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REVIEW

Activation of toll-like receptor signaling pathways leading to nitric oxide-mediated antiviral responses

Mohamed Sarjoon Abdul-Cader[1] · Aruna Amarasinghe[1] ·
Mohamed Faizal Abdul-Careem[1]

Abstract Toll-like receptors (TLRs), well-characterized pattern-recognizing receptors of the innate arm of the immune system, are vital in detecting pathogen-associated molecular patterns (PAMPs). The TLR-PAMP interaction initiates an intracellular signaling cascade, predominantly culminating in upregulation of antiviral components, including inducible nitric oxide synthase (iNOS). After activation, various TLR pathways can promote iNOS production *via* the myeloid differentiation primary response-88 (MyD-88) adapter protein. Subsequently, iNOS facilitates production of nitric oxide (NO), a highly reactive and potent antiviral molecule that can inhibit replication of RNA and DNA viruses. Furthermore, NO can diffuse freely across cell membranes and elicit antiviral mechanisms in various ways, including direct and indirect damage to viral genomes. This review emphasizes current knowledge of NO-mediated antiviral responses elicited after activation of TLR signaling pathways.

Introduction

The innate immune system, which mounts host responses against invading pathogens, is equipped with a broad range of germ-line-encoded host receptors referred to as pattern-recognition receptors (PRRs), including NOD-like receptors (NLRs), toll-like receptors (TLRs), RIG-like receptors (RLRs), and C-lectin-type receptors (CLRs) [94]. These innate receptors are capable of recognizing microbial pathogens (e.g., viruses, bacteria and fungi) due to the presence of pathogen-associated molecular patterns (PAMPs), molecules that are highly conserved among microbes. Of the aforementioned PRRs, TLRs are well characterized and indispensable in detecting PAMPs of viruses and other pathogens [81]. When a TLR recognizes a PAMP, that activates an intracellular signaling cascade [117], culminating in upregulation of gene transcription for production of innate antiviral components, including type-1 interferons (IFNs) and pro-inflammatory mediators, including inducible nitric oxide synthase (iNOS) [137]. The latter enzyme promotes production of nitric oxide (NO), which can inhibit viral replication, both directly and indirectly [5, 120].

In the last two decades, our understanding of TLR biology has progressed substantially and been the subject of many reviews [1, 64, 117, 135, 137, 140]. However, none of these reviews has focused on TLR-signaling-mediated production of NO leading to antiviral responses. Therefore, the primary purpose of this review is to discuss current knowledge of NO-mediated antiviral responses elicited following activation of TLR signaling pathways.

Toll-like receptors

The first TLR, identified in an insect (*Drosophila*), was a molecule with a critical role in antifungal responses. Subsequently, 13 types of TLRs (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12 and TLR13) have been identified in mammals. It is noteworthy that TLR1-9 are present in both humans and mice, whereas TLR11, TLR12 and TLR13 are present only in mice (Fig. 1) [117]. Finally, TLR10 was identified in

✉ Mohamed Faizal Abdul-Careem
faizal.abdulcareem@ucalgary.ca

1   Department of Ecosystem and Public Health, Faculty of
     Veterinary Medicine, University of Calgary, Health Research
     Innovation Center 2C58, 3330 Hospital Drive NW, Calgary,
     AB T2N 4N1, Canada

