoh et al., 2008; Watanabe et al., 2011), formation of dsRNA complexes with antimicrobial peptide LL-37 (Singh et al., 2013; Takahashi et al., 2018), and autophagic uptake of dsRNA from the cytosol and trafficking to endosomes with inhibited lysosomal degradation (Sørensen et al., 2018; Galluzzi and Green, 2019; Hase et al., 2020). Whether TLR3 can be activated from the cell surface is still unknown.

### 2.2.2 Structural basis of TLR3

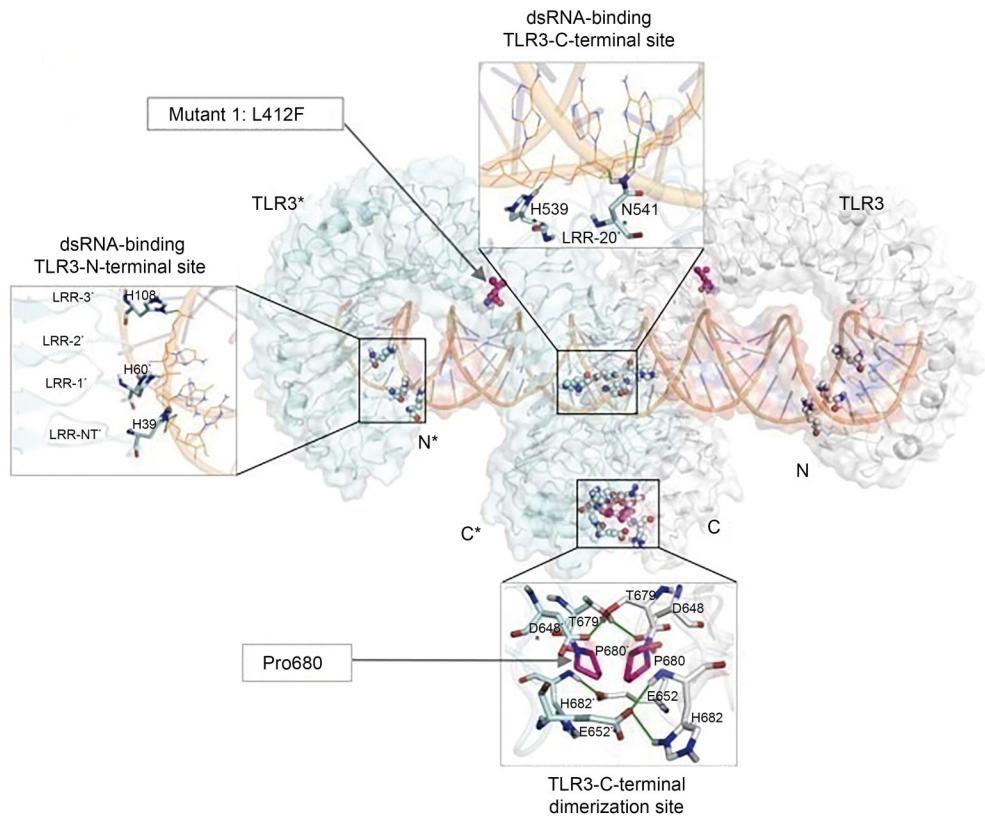
When TLR3 associates with dsRNA, a dsRNA-TLR3 signaling complex composed of one dsRNA

and two TLR3 molecules is formed (Liu et al., 2008; Peisley and Hur, 2013). The molecular structure of a signaling unit (SU) shows that dsRNA ligands bind two regions, one at the C-terminus (LRR19–LRR21) and the other near the N-terminus (LRR-NT and LRR1–LRR3) (Gao et al., 2015). TLR3 binds with ligands exclusively via surface contacts (mainly hydrogen bonding and electrostatic interactions), while the protein–protein interactions occur only at the LRR-CT in the TLR3-dsRNA complex (Gao et al., 2015). Mutational analysis of human TLR3 has revealed that His39, His60, and His108 residues at the N-terminus, and His539 and Asn541 residues at the C-terminus, interact with dsRNA. The C-terminal dimerization site is critical for dsRNA binding and TLR3 signaling (Liu et al., 2008; Wang Y et al., 2010). Gao et al. (2015) found a weak dimer interface at the TLR3 ECD C-terminal site, which is required for effective dsRNA binding, and Pro680 is crucial for maintaining the dimer interface.

Studies have shown that, like TLR9, once within endosomes, TLR3 is cleaved by several cathepsins within its ECD because the full-length (non-cleaved) proteins are unable to form contacts with one another essential for receptor dimerization (Ewald et al., 2011; Fitzgerald and Kagan, 2020). The cleaved fragments remain associated with each other, and both are important for the inflammatory activity of TLR9/3 (Fitzgerald and Kagan, 2020). However, more research is required to clarify the type of cathepsins required for cleaving TLR3 and how TLR3 is proteolytically processed. Furthermore, Luo et al. (2012) demonstrated that lateral SU clustering is necessary for productive TLR3 signaling. Three neutralizing Fab fragments (Fab15, Fab12, and Fab1068) of three antibodies prevent the lateral clustering of SUs along the length of the dsRNA ligand, resulting in antagonism towards TLR3 signaling. This indicates that lateral clustering of SUs is necessary for TLR3 signal transduction. The structure of the TLR3-dsRNA signaling complex is shown in Fig. 1.

### 2.2.3 Structural basis of dsRNA

The minor groove, which contains degenerate sequence information, and the phosphate backbone are the main determinants of dsRNA binding to receptors (Doyle and Jantsch, 2002; Leonard et al., 2008). The major groove, which contains sequence-specific



**Fig. 1 Structure of the human TLR3-dsRNA complex and three crucial interaction sites (Gosu et al., 2019).** Individual chains of the TLR3 complex are shown in white (TLR3) and cyan (TLR3\*). dsRNA, hydrogen bonds, and mutant residues are shown in orange, green, and magenta, respectively. For clarity, only the TLR3\* N- and C-terminal interaction sites of dsRNA (46 bp) binding are shown. TLR: Toll-like receptor; dsRNA: double-stranded RNA.

information, is a common site of double-stranded DNA (dsDNA)-protein interaction (Pabo and Sauer, 1984). Since dsRNA is also found in many cellular RNAs, three mechanisms have been proposed to be responsible for discrimination between viral and cellular dsRNAs by TLR3. First, the endosomal location of the TLR3 ECD restricts access of cellular dsRNAs to TLR3 (Sioud, 2006). Second, the presence of modified nucleotides such as *N*-methyladenosine and 2-thiouridine provides an additional physicochemical specificity for TLR3 to discriminate between self and non-self dsRNAs (Karikó et al., 2005). Third, TLR3 requires dsRNA to be longer than 40 bp for robust stimulation, which helps avoid inappropriate recognition of some cellular small interfering RNAs (siRNAs), microRNAs (miRNAs), or ssRNAs with short hairpin structures (Leonard et al., 2008). Other studies, however, showed that exogenously introduced 21-bp siRNA can also stimulate TLR3, suggesting that a high dose of RNA may compensate for the

low-affinity interaction with short dsRNA (Karikó et al., 2004; Kleinman et al., 2008). This evidence suggests that dsRNA length is not an absolute criterion used by TLR3 for self and non-self discrimination, but rather a relative condition that can be scaled by the abundance of RNA and receptors in the cell (Peisley and Hur, 2013). How shorter dsRNA ligands (21–39 bp) activate TLR3 signaling remains to be determined. It is also unclear how dsRNA ligands longer than 90 bp are sensed, which may induce higher-order oligomerization of the receptor (Luo et al., 2012).