Fig. 1 Common and specific TLRs in humans, mice and birds. In mammals, TLR2 can dimerize with TLR1 or TLR6, whereas in birds, TLR2 is divided into TLR2a and TLR2b and TLR1 is divided into TLR1La and TLR1Lb (due to gene duplication). TLR2s can form a heterodimer complex with TLR1Ls
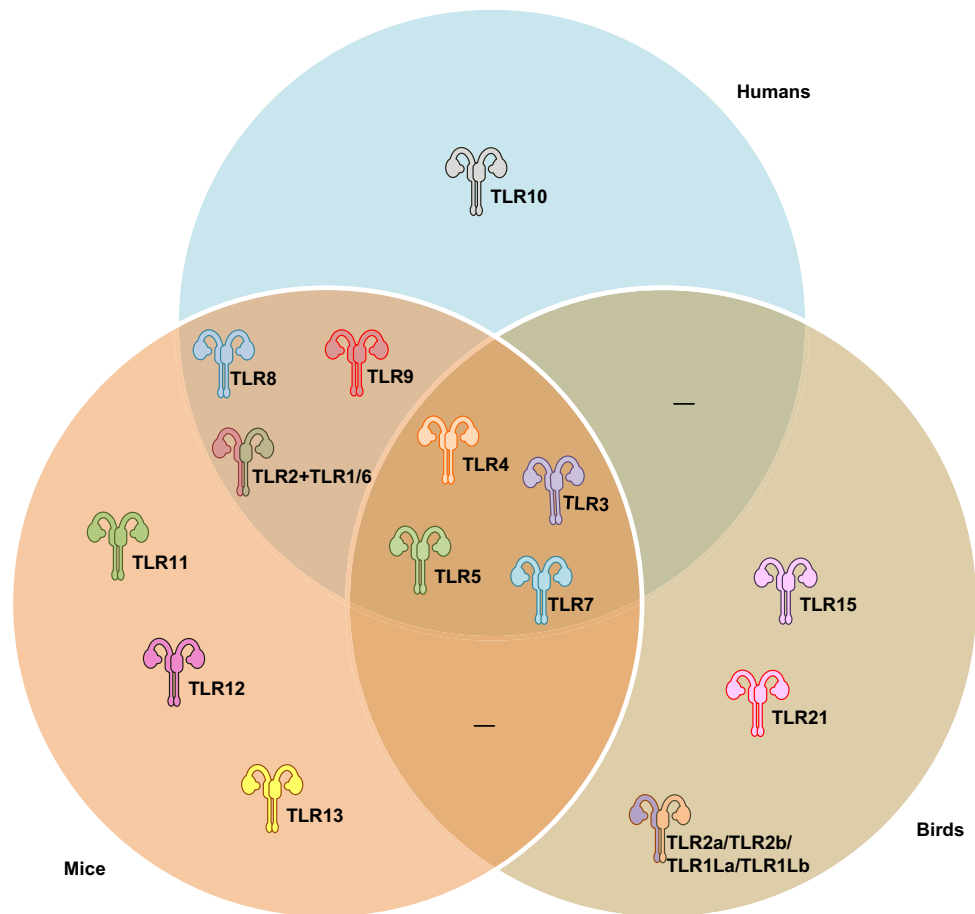
humans; mice have a TLR10 gene, but it is interrupted and nonfunctional [42]. In birds, TLR2a, TLR2b, TLR4, TLR5 and TLR7 are comparable to their counterparts in humans and mice, whereas TLR1La, TLR1Lb and TLR15 are exclusive to birds [17]. Furthermore, in birds, the TLR8 gene is apparently nonfunctional [106] and the TLR9 gene is missing [136, 148]. That notwithstanding, TLR21, which is unique in birds and fish, has functions similar to those of TLR9 in mammals [16].

Expression of TLRs

TLRs are expressed on various immune and non-immune cells, including macrophages, T and B lymphocytes, and epithelial cells, [9, 53]. In addition, cells in muscle, heart, brain and reproductive organs (testis, ovary, uterus and placenta) also express TLRs [38, 91]. That preferential expression of TLR types varies among cell types suggests activation of specific TLR signaling pathways depending on the type of cells involved. For example, most human peripheral blood mononuclear cells (PBMCs) express TLR1 and TLR6 [53], monocytes and B cells preferentially express TLR2 and TLR10, respectively, and B cells and

subsets of dendritic cells highly express TLR7 and TLR9 [53]. Furthermore, TLR7 and TLR9 are highly expressed in dendritic precursor cells following stimulation, and precursor cells of monocytes can be stimulated to upregulate TLR2 and TLR4 [58].

Expression of TLRs varies among host cells; they are expressed on the cell membrane, endosomal membrane, or both, depending on the type of TLRs (Fig. 2). For example, TLR1-2, 5-6 and 10-11 are expressed on the cell surface and distinguish PAMPs on the surface of microbes [132], whereas, TLR3, 7, 8, 9 and 21 are expressed intracellularly on the membrane of the endosomal compartment, and they strategically recognize microbial nucleic acid components during replication [7, 47, 48]. Additionally, TLR4 can be expressed on both cellular and endosomal membranes [133] and is capable of interacting with PAMPs that are on the surface of the microbes or are exposed during replication within the host cell.