#### 2.2.4 Relevant proteins

TLR3 presents a K63-linked poly-ubiquitination at K831 by the E3 ubiquitin ligase tripartite motif-containing protein 3 (TRIM3), which is located mainly in the Golgi apparatus (Li et al., 2020). Then, the polyubiquitinated TLR3 is distributed to endolysosomes to sense viral dsRNA and trigger an antiviral response. The cytoplasmic TIR domain of TLR3 is

tyrosine-phosphorylated upon ligand binding (Sarkar et al., 2003). Bruton's tyrosine kinase (BTK) was reported to phosphorylate the cytoplasmic domain of TLR3, particularly the critical Tyr759 residue (Lee et al., 2012).

Cluster of differentiation (CD14), a class-A scavenger receptor, and clathrin-mediated endocytic pathways participate in cellular uptake of extracellular dsRNA (Lee et al., 2006; Itoh et al., 2008; Limmon et al., 2008). However, a recent report indicated that human CD14 acts as a co-receptor only to human TLR9, not to TLR3, TLR7, or TLR8 (Weber et al., 2012). Mex3 RNA-binding family member B (Mex3B), an RNA-binding protein, is reportedly involved in the process of presenting endocytosed dsRNA to TLR3 in intracellular compartments (Yang et al., 2016; Zhu et al., 2016). Mex3B acts as a co-receptor of TLR3 in response to dsRNA, promoting dsRNA binding with endosomal TLR3 and proteolytic processing of TLR3 (Yang et al., 2016; Zhu et al., 2016). The zinc-finger FYVE domain-containing protein ZFYVE1 was revealed to promote the binding of TLR3 to poly(I:C) by associating with the ECD of TLR3 via its FYVE domain (Zhong et al., 2020). Ectopically expressed ZFYVE1 binds to the poly(I:C), and thus enhances the binding of TLR3 to poly(I:C) (Yamashita et al., 2012; Zhong et al., 2020). The relationship between Mex3B and ZFYVE1 in regulating the TLR3-mediated response is unclear. Luo et al. (2020) demonstrated that the Sh2 domain-containing leukocyte protein (SLP) adaptor is the universal TLR adaptor for TLR2, TLR3, TLR4, and TLR9, and the C-terminal Src kinase (CSK)-interacting membrane protein (SCIMP) presents Lyn and other effectors, such as Csk, Gdb2, and Slp65, to TLRs during cellular activation.

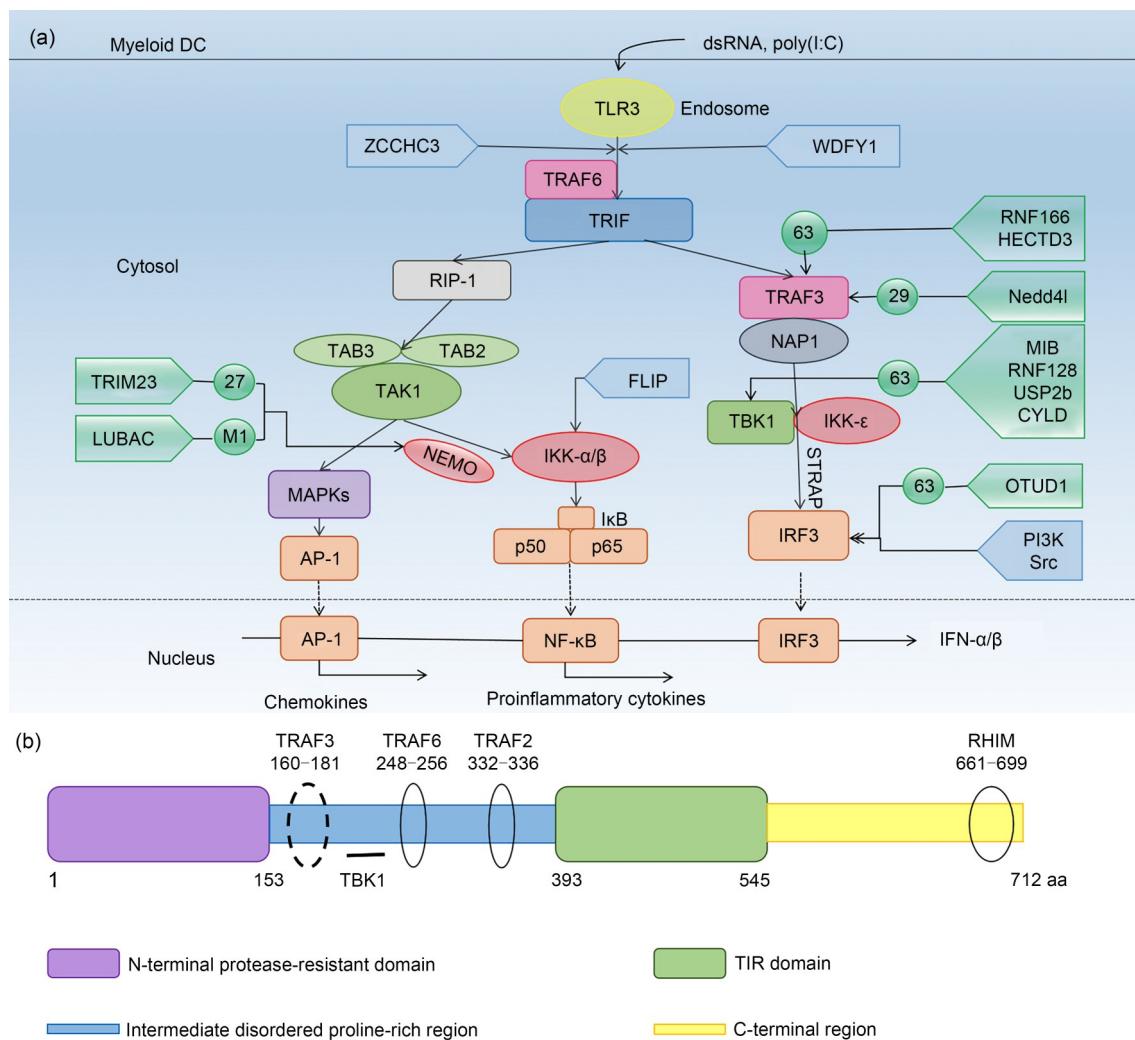
### 3 TLR3-TRIF signaling pathway

#### 3.1 Overview of TLR3-TRIF signaling pathway

Upon activation, TLR3 recruits TRIF to trigger a downstream signaling cascade (Matsumoto et al., 2011). Unlike other TLR family members, TLR3 is utterly dependent on TRIF (Yang and Shu, 2020). The TLR3-TRIF signaling pathway involves signal transduction by TRAF3/6, TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), inhibitor of κB (IκB) kinase-related kinase-ε (IKK-ε;

also called IKK-i), receptor-interacting protein-1 (RIP-1), and NF-κB-activating kinase (NAK)-associated protein 1 (NAP1). This signaling pathway ultimately activates transcription factors, namely IRF3/7, NF-κB, and the activator protein 1 (AP-1), thus mediating the production of type I IFNs, proinflammatory cytokines, and chemokines, respectively, following TLR3 activation. Phosphoinositide 3-kinase (PI3K), p38-mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) are also activated by TLR3 signaling (Schröder and Bowie, 2005). TLR3-induced MAPKs are responsible for activating AP-1, and PI3K is crucial for full phosphorylation and activation of IRF3 (Ameyar et al., 2003; Sarkar et al., 2004). Ubiquitination and phosphorylation play an indispensable role in the fine-tuning of the TLR3 signaling cascade. The general scenario of the TLR3-TRIF signaling pathway is described in Fig. 2a.