TLR ligands

Each TLR binds to a unique set of ligands (PAMPs of microbes or synthetic compounds) in order to activate
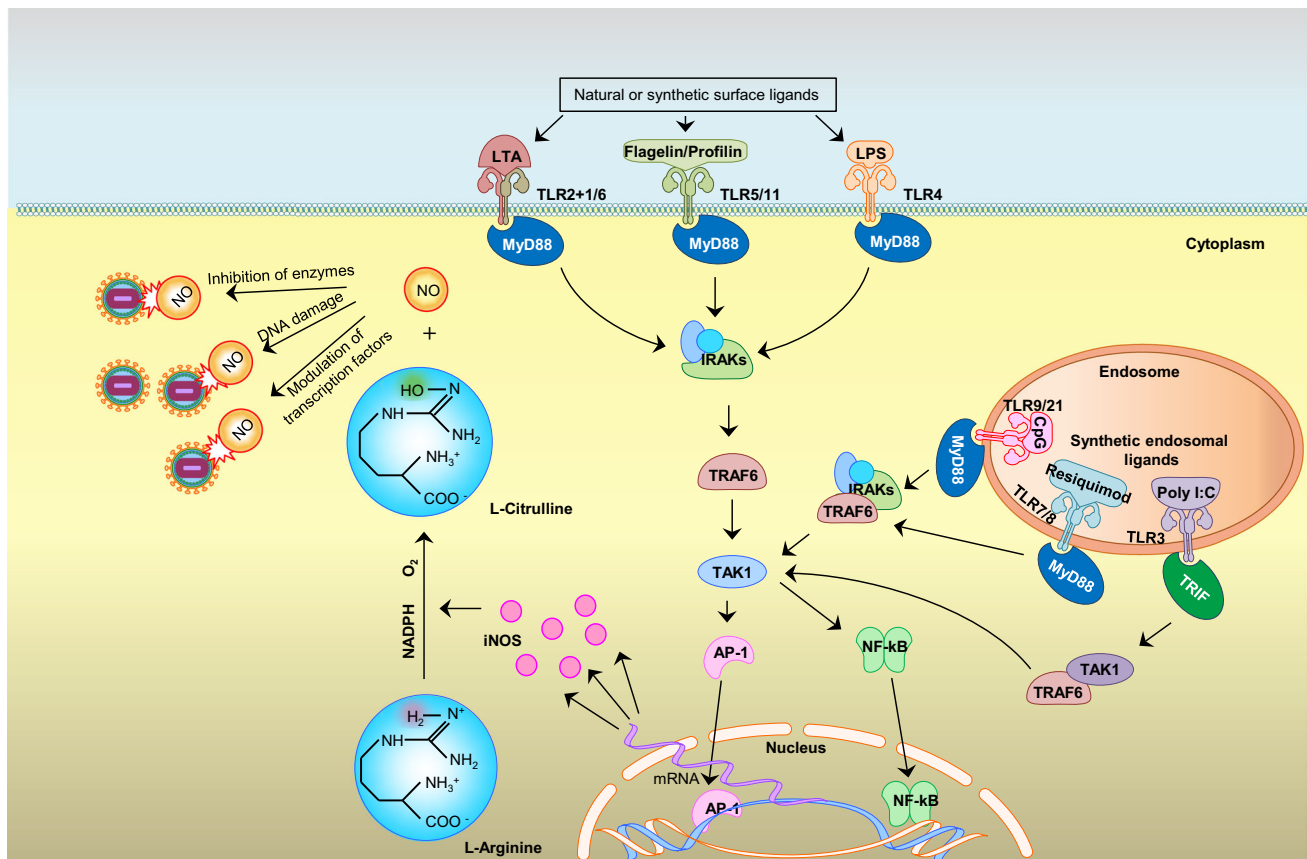
Fig. 2 Illustration of potential synthesis of NO *via* TLR signaling leading to NO-mediated antiviral activity. The TLRs are expressed on the cell surface or inside cells. Among those expressed on the surface, TLR2, 4, 5 and 11 are well studied with respect to iNOS expression. TLR3, 7, 8 and 9/21 are expressed on the membrane of the endosomal compartment and recognize nucleic-acid-based PAMPs, leading to expression of iNOS (among many other mediators). Most of these TLRs use MyD-88 for downstream signaling, but TLR3 uses TRIF protein as an adaptor molecule. In downstream signaling, activated NF-κB or AP-1 enters the nucleus and upregulates gene transcription for iNOS, which facilitates conversion of L-arginine to L-citrulline (using NADPH as an electron donor) to generate highly reactive NO, which has various antiviral effects. TLR, toll-like receptor; LTA, lipoteichoic acid; LPS, lipopolysaccharide; CpG, CpG motif of unmethylated DNA; Poly I:C, polyinosine-polycytidylic acid; MyD-88, myeloid differentiation primary response 88; TRIF, TIR-domain-containing adaptor inducing IFN; IRAKs, IL-1 receptor-associated kinases; TRAF, TNF-receptor-associated factor; TAK1, transforming growth factor beta-activated kinase-1; IKKε, IkappaB kinase-epsilon; RIP1, receptor-interacting protein kinase 1; NF-κB, nuclear factor kappa B; AP-1, activator protein-1; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate H; NO, nitric oxide

signaling pathways. Of the surface-expressing TLRs, TLR2 mainly recognizes the peptidoglycan and lipoteichoic acid (LTA) present in Gram-positive bacteria [66, 138]. Additionally, TLR2 can recognize zymosan, a cell wall component of yeast [119], hemagglutinin protein of measles virus [14], core protein and nonstructural-3 protein of hepatitis C virus [29], and surface glycoproteins (gH, gL and gB) of herpes simplex virus (HSV) [79]. It appears that TLR2 is mainly capable of recognizing lipoproteins or lipopeptides; perhaps at least some of the other putative TLR2 ligands were misclassified due to contamination with highly active natural lipoproteins or lipopeptides [149]. Furthermore, TLR2 can form a heterodimer complex with TLR1 or TLR6 and recognize lipopeptides present in various bacteria [121]. Similarly, TLR1 associated with TLR2 recognizes triacyl lipopeptides, whereas a TLR6-TLR2 complex recognizes diacyl lipopeptides [55]. In addition, TLR4 binds to bacterial endotoxin and lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria [11]. However, for initiation of LPS signaling, TLR4 requires association with another surface molecule, myeloid differentiation factor 2 (MD-2) [124], and CD14 [109]. Moreover, TLR4 can recognize cell wall components of viruses, fungi and helminths, including the envelope protein of murine retroviruses, HIV-1 and human endogenous retrovirus [96, 111, 114], the fusion protein of respiratory syncytial virus [43], α-glucan and mannan of fungus [15, 127], and lacto-N-fucopentaose III of helminths [139]. Expression of TLR5 occurs in intestinal epithelial cells, mainly at the basolateral surface; it

recognizes invading flagellated pathogenic bacteria by identifying a protein called flagellin [33]. The amino acid sequence of TLR5 appears to be similar to those of TLR11 and TLR12 [115]; the latter two TLRs form a complex and recognize profilin-like proteins in *Toxoplasma gondii* (protozoan parasite) [108]. Finally, TLR10 may sense ligands derived from within the host rather than from microbes [102].

Considering endosomal TLRs, TLR3 binds to viral double-stranded RNA (dsRNA) and polyinosinic poly-cytidylic acid (polyI:C; a synthetic compound, structurally similar to viral dsRNA) [86], whereas TLR7 and 8 bind to viral single-stranded RNA (ssRNA) [47]. Two receptors, TLR9 in mammals and TLR21 in birds, are the only ones known to detect both bacterial and viral DNA containing unmethylated cytosine-guanosine deoxynucleotides (CpG) motifs, which are generally methylated in vertebrate genomes [48]. Consequently, the frequency of CpG motifs is negligible in vertebrate DNA, although it occurs with high frequency in microbial genomes [71]. Differences in methylation and the prevalence of unmethylated CpG motifs in DNA of microbes (bacteria, fungus and viruses) allow selective host responses against DNA of microbial origin. CpG DNA has three major classes (A, B and C) based on structural variations and effects on PBMCs [25]. Class A CpG DNA (also known as ODN 2216) predominantly activates dendritic cells and natural killer (NK) cells, with effects mediated *via* interferon regulating factor (IRF) 7 signaling pathways from early endosomes that promote production of type 1 IFN. Class B (ODN 2007) is a strong activator of B cells and monocytes and operates *via* nuclear factor kappa (NF-κ) B signaling pathway from late endosomes, leading to production of pro-inflammatory mediators. Finally, class C CpG DNA has characteristics of both class A and B [72] in terms of both structure and function.

Detailed understanding of TLR structure and signaling mechanisms have enabled development of specific synthetic ligands with therapeutic potential [144]. In that regard, these ligands can be used to manipulate the host immune system [51, 144].

Structure and signaling mechanism of TLRs

Structure of TLRs

The TLRs are in the type 1 transmembrane protein family. Structurally, each TLR expressed on a cell membrane consists of a cytoplasmic C-terminal domain, a transmembrane component, and an N-terminal domain that is exposed to the outside of the cell [37]. In direct contrast, in endosomal TLRs, the N-terminal region is exposed internally and the C-terminal region is exposed externally [55]. The C-terminal domain is conserved and is homologous to the internal domain of an interleukin 1 receptor (IL-1R) named Toll/IL-1R (TIR); therefore, TLRs are classified as a subfamily of the interleukin-1 receptor/toll-like receptor superfamily [100]. Although the N-terminal domain of most TLRs has a horseshoe shape, extracellular regions of TLR8 and TLR9 are ring-shaped [101, 134]. The extracellular component of TLR2 can form a heterodimer with the extracellular domain of TLR1 or TLR6, although other TLRs can form only homodimers [28]. Furthermore, before and after ligand binding, TLR9 is a monomer and a dimer, respectively [101]. Cluster of differentiation-14 (CD-14) molecules can be associated with TLR4, TLR1/TLR2 or TLR2/TLR6 [12], and a concerted effort is required for recognition of certain ligands. Conversely, the curved N-terminal domain of TLRs interacts with ligands, and the ligand binding sites consist of many leucine-rich repeats (LRR). The length and the sequence of LRRs vary among TLRs [20]. In contrast to other TLRs, TLR7,8 and 9 contain a long insertion loop region (Z-loop) of ∼40 amino acids between LRR14 and 15 [134]. Recognition of various PAMPs by the N-terminal domain causes TLR molecules to undergo conformational changes in the TIR domain that facilitate binding of various intracellular adaptor molecules with the TIR domain, thereby triggering a cascade signaling mechanism [99].