The *TRIF* gene of humans is located on chromosome 19p13.3 and encodes 712 amino acids, making it much larger than the other TIR domain-containing linker proteins (Mahita and Sowdhamini, 2017). TRIF consists of an N-terminal protease-resistant domain (1–153 amino acids (aa)), an intermediate disordered proline-rich region (154–392 aa), a TIR domain (393–545 aa), and a C-terminal region (containing an RIP homotypic interaction motif (RHIM) domain, 661–699 aa) (Mahita and Sowdhamini, 2017) (Fig. 2b). The N-terminal region is involved in TRIF-mediated IRF3 activation, the C-terminal region is crucial for NF-κB activation and apoptosis, while the TIR domain is essential for binding to TLR3 (Tatematsu et al., 2010; Kumeta et al., 2014; Patra et al., 2020). The disordered region between the N-terminal domain and the TIR domain contains binding sites for many downstream proteins such as TBK1, TRAF2 (332–336 aa), and TRAF6 (TRAF6-binding motif 2 (T6BM) domain, 248–256 aa) (Sasai et al., 2010). Mahita and Sowdhamini (2017) proved that the N-terminal domain binds to the BB-loop region of the TIR domain to prevent TLR3 homodimerization. The physical association between TRAF3 and TRIF has always been controversial. Many conflicting data about their physical association have been reported (Sasai et al., 2010). However, a recent report indicated that the 21 amino acid sequences (160–181 aa) from the amino-terminal half of TRIF are crucial for the TRAF3



**Fig. 2 Mechanism of the Toll-like receptor 3 (TLR3)-Toll-interleukin-1 receptor (TIR)-domain-containing adaptor-inducing interferon- $\beta$  (IFN- $\beta$ ) (TRIF) signaling pathway and structure of human TRIF. (a) Mechanism of the TLR3-TRIF signaling pathway.** After double-stranded RNA (dsRNA) activates endosomal TLR3, TRIF transiently colocalizes with TLR3; it dissociates from the receptor and forms a speckle-like structure that relocates with downstream-signaling molecules such as tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2), TRAF6, and receptor-interacting protein-1 (RIP-1) (Funami et al., 2004, 2007). Then, with the cooperation of TRAF3, TRAF family member-associated nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B) activator (TANK)-binding kinase 1 (TBK1), inhibitor of  $\kappa$ B (IkB) kinase-related kinase- $\epsilon$  (IKK- $\epsilon$ ; also called IKK-i), and NF- $\kappa$ B-activating kinase (NAK)-associated protein 1 (NAP1), this signaling pathway ultimately activates transcription factors, namely IFN-regulatory factor 3/7 (IRF3/7), the TRIF-dependent NF- $\kappa$ B, and the activator protein 1 (AP-1), thus mediating the production of type I IFNs, proinflammatory cytokines, and chemokines, respectively. **(b) Schematic structure of human TRIF.** TRIF consists of an N-terminal protease-resistant domain (1–153 amino acids (aa)), an intermediate disordered proline-rich region (154–392 aa), a TIR domain (393–545 aa), and a C-terminal region (containing a RIP homotypic interaction motif (RHIM) domain, 661–699 aa) (Mahita and Sowdhamini, 2017). The disordered region between the N-terminal domain and the TIR domain contains binding sites for many downstream proteins such as TBK1, TRAF2 (332–336 aa), and TRAF6 (248–256 aa) (Sasai et al., 2010). The physical association between TRAF3 and TRIF is controversial (Nguyen et al., 2014). ZCCHC3: zinc finger CCHC-type containing 3; WDFY1: WD-repeat- and FYVE-domain-containing protein 1; TRIM23: tripartite motif-containing protein 23; LUBAC: linear ubiquitin chain assembly complex; RNF166: ring finger protein 166; HECTD3: E6-associated protein carboxyl terminus domain containing 3; Nedd4l: neural precursor cell expressed developmentally down-regulated 4-like; MIB: mind bomb; RNF128: RING finger 128; USP2b: ubiquitin-specific protease 2 isoform b; CYLD: conserved cylindromatosis; OTUD1: termed OTU domain-containing protein 1; PI3K: phosphoinositide 3-kinase; NEMO: NF- $\kappa$ B essential modifier; MAPK: p38-mitogen-activated protein kinase; FLIP: viral FLICE-like inhibitory protein.

association and modulating TRIF ubiquitination and degradation (Nguyen et al., 2014). TRIF with a 21-residue deletion ( $\Delta 160-181$ ) inefficiently transactivated the IFN pathway, and its association with TRAF3 was weaker than that of wild-type (WT) TRIF (Nguyen et al., 2014).

Funami et al. (2016) demonstrated that 14-3-3- $\zeta$  acts as a novel component of the TRIF signalosome that functions in TLR3-mediated signaling. With the ablation of the TRIF signalosome in 14-3-3- $\zeta$ -knockdown cells, the synthesis of IFN and inflammatory cytokines decreased, and TLR3-mediated IRF3 translocation, as well as I $\kappa$ B $\alpha$  phosphorylation, was diminished. The identification of 14-3-3- $\zeta$  has shed new light on TRIF signaling.

### 3.2 Activation mechanism of the TLR3-TRIF signaling pathway

#### 3.2.1 Recruitment of TRIF

TRIF is expressed at a low level in most tissues and cells, and exists diffusely in the cytoplasm of resting cells (Funami et al., 2016). After dsRNA activates endosomal TLR3, TRIF transiently colocalizes with TLR3; it dissociates from the receptor and alters its distribution profile from a diffuse cytoplasmic pattern to a speckle-like structure that relocates with downstream-signaling molecules (Funami et al., 2004, 2007). Both TLR3 and TRIF contain a TIR domain, which is crucial to their interaction once TLR3 binds to the ligand (Oshiumi et al., 2003). The TIR domain of TLR3 has three highly conserved sequences, Box1, Box2, and Box3; Box2 constitutes the BB loop, which is crucial for binding downstream linker proteins (Singh et al., 2014). Unlike other TLRs, the BB loop in binding TRIF does not contain conservative proline residues, but is replaced by alanine, which is critical for binding TRIF. The TLR3 mutant A795H (TLR3 with Ala795His mutation) has lost the ability to bind to TRIF (Oshiumi et al., 2003).

A recent report indicated that WD-repeat- and FYVE-domain-containing protein 1 (WDFY1) acts as a critical adaptor protein during TLR3 recruitment of TRIF by associating with the TIR domain of TLR3 (Hu et al., 2015). Their interaction depends on the tyrosine phosphorylation of TLR3. Zinc finger CCHC-type containing 3 (ZCCHC3) is also a critical component of the TLR3-TRIF signaling pathway, and facilitates the recruitment of TRIF to TLR3 after

poly(I:C) stimulation (Zang et al., 2020). The N-terminal domain (1–300 aa) and C-terminal domain (300–404 aa) of ZCCHC3 are functional for its interaction with the N-terminal/TIR domains of TRIF and the TIR domain of TLR3, respectively.

#### 3.2.2 TRIF-dependent activation of IRF3

IRF3 and another IRF family member IRF7, are known to be IFN regulatory factors (Wu and Chen, 2014). Both mediate the activation of NF- $\kappa$ B, but IRF3 is activated by the TRIF pathway and IRF7 by the MyD88 pathway (Kawai and Akira, 2010). In inactivated cells, IRF3 is phosphorylated to form the IRF3:IRF3 homodimer or the IRF3:IRF7 heterodimer, which enters the nucleus and leads to specific gene expression (Schmid et al., 2014). TRIF-mediated IRF3 activation is regulated by the protein kinases IKK- $\epsilon$  and TBK1. Activation may depend on upstream linker proteins, NAP1, TANK, and SINTBAD (similar to NAP1 TBK1 adaptor) (Tatematsu et al., 2010; Schmid et al., 2014). NAP1, TANK, and SINTBAD are similar in structure. NAP1 participates in the recruitment of IRF3 kinases to the N-terminal region of TRIF. TANK, as a binding ligand of TBK1, IKK- $\epsilon$ , and the TRAF family, is responsible for associating TRAF3 with TBK1 and IKK- $\epsilon$  (Ryzhakov and Randow, 2007).

TRIF is homo-oligomerized at the Pro434 residue in the TIR domain and the C-terminal region. The recruitment of TRAF3 by TRIF is essential for the activation of IRF3 (Funami et al., 2008). TRAF3 undergoes a Lys63-linked poly-ubiquitination with the synergy of the E2 ubiquitin-conjugating enzyme Ubc13/Uev5 (Zeng et al., 2009; Tseng et al., 2010). Then, ubiquitinated TRAF3 mediates oligomerization of TBK1 and IKK- $\epsilon$  via adaptor proteins such as TANK and NAP1 (Guo and Cheng, 2007; Tatematsu et al., 2010). TBK1 and IKK- $\epsilon$  are responsible for phosphorylation and activation of IRF3 (Zhou et al., 2020). The phosphorylated IRF3 dimerizes and translocates to the nucleus to initiate the transcription of the *IFN- $\beta$*  gene (Honda et al., 2006). The activation, regulation, substrate, and function of TBK1 and IKK- $\epsilon$  remain to be elucidated.

c-Src tyrosine kinase is activated by dsRNA in human DCs, and then is recruited to TLR3 (Johnsen et al., 2006). In Src kinase-deficient cells, dsRNA-induced activation of IRF3 and activator of transcription 1

is abolished. Phosphorylation of TLR3 on tyrosine 759 increases in parallel to Src-dependent IFN- $\beta$  production (Zhang et al., 2016). Serine/threonine kinase receptor-associated protein (STRAP) acts as a scaffold protein in TLR3-triggered signaling (Huh et al., 2017), and strongly interacts with TBK1 and IRF3 to enhance IFN- $\beta$  production. K63-linked polyubiquitination of TRAF3 is promoted by overexpression of the E3 ligase ring finger protein 166 (RNF166) following Sendai virus (SeV) infection (Chen et al., 2015). The E6-associated protein carboxyl terminus domain containing 3 (HECTD3) also acts as an E3 ligase catalyzing this polyubiquitination during bacterial infection, but the exact function of HECTD3 during viral infection is poorly understood (Li et al., 2018). Neural precursor cell-expressed developmentally down-regulated 4-like (Nedd4l) promotes TRAF3-mediated signal transduction by catalyzing K29-linked ubiquitination at the C56 and C124 cysteine residues of TRAF3 (Liu, 2019). E3 ubiquitin ligases, mind bomb (MIB) and RING finger 128 (RNF128), promote the K63-linked polyubiquitination of TBK1 for its activation (Li et al., 2011; Song et al., 2016). In addition to E3 ligases, the deubiquitinating enzymes (DUBs) ubiquitin-specific protease 2 isoform b (USP2b) and the conserved cylindromatosis (CYLD) target TBK1 for K63-linked deubiquitination, and OTU domain-containing protein 1 (OTUD1) cleaves the K63-linked polyubiquitin chains from IRF3 (Lu et al., 2018).

TRIF and two other adaptor proteins, stimulator of *IFN* genes (STING) and mitochondrial antiviral signaling (MAVS), mediate the recruitment of IRF3 via a conserved pLxIS motif, the same motif as that of IRF3 (Zhao BY et al., 2016). The pLxIS motifs of the three adaptor proteins are phosphorylated by TBK1 or IKK- $\epsilon$  (Zhao BY et al., 2016). A recent paper demonstrated that phosphorylated TRIF, MAVS, and STING bind to a positively charged surface of IRF3 and thus recruit IRF3 for activation by TBK1 (Liu et al., 2015). TRIF is phosphorylated at its consensus motif S210/S212/T214 by TBK1, and TRIF S210 is the critical phosphorylation site for IRF3 activation.

### 3.2.3 TRIF-dependent activation of NF- $\kappa$ B

NF- $\kappa$ B is a central transcription factor crucial to innate and adaptive immunities, cell proliferation, apoptosis, and the stress response (Cartwright et al., 2016). The p50/p65 heterodimer of NF- $\kappa$ B activates the

expression of IFN- $\beta$  and proinflammatory cytokines (Kawai and Akira, 2007; Kohl et al., 2019). The K63-linked polyubiquitin chains of RIP-1 and TRAF6 can recruit the transforming growth factor- $\beta$  (TGF- $\beta$ )-activated kinase 1 (TAK1)-TAK1-binding protein 2 (TAB2)-TAB3 complex via the ubiquitin-binding activity of TAB2/3, causing TAK1 autophosphorylation and activation (Yang and Shu, 2020). TAK1 activates the I $\kappa$ B kinase complex, which consists of the IKK- $\alpha$  and IKK- $\beta$  kinases scaffolded by NF- $\kappa$ B essential modifier (NEMO), ultimately activating NF- $\kappa$ B or stimulating MAPK-mediated AP-1 transcriptional responses (Emmerich et al., 2013; Mitchell et al., 2016). RIP-1 associates with TRIF via the RHIM domain at the C-terminal of TRIF followed by RIP-1 interaction with TNF receptor-associated death domain (TRADD) protein. TRADD is an adaptor protein related to the ubiquitination of RIP-1, via the homotypic death domain (Meylan et al., 2004; Ermolaeva et al., 2008; Park et al., 2015). TRAF6 binds with the T6BM domain in the N-terminal region of TRIF to mediate NF- $\kappa$ B activation (Mahita and Sowdhamini, 2017). In addition, TRAF6 mediates the recruitment of meddlesome-associated TBK1 to stimulate the rapid induction of glycolysis following TLR3 activation (Tan and Kagan, 2019). The rapid glycolysis drives metabolic changes in the cell, resulting in an increased need for histone modifications related to durable transcriptional activities in the nucleus or enhanced protein synthesis and secretory activities associated with TLR3 signaling (Corcoran and O'Neill, 2016; Langston et al., 2019).

The activation of the NF- $\kappa$ B signaling pathway is a sign of viral infection. Once NF- $\kappa$ B is activated, it up-regulates many cytokines and chemokines (such as TNF- $\alpha$  and IL-6), which give positive feedback through the NF- $\kappa$ B pathway, thereby amplifying the initial inflammatory signal. The activation of NF- $\kappa$ B is influenced by many regulators that act on adaptor proteins and kinases of the NF- $\kappa$ B pathway. The E3 ligase TRIM23 regulates the activation of NEMO by atypical K27-linked polyubiquitination, and the linear ubiquitin chain assembly complex (LUBAC), another E3 ligase, catalyzes the linear ubiquitination of NEMO at K285 and K309 (Zheng and Gao, 2020). The viral FLICE-like inhibitory protein (FLIP) forms a stable complex with a central region of the inhibitor of IKK- $\gamma$  and activates the NF- $\kappa$ B pathway via IKK activation (Baratchian et al., 2016).