Initially, TLR9 is present in the endoplasmic reticulum (ER) [78], but after exposure of host cells to PAMPs, it migrates to endosomes [77]. In general, TLRs expressed in an endosomal membrane need to interact with a transmembrane protein of the ER, namely, Unc-93 homolog B1 (UNC-93B) as a prerequisite for endosomal translocation from the ER and subsequent recognition of PAMPs [22]. Such translocated TLRs, particularly TLR7 and TLR9, need further activation through cleavage of their N-terminal domain by endosomal proteases [31]. Activation of TLR9 is mediated by binding a stimulatory sequence of microbial DNA containing CpG motifs; however, a non-stimulatory sequence of microbial DNA can competitively block this activation [77, 110].

TLR signaling pathways

Activation of TLR signaling pathways results in maturation, differentiation and expansion of a number of immune cells, including macrophages, B cells, NK cells and T cells. Innate mediators activated downstream of TLR signaling coordinate recruitment of immune cells. In a recent study using a microarray to evaluate thousands of genes, CpG DNA treatment of host cells upregulated 77 genes, including IFNs, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-10, IL-12, cyclooxygenase-2 (COX-2),

iNOS and granulocyte-macrophage colony-stimulating factor (GM-CSF) [62].

A TLR-ligand interaction initially stimulates conformational changes in the TIR domain, facilitating recruitment of various adaptor molecules, e.g., myeloid differentiation primary response-88 (MyD-88) protein, TIR domain-containing adaptor protein (TIRAP), and a TIR-domain-containing adaptor inducing IFN (TRIF) to initiate downstream signaling [52, 128]. Furthermore, MyD-88 is a key adaptor molecule involved in most of the activating signaling pathways of cell-surface and endosomal TLRs that are denoted MyD-88-dependent signaling pathways (Fig. 2) [88]. However, in studies with MyD-88 knockout mice/cell lines, NF-κB was not activated following TLR2, TLR7 and TLR9 stimulation [49, 63, 122]. However, ligands of TLR3 and TLR4 produce type 1 IFNs and delay activation of NF-κB, suggesting involvement of a MyD-88-independent signaling pathway [7, 30]. Furthermore, a TRIF protein is a key adaptor molecule for TLR3 activation pathway (designated as a TRIF-dependent signaling pathway or MyD-88-independent signaling pathway) [128]. In contrast, TLR4 may initiate both MyD-88-dependent and TRIF-dependent signaling pathways in mammals [152]. In chickens, TLR4 activation may upregulate TRIF mRNA expression [59], although an LPS-TLR4 interaction selectively activates only a MyD-88-dependent signaling pathway in chickens, and not both signaling pathways, as in mammals [65].

In the MyD-88-dependent signaling pathway, signaling initiated by TLR-associated MyD-88 molecules activates many cytoplasmic mediators, including IL-1 receptor-associated kinases (IRAKs) and TNF receptor-associated factor (TRAF) 6, resulting in activation of transforming growth factor beta-activated kinase-1 (TAK1). The latter protein ultimately has a dual role, activating either NF-κB or activator protein (AP) 1 through a series of reactions (Fig. 2) [145]. Thereafter, NF-κB or AP-1 enters the nucleus and upregulates gene transcription of innate and pro-inflammatory mediators including iNOS, IL-1, TNF-α and IL-6 (Fig. 2) [4, 145]. Furthermore, activation of the MyD-88-IRAK-TRAF6 complex by endosomal TLRs sequentially activates an additional pathway via activation of TRAF3, IRAK1 and IkappaB kinase-α (IKKα), ultimately leading to activation of IRF-7 [20, 141], which enters the nucleus and upregulates gene transcription for type 1 IFNs (potent antiviral cytokines) [142].