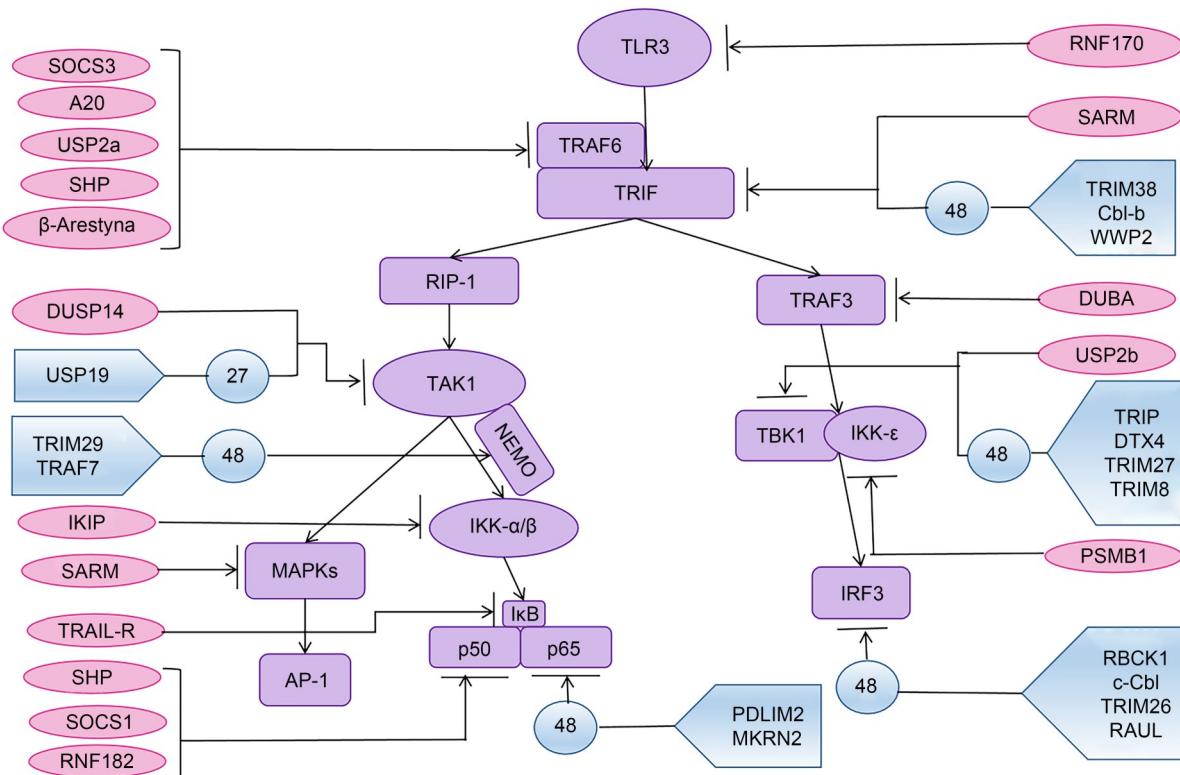
## 4 Negative regulation of TLR3 signaling

The PRR pathways are controlled by many external and intracellular molecules to maintain a balance between activation and inhibition, thus balancing the beneficial and adverse effects of antigen detection (Antosz and Choroszyńska, 2013). The mechanism of negative regulation with TLR is most developed in immune cells. It is strictly limited to a specific TLR and operates at multiple levels of TLR signaling. Here, we focus on TLR3-negative regulators, which inhibit signals at different stages of activation (Fig. 3).

### 4.1 Negative regulation of ubiquitination modifying enzymes

E3 ubiquitin ligases and deubiquitinases (DUBs), as the master regulators of TLR signaling, cooperatively regulate the dynamic and reversible ubiquitination process (Zheng and Gao, 2020).

The TLR3-binding E3 ligase RNF170 mediates the K48-linked poly-ubiquitination of K766 in the TIR domain of TLR3 (Song et al., 2020). RNF170 promotes the degradation of TLR3 via the proteasome pathway, thus selectively inhibiting TLR3-mediated pathways (Song et al., 2020). TRIM38 associates with



**Fig. 3** Negative regulation of TLR3 signaling. The trunk of the TLR3-TRIF signaling pathway is shown in purple; ubiquitination modifying enzymes that negatively regulate the pathway are in blue; other negative regulators, such as membrane proteins, adaptor proteins, and phosphatases, are in pink. AP-1: activator protein 1; c-Cbl: Casitas B-lineage lymphoma; DTX4: Deltex 4; DUBA: deubiquitinating enzyme A; DUSP14: dual-specificity phosphatase 14; IκB: inhibitor of κB; IKK: IκB kinase-related kinase; IKIP: IKK-interacting protein; IRF3: interferon (IFN)-regulatory factor 3; MAPK: mitogen-activated protein kinase; MKRN2: makorin ring finger protein 2; NF-κB: nuclear transcription factor-κB; NEMO: NF-κB essential modifier; PDLIM2: PDZ and LIM domain-containing protein 2; PSMB: proteasome β subunit; RAUL: transcriptional activator (RTA)-associated ubiquitin ligase; RBCK1: RBCC protein interacting with PKC1; RIP-1: receptor-interacting protein-1; RNF: ring finger protein; SARM: sterile α and HEAT/Armadillo motif; SHP: small heterodimer partner; SOCS: suppressor of cytokine signaling; TGFB-β: transforming growth factor-β; TAK1: TGF-β-activated kinase 1; TLR3: Toll-like receptor 3; TNF: tumor necrosis factor; TRAF: TNF receptor-associated factor; TBK1: TRAF family member-associated NF-κB activator (TANK)-binding kinase 1; TRAIL: TNF-related apoptosis-inducing ligand; TRIF: Toll-interleukin-1 receptor (TIR)-domain-containing adaptor-inducing IFN-β; TRIM: tripartite motif-containing protein; TRIP: TRAF-interacting protein; USP2a: ubiquitin-specific protease 2 isoform a; USP19: ubiquitin-specific protease 19; WWP2: WW domain-containing protein 2.

the N-terminus of TRIF through its PRYSPRY domain to mediate K48-linked poly-ubiquitination and degradation of TRIF. Other proteins involved in this process are WW domain-containing protein 2 (WWP2) and Casitas B-lineage lymphoma (c-Cbl) (Han et al., 2010; Xue et al., 2012; Yang et al., 2013).

The E3 ubiquitin ligases TRAF-interacting protein (TRIP), Deltex4 (DTX4), TRIM27, and TRIM8 are responsible for modulating the turnover of TBK1 through K48-linked poly-ubiquitination (Cui et al., 2012; Zhang et al., 2012; Zheng et al., 2015; Ye et al., 2017). The E3 ligases RBCC protein interacting with PKC1 (RBCK1), c-Cbl, TRIM26, and RNA transcriptional activator (RTA)-associated ubiquitin ligase (RAUL) target the nuclear IRF3 for K48-linked poly-ubiquitination and degradation (Yu and Hayward, 2010; Wang et al., 2015; Zhao XB et al., 2016).

E3 ligase ubiquitin-specific protease 19 (USP19) induces deconjugation of K63- and K27-linked polyubiquitin chains from TAK1, resulting in disruption of the TAK1-TAB2/3 complex (Lei et al., 2019). Additionally, USP19 impairs the recruitment of TRIF to TLR3/4 by catalyzing the removal of TRIF K27-linked polyubiquitin moieties (Wu X et al., 2019). TRIM29 and TRAF7 promote the turnover of NEMO by facilitating the K48- and K29-linked poly-ubiquitination of NEMO, respectively (Xing et al., 2016). Makorin ring finger protein 2 (MKRN2) and PDZ and LIM domain-containing protein 2 (PDLIM2) cooperatively promote the K48-linked poly-ubiquitination and proteasomal degradation of nuclear p65 (Shin et al., 2017). RNF182 can also contribute to the degradation of p65 via K48-linked ubiquitination, thus inhibiting TLR3-triggered proinflammatory responses (Cao Y et al., 2019).

DUBs, such as A20, deubiquitinating enzyme A (DUBA), and USP2, are reported to be involved in the type I IFN pathway. Zinc-finger protein A20 is a negative regulator that regulates both MyD88- and TRIF-dependent TLR signaling pathways (Saitoh et al., 2005). Saitoh et al. (2005) reported that A20 negatively regulates TLR3-mediated *IFN-β* gene transcription by inhibiting IRF3 activation. A20 also prevents NF-κB activation via the A20–TRAF6 axis (Boone et al., 2004). DUBA negatively regulates TLR3-mediated type I IFN production by selectively cleaving the Lys63-linked polyubiquitin chains on TRAF3 (Kaya-gaki et al., 2007). USP2a negatively regulates NF-κB

activation by cleaving K63-linked polyubiquitin chains on TRAF6 (He et al., 2013). USP2b targets TBK1 and deubiquitinates TBK1 K63-linked poly-ubiquitination, thus negatively modulating the TLR3/4 signaling pathway (Zhang et al., 2014).