In the MyD-88-independant pathway (TRIF-dependent signaling pathway) [128], the activated TIR domain binds to TRIF (an adaptor molecule), which recruits receptor-interacting protein kinase 1 (RIP1) and TRAF6 molecules to activate TAK1, ultimately leading to activation of NF-κB, as in the MyD-88-dependent pathway [7, 147]. Alternatively, TRIF can activate the TRAF3-TANK-binding kinase 1 (TBK1)-IKKε complex, causing phosphorylation and activation of IRF3 and IRF7 [30, 118], which move into the nucleus and upregulate transcription for antiviral type-1 IFNs [118].

Cellular production and antiviral mechanisms of NO

NO production

It is well known that NO is a highly diffusible free radical molecule derived from L-arginine via NO synthase (NOS) enzyme activity in the presence of NADPH [95] and that it is widely involved in the regulation of various physiological mechanisms, including the immune, circulatory and nervous systems (Fig. 2). There are three isoforms of NOS: neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS or NOS2) and inducible NOS (iNOS or NOS3) [143]. Both nNOS and eNOS are classified as constitutive NOS; they are less responsive to stimulation, have calmodulin/calcium-dependent enzyme systems [6], and produce low concentrations of NO [5]. Conversely, iNOS is mainly involved in the innate arm of the immune system, and its enzyme activity is calmodulin/calcium-independent [6, 87]. Furthermore, immunological stimulation (e.g., TLR signaling) is necessary for it to produce NO [87]. However, once stimulated, iNOS produces large quantities of NO for prolonged intervals, thereby facilitating innate host responses [75, 120].

Activation of TLR signalling pathways leading to NO production

Production of NO in host cells can be activated via various TLR pathways (Fig. 2). Of the cell-membrane-expressed TLRs, TLR2, 3, 4 and 5 are involved in signaling leading to NO production via activation of the MyD-88-dependent pathway [45]. Binding of LPS to TLR4 induces a strong NO response [45]. In a mouse macrophage cell line, LPS in combination with IFN-γ elicited production of much more NO than LPS alone [84], suggesting that mouse macrophages require priming with IFN-γ to enhance NO production. Similarly, in avian macrophages, priming with IFN-γ [23] or a viral infection [90] is a prerequisite for an LPS-mediated NO response. Gram-negative bacterial flagellin induces NO production in macrophages by a pathway involving both TLR4 and TLR5. Furthermore, TLR4 may promote binding of flagellin to TLR5/TLR4 complexes, as flagellin failed to induce NO production in cells with non-functional TLR4 [93]. The TLR2 ligand LTA induces iNOS expression and NO release from mouse macrophage cell line (RAW 264.7), and this is mediated

via two pathways leading to NF-κB activation. An early response (minutes) is mediated by NF-κB activation *via* phosphatidylcholine-phospholipase activation, whereas in a late response (hours), NF-κB production is promoted by a COX2-prostaglandin E2-mediated pathway [19].

Of the endosomal-membrane-expressed TLRs, both TLR9/21 signaling and TLR3 signaling induce NO production. The presence of DNA containing CpG motifs, which serve as TLR9/21 ligands, induces NO production in macrophage cell lines; the stimulatory effect is positively correlated with a number of motifs in CpG DNA, e.g., GTCGTT [44]. Production of NO by avian macrophages was studied using various classes of CpG, *viz.* CpG 2216, CpG 2395, CpG 1826 and CpG 2007. Nearly all CpG classes (except CpG 2216) induced significant production of NO in comparison to non-CpG controls [8]. Furthermore, poly I:C, the TLR3 ligand, stimulated mouse bone marrow macrophages to produce NO [76], and it also activated iNOS in human monocyte-derived macrophages [125].

In addition to TLR signaling using single TLR ligands, additive effects of induction of multiple TLR pathways for NO production have been reported. For example, production of NO in avian PBMCs was higher with a combination of ligands than with only a single ligand, confirming previous observations [46]. Also LPS and microbial DNA had a synergistic effect in enhancing iNOS expression and production of NO in macrophages [36]. Similarly, a combination of recombinant flagellin and LPS synergistically induced NO production in avian PBMCs [40]. In addition, peptidoglycan (PepG) and LTA from *Staphylococcus aureus* synergistically induce iNOS. Finally, the level of NO production by murine macrophages in response to PepG, LTA and PepG plus LTA was much higher if two or more of these ligands were present [27].

Antiviral mechanisms of NO

A highly reactive free radical, NO has key roles in innate immune responses against numerous viruses [13, 24, 120] that infect mammals and birds [41, 70, 146]. It is well documented that induction of iNOS expression or providing NO by adding NO donors such as S-nitroso-N-acetylpenicillamine (SNAP) inhibits replication of various RNA or DNA viruses. Due to its hydrophobicity, NO diffuses freely across cell membranes (without receptors or carrier proteins) [21, 83]. An unpaired electron makes NO highly chemically reactive, and its antiviral mechanism is mainly a paracrine effect [73]. After diffusion through the cell membrane, NO may have several antiviral mechanisms. First, viruses with a DNA genome may undergo direct damage *via* nitrosation of primary amines [97, 131]

in addition to indirect damage by endogenously produced NO-mediated N-nitrosamines, including N_2O_3 [92]. Second, NO indirectly reduces synthesis of viral genome and viral proteins in host cells by inactivating or modifying molecules involved in viral replication, including viral proteases [120], ribonucleotide reductase [80], reverse transcriptase [21, 104], transcriptional factors [123], and tyrosine- or heme-containing enzymes [54], through nitrosylation of these enzymes (Fig. 2) [130, 151].

This NO-mediated inhibition has been demonstrated *in vitro* against several viruses, including influenza virus [112, 113], dengue virus [129], herpes simplex virus [24, 89], vesicular stomatitis virus (VSV) [13], Japanese encephalitis virus [82], infectious laryngotracheitis virus [41], Marek's disease virus (MDV) [146], coxsackievirus [34, 151], vaccinia virus [60], porcine respiratory coronavirus [57], rhinovirus [116], flavivirus [70], and hantavirus [67]. Similarly, based on *in vivo* studies, NO-mediated antiviral responses have been demonstrated against influenza virus [56], dengue virus [32], herpes simplex virus [10, 35], mouse hepatitis virus [74], Friend murine leukemia virus [3], hepatitis B virus [39], respiratory syncytial virus [126], infectious bursal disease virus [107], murine cytomegalovirus [98], MDV [146], coxsackievirus [34, 85], hantavirus [67], adenovirus [18] and VSV [69]. Antiviral responses observed in those *in vitro* and *in vivo* studies were based on exogenous NO supplied *via* NO donors. In addition to an antiviral effect, NO also has cytostatic effects in infected host cells, including inhibition of host DNA synthesis, protein synthesis and mitochondrial metabolism [105].

TLR activation leading to NO-mediated antiviral activity

Although there are numerous reports regarding NO-mediated antiviral responses, there is a paucity of literature on TLR-signaling-induced NO (endogenously produced)-mediated antiviral responses in mammals [3, 24, 50, 68, 85, 89] or birds [41, 105, 146]. A few *in vitro* studies have described NO-mediated antiviral activity following administration of TLR4 ligand against many viruses, including MDV, herpes simplex virus, infectious laryngotracheitis virus, coxsackievirus, and reovirus (Table 1). Similarly, an *in vitro* experiment with IRF3$^{-/-}$IRF9$^{-/-}$ mouse embryo fibroblasts demonstrated NO-mediated antiviral activity using the TLR3 ligand poly I:C. In that study, iNOS inhibitors, including aminoguanidine hydrochloride (AMG) and N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL), reversed the antiviral effect of poly I:C [89]. In an *in vivo* study in mice, coxsackievirus infection in combination with LPS (TLR4 ligand) induced expression of iNOS in macrophages. In addition, inhibition

Table 1 Viruses that activate TLR signaling pathways leading to NO-mediated antiviral activity

Virus name	Type of virus	Common host	Model	Ligand (TLR)	Outcome	Reference
Herpes simplex virus-1	dsDNA	Humans	*In vitro*	LPS (TLR4)	TLR4 (LPS)-mediated, NO-dependent antiviral response in a murine macrophage cell line	[24]
				Poly I:C (TLR3)	TLR3 (Poly I:C)-mediated, NO-dependent antiviral response in murine embryonic fibroblasts	[89]
Herpesvirus of turkeys	dsDNA	Birds	*In vitro*	LPS (TLR4)	TLR4 (LPS)-mediated, NO-dependent antiviral response in avian embryonic fibroblasts	[146]
Marek's disease virus	dsDNA	Birds	*In vitro*	LPS (TLR4)	TLR4 (LPS)-mediated, NO-dependent antiviral response in avian embryonic fibroblasts	[146]
			In vivo	LPS (TLR4)	TLR4 (LPS)-mediated, NO-dependent antiviral response	
Coxsackie virus	ssRNA	Humans	*In vitro*	LPS (TLR4)	TLR4 (LPS)-mediated, NO-dependent antiviral response in murine macrophage cell line.	[50]
			In vivo	LPS (TLR4)	TLR4 (LPS)-mediated NO dependent antiviral response shown in a mouse model	[85]
Friend murine leukemia virus	ssRNA	Murine	*In vitro*	LPS (TLR4)	TLR4 (LPS)-mediated, NO-dependent antiviral response in murine macrophages	[3]
Laryngo-tracheitis virus	dsDNA	Birds	*In vitro*	LPS (TLR4)	TLR (LPS)-mediated, NO-dependent antiviral response in an avian macrophage cell line	[41]
Reovirus	dsRNA	Birds	*In vitro*	LPS (TLR4)	TLR (LPS)-mediated, NO dependent antiviral response in an avian macrophage cell line	[105]

of NO production by NG-monomethyl-L-arginine (NMMA) increased viral load and mortality [85].