The members of the suppressor of cytokine signaling (SOCS) family also participate in immune response regulation (Antosz and Choroszyńska, 2013). SOCS1 targets the subunit p65 (RelA)-NF-κB and leads to proteolysis of the NF-κB molecule by ubiquitination (Ryo et al., 2003). SOCS3 represses TRAF6 activation to block the subsequent activation of TAK1, which must activate both the NF-κB and MAPK pathways (Frobøse et al., 2006).

#### 4.2 Negative regulation of membrane proteins

The TNF-related apoptosis-inducing ligand (TRAIL) cytokine was identified as a member of the TNF superfamily which initiates the apoptotic pathway in many cancer cell lines (Yuan et al., 2018). Diehl et al. (2004) claimed that TRAIL-R inhibits TLR signaling by stabilizing IkBα and decreasing the expression of the transcription factor NF-κB, and showed that TLR2, TLR3, and TLR4 ligands bolster the expression of TRAIL-R. In contrast, in the case of TRAIL-R deficit, there is an increased production of cytokines in response to these ligands (Diehl et al., 2004).

#### 4.3 Negative regulation of adaptor proteins

β-Arrestin 1 and β-arrestin 2 are ubiquitously expressed multifunctional scaffolding proteins that affect inflammatory signaling in various cell lines (Freedman and Shenoy, 2018). β-Arrestins participate in intracellular signaling by playing a role as MAPK scaffolds or mediating Src activation (Laporte and Scott, 2019). Witherow et al. (2004) indicated that β-arrestin is involved in modulating TLR signaling by affecting NF-κB activation. Research by Wang et al. (2006) showed that β-arrestin interacts with TRAF6 via the TIR domain, thus preventing the auto-ubiquitination and oligomerization of TRAF6 required for the activation of NF-κB and AP-1.

Sterile α and HEAT/Armadillo motif (SARM), the most conserved member of the TIR adaptor family, is located in the cytoplasm and directly affects the cytosol TRIF, decreasing NF-κB and IRF3, and thus negatively regulates TRIF-dependent signaling

induced by TLR3 and TLR4 (Carty and Bowie, 2019). Research by Peng et al. (2010) showed that, in human cells, SARM blocks the TRIF- and MyD88-dependent activation of AP-1 and the endogenous form of AP-1.

#### 4.4 Negative regulation of phosphatases

Small heterodimer partner (SHP) is also a negative TLR signaling regulator (Zhang and Shen, 2011). In resting cells, SHP inhibits NF- $\kappa$ B-dependent signaling by interacting with p65-NF- $\kappa$ B (Zhang and Shen, 2011). Upon stimulation, SHP decreases the TRAF6 Lys63-link polyubiquitin by interacting with the RING TRAF6 domain (Yuk et al., 2011). DUSP14, a member of the dual-specificity phosphatase (DUSP) family, represses TNF- and IL-1 $\beta$ -triggered NF- $\kappa$ B activation by dephosphorylating TAK1 T187 (Zheng et al., 2013).

#### 4.5 Other negative regulators

PSMB1 is a proteasome  $\beta$  subunit (PSMB) family member, which inhibits the TLR and RLR signaling pathways (Sorokin et al., 2009; Wu FY et al., 2019). The silencing of PSMB1 increases IFN- $\beta$  production, whereas overexpression of PSMB1 inhibits activation of virus-induced IFN- $\beta$  promoter (Wu FY et al., 2019). PSMB1 promotes the degradation of IKK- $\epsilon$  by interacting with IKK- $\epsilon$  through the ubiquitin-proteasome system (Wu FY et al., 2019). IKK-interacting protein (IKIP) inhibits the formation of IKK complex by binding to IKK- $\alpha/\beta$  in competition with NEMO, thus hindering the phosphorylation of IKK- $\alpha/\beta$  and negatively regulating the activation of the downstream NF- $\kappa$ B signaling pathway (Wu HF et al., 2020). In the presence

of lipopolysaccharide (LPS), poly(I:C), TNF- $\alpha$ , and IL-1 $\beta$  stimulation, the phosphorylation of IKK- $\alpha/\beta$ , I $\kappa$ B, and p65 is enhanced, and the expression of TNF- $\alpha$  and IL-6 is increased in the macrophages of IKIP-deficient mice (Wu HF et al., 2020).

The signals transmitted from TLR are controlled by specific inhibitors, which show tissue and cellular specificity in many cases. However, the mechanisms underlying this specificity are poorly understood. Many inhibitory factors known to affect TLR signaling have yet to be evaluated.

### 5 Antiviral effects of TLR3

TLR3 may be a double-edged sword that functions in ensuring or compromising host immunity against viruses (Perales-Linares and Navas-Martin, 2013) (Table 1). TLR3-mediated signaling during viral infections protects against correlative diseases by reducing the viral loads and modulating immune responses, while over-activation of the pathogenic immune response induced by TLR3 signal transduction can lead to pathogenesis (Perales-Linares and Navas-Martin, 2013). Thus, the tight balance between a controlled antiviral response and excessive immune activation determines the pathological outcome of TLR3-related diseases. The TLR3 pathway can control immunity to most of the clinically relevant viral infections in humans, including those caused by flaviviruses, hepatitis viruses, RV, herpesvirus, retroviruses, encephalomyocarditis virus, orthomyxoviruses, and the currently epidemic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

**Table 1 Roles of TLR3 in antiviral responses**

Effect	Virus	Target organ	Disease	Role of TLR3
Protection	Herpes simplex virus type 1 (HSV-1)	Central nervous system (CNS)	Encephalitis or others	TLR3 recruits the metabolic checkpoint kinase complex mTORC2, thus enabling the activation of molecules (including mTORC1) required for type I IFN induction (Mielcarska et al., 2018).
	Encephalomyocarditis virus (EMCV)	Myocardium, brain	Encephalomyocarditis	TLR3 decreases viral replication in the heart, and decreases myocardial injury (Hardarson et al., 2007).
	Coxsackievirus strain B serotype 3 (CVB3)	Myocardium, meninges, pancreas	Acute and chronic myocarditis, meningitis, and pancreatitis	TLR3 resists CVB3 infection and prevents the progression from myocarditis to iDCM (Abston et al., 2013; Sesti-Costa et al., 2017).
	Murine cytomegalovirus (MCMV)	Liver	Hepatitis	Cytokine (type I IFN, IFN- $\gamma$ , and IL-12p40) production, and NK cell and NKT cell activation are impaired in TLR3-deficient mice compared with wild-type mice (Matsumoto et al., 2011).

To be continued

Table 1

Effect	Virus	Target organ	Disease	Role of TLR3
	Rotavirus (RV)	Kidney	Glomerulonephritis	TLR3 plays a synergistic role with protective factors and down-regulates the expression of cytokines induced by RV (Jiang et al., 2017).
	Poliovirus (PV)	Bone marrow	Poliomyelitis	TLR3-TRIF signaling pathway governs IFN induction and host protection against PV infection (Oshumi et al., 2011).
	Human immunodeficiency virus 1 (HIV-1)	CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells, macrophages, others	Immunodeficiency, encephalitis, and others	TLR3 decreases HIV-1 infection in macrophages and enhances the development of HIV-specific CD4 <sup>+</sup> and CD8 <sup>+</sup> cytotoxic T lymphocytes (Cheng et al., 2018; Nguyen et al., 2020).
	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	Respiratory tract	Coronavirus disease 2019 (COVID-19)	TLR3- and IRF7-dependent type I IFN immunity may be essential for preventing life-threatening COVID-19 pneumonia (Zhang et al., 2020).
	Influenza A virus (IAV)	CNS, brain	Encephalopathy	TLR3 signaling pathways are activated preferentially following IAV infection to release a range of proinflammatory cytokines (Huo et al., 2018).
Deterioration	Phlebovirus	Liver	Hepatopathy	Compared with TLR3-deficient mice, wild-type mice demonstrate decreased resistance to lethal infection. The result may be caused by overproduction of inflammatory mediators via TLR3 signaling (Gowen et al., 2006).
Bi-direction	Hepatitis C virus (HCV)	Liver	Hepatitis C	Protection: TLR3 inhibits HCV replication in HuH7 cells (Zhou et al., 2016). Deterioration: TLR3 possibly contributes to the intrahepatic and unbalanced proinflammatory response (Li et al., 2012).
	Hepatitis B virus (HBV)	Liver	Hepatitis	Protection: TLR3-knockout mice are unable to express IL-8 and other requisite molecules to activate immune responses against HBV (Maire et al., 2008). Activation of mDC using TLR3 ligands leads to improved NK cell function in CHB infection (Tjwa et al., 2012). Deterioration: TLR3 polymorphism rs3775291 was associated with an increased risk of developing CHB (Geng et al., 2016; Fischer et al., 2018).
	West Nile virus (WNV)	Brain	Encephalitis	Protection: TLR3 restricts WNV replication in neurons and antagonizes against NS1 (Daffis et al., 2008; Wilson et al., 2008). Deterioration: compared with TLR3-deficient mice, wild-type mice are less resistant to lethal WNV infection (Wang et al., 2004). Increased frequency of WNV encephalitis in elderly humans may be related to increased TLR3 signaling (Kong et al., 2008).
Unclear	Influenza A (H1N1)	Lung	Pneumonia	The presence of TLR3 rs5743313/CT polymorphism has a close relationship with the increased risk of pneumonia in children infected by the pandemic A/H1N1/2009 (Esposito et al., 2012). TLR3 does not worsen the pathogenesis of pH1N1 infection (Leung et al., 2014).
	Tick-borne encephalitis virus (TBEV)	CNS	Encephalitis	TLR3 may be a risk factor for TBEV infection (Kindberg et al., 2011).

CD: cluster of differentiation; CHB: chronic hepatitis B; iDCM: inflammatory dilated cardiomyopathy; IFN: interferon; IL: interleukin; IRF: IFN-regulatory factor; mDC: myeloid dendritic cell; mTORC: mammalian target of rapamycin complex; NK: natural killer; NKT: natural killer T; NS1: non-structural protein 1; pH1N1: pandemic H1N1 influenza; TLR3: Toll-like receptor 3; TRIF: Toll-IL-1 receptor (TIR)-domain-containing adaptor-inducing IFN- $\beta$ .

An essential role for TLR3 in protection from herpes simplex virus type 1 (HSV-1) infection has been demonstrated (Zhang et al., 2007). HSV-1 is a

prevalent neurotropic virus that infects the central nervous system (CNS) and generates herpes simplex encephalitis (HSE) in children with inborn errors of

TLR3 immunity (Sancho-Shimizu et al., 2011). The pathogenesis of HSE in children with TLR3-pathway deficiencies is related to impaired TLR3- and Unc-93 homolog B (UNC-93B)-dependent IFN- $\alpha/\beta$  intrinsic immunity against HSV-1 in the CNS (Lafaille et al., 2012). Sato et al. (2018) demonstrated that TLR3 was required for innate immune responses to HSV-1 in neurons and astrocytes. Upon HSV-1 infection, TLR3 recruited the metabolic checkpoint kinase complex mammalian target of rapamycin complex 2 (mTORC2), which led to chemokine induction and TLR3 trafficking to the cell periphery, thus enabling the activation of molecules (including mTORC1) required for type I IFN induction (Mielcarska et al., 2018). In contrast, the failure to express a functional TLR3 disrupted signaling mechanisms that induced antiviral response during infection with HSV-1, indicating that TLR3 is essential for effective antiviral immunity in HSV infection (Mielcarska et al., 2018).

Coxsackievirus strain B serotype 3 (CVB3) is a positive-sense ssRNA virus of the Picornaviridae family (Esfandiarei and McManus, 2008). CVB3 is one of the main causes of myocarditis, and causes a wide range of other infections such as meningitis and pancreatitis (Corsten et al., 2012). It has been reported that TLR3-deficient mice are more susceptible to acute CVB3-induced myocarditis, which is externalized as increased viral load and myocardial tissue damage (Negishi et al., 2008). Abston et al. (2013) revealed that TLR3 prevented the progression from myocarditis to inflammatory dilated cardiomyopathy (iDCM) following CVB3 infection by reducing the IL-4 level and acute viral replication in the heart. Sesti-Costa et al. (2017) found that TLR3 up-regulated CD80 and CD86 in DCs to resist CVB3 infection. In the absence of TLR3, DCs secreted higher levels of the inhibitory molecule programmed death-ligand 1 (PD-L1), while lowering the levels of TNF- $\alpha$  and IL-10 (Sesti-Costa et al., 2017).

The up-regulation of epithelial TLR3 expression during infancy might contribute to age-dependent susceptibility to RV infection (Pott et al., 2012). Jiang et al. (2017) demonstrated that probiotics acted on the TLR3/NF- $\kappa$ B signaling pathway during treatment for diarrhea caused by RV. This not only played a synergistic role with protective factors, but also down-regulated the expression of cytokines induced by RV, thus protecting small intestinal epithelial cells and

repairing small intestinal injury. Sander et al. (2017) found that prostaglandin E2 (PGE2) directly induced autoimmunity in RV infection and triggered TLR signals, thereby inhibiting viral binding and stimulating viral gene expression.

Based on existing data, it is postulated that TLR3 may contribute to the progression toward the acquired immune deficiency syndrome (AIDS). Myeloid DCs are the primary targets of human immunodeficiency virus 1 (HIV-1) lentiviral transduction following subcutaneous immunization (Martin-Gayo and Yu, 2017). The lentiviral activation of DCs depends on TLR3/7 (Breckpot et al., 2010). Bhargavan et al. (2016) demonstrated that TLR3 activation increased HIV-1 transactivation via the NF- $\kappa$ B and JNK pathways. Other evidence suggests that selective TLR3 activation promotes the production of type I IFNs,  $\beta$ -chemokines, and miRNA-155, which preferentially target the 3' untranslated region (UTR) of HIV-1 transcript. This significantly decreases HIV-1 infection in macrophages and enhances the development of HIV-specific CD4 $^{+}$  and CD8 $^{+}$  cytotoxic T lymphocytes in humanized mice (Cheng et al., 2018; Nguyen et al., 2020). The gene expression of TLR3 is markedly increased in advanced HIV-1-infected human macrophages, but shows no significant difference in chronic HIV-1-infected and healthy ones (Alvarez-Carbonell et al., 2017; Liu and Gack, 2020). In addition, triggering TLR3 with specific ligands could have therapeutic potential against HIV-1 infection in humans. A combined TLR3 agonist and CD40-targeting HIV-1 vaccine therapy is being established against HIV-1 disease (Cheng et al., 2018; Saxena et al., 2019). The HIV5pep with poly(I:C) vaccination approach was demonstrated to activate the replication of HIV-1 reservoirs and enhance the anti-HIV-1 T-cell response, resulting in reduced HIV-1 pools (Cheng et al., 2018).