Viral constituents that activate the TLR pathways leading to NO-mediated antiviral responses are shown in Figure 3. In addition to viral nucleic acid, components of viral envelopes are also capable of inducing NO

Fig. 3 Viral components that activate TLRs signaling leading to NO-mediated antiviral activity in mammals and birds. In birds, TLR8 and TLR9 are absent; however, TLR21 replaces the function of TLR9 in mammals. TLR3, TLR7, TLR8, TLR9 and TLR21 are endosomal TLRs that recognize viral nucleic acids, whereas TLR2 and TLR4 are surface TLRs that detect viral surface molecules

production, leading to antiviral activity. Induction of NO following viral replication in the host may curtail ongoing viral replication. However, prior induction of NO- mediated antiviral responses *via* induction of the TLR pathway appears to be more effective in preventing virus-induced pathology.
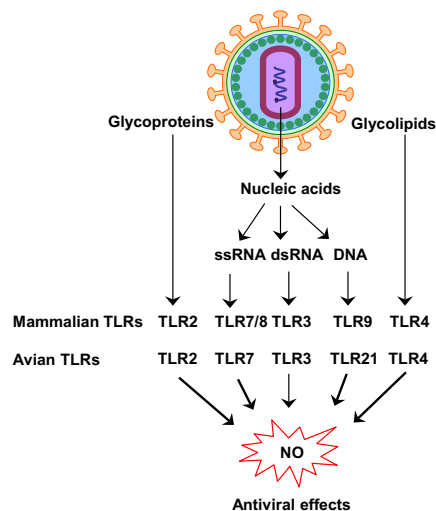
NO and influenza virus

Although the role of NO against influenza virus infection is not well defined, *in vitro*, there are clearly beneficial effects of NO against influenza virus infections. Adding SNAP (an NO donor) to Madin-Darby canine kidney (MDCK) cells immediately after infection with influenza A and B viruses inhibited replication of both viruses in a dose-dependent manner during initial stages of infection [113]. Similarly, when the MDCK cell line was exposed to gaseous NO before and after infection with influenza A and B viruses, there was inactivation of viral neuraminidase activity and inhibition of viral infectivity by both pre- and post-infection NO exposures [112]. Unfortunately, studies conducted *in vivo* do not necessarily support observations made in *in vitro* systems. In a mouse model of influenza infection, inhalation of NO prior to influenza infection may decrease mouse survival, with no change in lung viral loads [26]. Using iNOS-knockout and wild-type mice, it was shown that NO production is not essential to clear infections with influenza virus A and reduce pulmonary pathology. In that

experiment, wild-type mice that produced NO had severe pneumonitis [61], suggesting that NO was a detrimental factor exacerbating pneumonia during influenza virus infection [2]. Apparent discrepancies in observations between *in vitro* and *in vivo* studies regarding the benefits of NO against influenza virus infection in mammalian models may be explained by NO-mediated increases in pulmonary inflammation, which could be detrimental to the host *in vivo* [150]. Perhaps an appropriate balance between antiviral and inflammatory effects of NO is required for successful protective immunity against influenza virus infection *in vivo* [103].

Conclusions

Of the two TLR signaling pathways, the MyD-88-dependent signaling pathway is the one that predominantly leads to NO-mediated antiviral responses. Analysis of the current knowledge in the area of TLR-signaling-mediated NO-dependent antiviral responses revealed knowledge deficits in three major areas. Firstly, despite many reports regarding antiviral activity of NO against numerous mammalian and avian viruses based on the use of external NO donor compounds, there is a paucity of information on activation of TLR signaling pathways resulting in endogenous NO production leading to innate antiviral responses in mammalian and avian hosts. Secondly, of the studies that describe activation of TLR signaling pathways leading to NO-mediated antiviral responses, there are very few on NO-mediated antiviral responses *in vivo* [85]. Consequently, the therapeutic or prophylactic potential of activation of TLR signaling leading to NO-mediated antiviral responses is not well defined for either mammals or birds. Finally, although various MyD-88 signaling pathways are potentially capable of eliciting a NO response, most studies have focused on TLR4 and TLR3 signaling leading to NO-mediated antiviral responses, and further studies are therefore required to clarify the role of other MyD-88 signaling pathways in NO-mediated antiviral responses.

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