Studies have shown that TLR3 is closely related to the current pandemic of coronavirus disease 2019 (COVID-19) caused by a novel virus strain, 2019 novel coronavirus (2019-nCoV)/SARS-CoV-2. SARS-CoV-2 is a positive-sense, ssRNA,  $\beta$ -coronavirus of the Coronaviridae family, which disrupts the host innate immune response and causes fatal acute respiratory distress syndrome (ARDS) (Guan et al., 2020; Wu F et al., 2020). An enrichment in rare variants predicted to be loss-of-function (LOF) at the 13 human loci known to govern TLR3- and IRF7-dependent

type I IFN immunity to influenza virus has been found in patients with lethal COVID-19 relative to those with asymptomatic or benign infection (Zhang et al., 2020). Therefore, TLR3- and IRF7-dependent type I IFN immunity may be essential for preventing life-threatening COVID-19 pneumonia. Furthermore, TLR3 agonists, like chloroquine, can be considered potential drugs for repurposing in COVID-19 treatment (Gao et al., 2020; Prasad et al., 2020). TLR3 is also a critical antigenic receptor for binding newly designed multi-epitope vaccines for COVID-19 (Dong et al., 2020; Ismail et al., 2020).

Hidaka et al. (2006) found a missense mutation (F303S) in the *TLR3* gene related to encephalopathy caused by the influenza A virus (IAV). Meng et al. (2016) reported that after IAV infection, mouse mastocytoma cell line (P815) cells mediate hyper-induction of proinflammatory cytokines and chemokines, with TLR3 playing a key role in the expression of proinflammatory cytokines. Huo et al. (2018) showed that, compared with other virus infections and inflammation, the TLR3 signaling pathways are activated preferentially following IAV infection to release a range of proinflammatory cytokines.

Evidence suggests that TLR3 shows both protective and damaging functions in the context of some human viral infections. Hepatitis C virus (HCV), a ssRNA virus, induces hepatitis C, probably along with developing complications such as cirrhosis, liver failure, and hepatocellular carcinoma (Spearman et al., 2019). Li et al. (2012) reported that TLR3 may contribute to the intrahepatic and unbalanced proinflammatory response. In contrast, a recent report showed that TLR3 activates macrophages to release exosomes that contain anti-HCV microRNA-29 (miR-29) family members, thereby inhibiting HCV replication in Huh7 cells (Zhou et al., 2016). Mosaad et al. (2019) further proved that the heterozygous CT genotype of TLR3 rs3775290 might be a susceptibility risk factor for chronic HCV infection. In contrast, the combination of female CC-AT-GA and the homozygous CC genotypes may be protective.

Hepatitis B virus (HBV) is a prevalent infectious agent which causes impaired liver function in humans (Assar et al., 2012). The role of TLR3 in HBV infection is also bi-directional. It has been reported that TLR3-knockout mice are unable to express IL-8 and other requisite molecules to activate immune responses

against HBV (Maire et al., 2008). An et al. (2007) found that patients with chronic HBV infection presented a slower elevation of TLR3 expression than healthy controls. Ondondo et al. (2009) revealed that patients with chronic hepatitis B (CHB) expressed lower TLR3 in monocyte-derived DCs (MoDCs) than normal controls. Based on the above two research findings, impairment of TLR3 expression and function was thought to be a major reason for the persistence of HBV infection (Karimi-Googheri and Arababadi, 2014). Additionally, Tjwa et al. (2012) demonstrated that activation of myeloid DC using TLR3 ligands led to improved natural killer (NK) cell function in CHB infection. Wang K et al. (2010) found that messenger RNA (mRNA) levels of TLR3 increased in CHB in the active phase, suggesting that TLR3 may play a vital role in starting innate immunity. Nevertheless, recent reports showed that the TLR3 polymorphism rs3775291 was associated with reduced spontaneous hepatitis B surface antigen (HBsAg) seroclearance (SC) of HBV infection and an increased risk of developing CHB (Geng et al., 2016; Fischer et al., 2018).

WNV is a flavivirus transmitted by mosquitoes, which causes encephalitis, especially in the elderly and immunocompromised individuals (Kramer et al., 2008). WNV non-structural protein 1 (*NS1*) is a crucial gene required for viral RNA replication and inhibiting TLR3-mediated signal transduction (Wilson et al., 2008). However, data on the role of TLR3 signaling in WNV protection or pathogenesis are controversial. While some studies have suggested that TLR3 serves a protective role against WNV by restricting WNV replication in neurons and through its antagonism against *NS1* (Daffis et al., 2008; Wilson et al., 2008), others have demonstrated that compared with TLR3-deficient mice, the WT mice were less resistant to lethal WNV infection (Wang et al., 2004). Kong et al. (2008) found that during WNV infection, the expression of TLR3 was reduced in macrophages of young donors, but increased in those of the elderly. This change with aging suggested that increased incidence of WNV encephalitis in elderly humans may be linked to increased TLR3 signaling, which may lead to elevated cytokine levels and contribute to the permeability of the blood-brain barrier. Recent research showed that immunization with *NS1* might reduce brain inflammation in the context of TLR3 deficiency (Patel et al., 2019).

The presence of the TLR3 rs5743313/CT polymorphism is closely related to an increased risk of pneumonia in children infected by the pandemic A/influenza A (H1N1)/2009 influenza virus (Esposito et al., 2012). A recent study showed that A/HK/415742/09 (pandemic H1N1 influenza (pH1N1)) virus-infected TLR3<sup>-/-</sup> mice did not have better survival than pH1N1-infected WT mice and showed no difference in viral titer and leukocyte infiltration in the lungs, suggesting that TLR3 did not worsen the pathogenesis of pH1N1 infection (Leung et al., 2014). The precise role of TLR3 against influenza in humans remains to be determined.

The effect of TLR3 on susceptibility to tick-borne encephalitis virus (TBEV) infection is poorly understood. Research by Kindberg et al. (2011) showed that the WT rs3775291 TLR3 allele was more common among TBEV patients than in healthy controls, suggesting that TLR3 might be a risk factor for TBEV infection. It was recently reported that polymorphisms in *TLR3* have a statistically significant impact on TBEV infection (Mickiené et al., 2014).

## 6 Conclusions and future directions

In this review, we discussed the TLR3-mediated antiviral response based on the activation and regulation of the TRIF signaling pathway. The TRIF and TRIF-mediated signaling pathways are critical for understanding the role of TLR3 in the immune response, which could give us a new direction to clarify the pathogenesis of diseases and find cures. Significant progress has been made in dissecting the mechanisms of TRIF-dependent activation of IRF3 and NF-κB, but how the TRIF/AP-1 pathway works needs further study. Recent work has focused on proteins that act as adaptors or mediate the activation of adaptors on the TRIF pathway, but some of their mechanisms remain to be further determined. A variety of enzymes that act as negative regulatory agencies of TLR3 have been found, but how these regulators are activated during viral infection and their cell type- and species-specific roles remain unclear.

Moreover, the dual role of TLR3 in ensuring or compromising host immunity against viruses is still largely unknown. In the case of chronic RNA viral infections that lead to sustained IFN-α/β signaling, the

TLR3-TRIF axis may be crucial in determining how the balance between antiviral and immune regulatory pathways affects defensive versus offensive responses. In future work, the TLR3-TRIF pathway may be essential for the establishment of specific therapeutic approaches to diminish TLR3-driven disease.

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## Author contributions

Huashan YI, Yujuan CHEN, and Xianping MA were responsible for conceptualization; Yujuan CHEN, Junhong LIN, and Yao ZHAO for article writing, data collection, and manuscript revision; Yujuan CHEN for picture design; and Xianping MA and Huashan YI for supervision. All authors have read and approved the final manuscript.

## Compliance with ethics guidelines

Yujuan CHEN, Junhong LIN, Yao ZHAO, Xianping MA, and Huashan YI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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