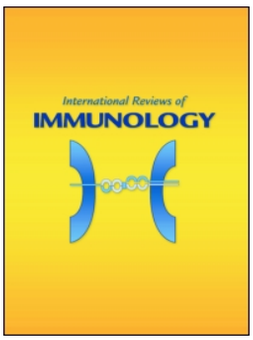

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Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals

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ABSTRACT

This review attempts to cover the implication of the toll-like receptors (TLRs) in controlling immune functions with emphasis on their significance, function, regulation and expression patterns. The tripartite TLRs are type I integral transmembrane receptors that are involved in recognition and conveying of pathogens to the immune system. These paralogs are located on cell surfaces or within endosomes. The TLRs are found to be functionally involved in the recognition of self and non-self-antigens, maturation of DCs and initiation of antigen-specific adaptive immune responses as they bridge the innate and adaptive immunity. Interestingly, they also have a significant role in immunotherapy and vaccination. Signals generated by TLRs are transduced through NFκB signaling and MAP kinases pathway to recruit pro-inflammatory cytokines and co-stimulatory molecules, which promote inflammatory responses. The excess production of these cytokines leads to grave systemic disorders like tumor growth and autoimmune disorders. Hence, regulation of the TLR signaling pathway is necessary to keep the host system safe. Many molecules like LPS, SOCS1, IRAK1, NFκB, and TRAF3 are involved in modulating the TLR pathways to induce appropriate response. Though quantification of these TLRs helps in correlating the magnitude of immune response exhibited by the animal, there are several internal, external, genetic and animal factors that affect their expression patterns. So it can be concluded that any identification based on those expression profiles may lead to improper diagnosis during certain conditions.

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Cancer immunotherapy; innate immunity; PAMPs; pattern recognition receptors; tissue repair; TLRs

Abbreviations

APCs	antigen-presenting cells
CD	complementarity determination
CNV	copy number variants
CpG	cytosine-phosphate-guanosine
DAMPs	danger associated molecular patterns
DCs	dendritic cells
DNA	deoxyribo nucleic acid
DREDD	death related ced-3/Nedd2-like protein
dsRNA	double-stranded RNA
EMT	epithelial-mesenchymal transition
ET	endotoxin tolerance
FADD	Fas-associated death domain
IFN	interferon
IFN-β	interferon beta
IKK	IκB kinase
IL-1R	interleukin-1 receptor
IRAK	IL-1R-associated kinase
IRF	interferon response factor
LPS	lipopolysaccharide
LRR	leucine rich repeats
MAL	MyD88 adaptor-like molecule
MAPKs	mitogen-activated protein kinases
MHC	mono-histocompatibility complex
MSU	monosodium-urate monohydrate
MyD88	myeloid differentiation primary response protein 88
NEC	necrotizing enterocolitis
NFκB	nuclear factor kappa B
NK	natural killer cells
NLR	NOD like receptors
NOD	nucleotide-binding oligomerization domain
PAMPs	pathogen associated molecular patterns
PRRs	pattern recognition receptors
RIG	retinoic acid inducible gene
RING	really interesting new gene
RIP-1	receptor-interacting protein 1
RLR	RIG like receptors
RNA	ribo nucleic acid
ROS	reactive oxygen species

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SARM	selective androgen receptor modulators
SH2	Src homology 2
SHP1	SH2-containing protein tyrosine phosphatase 1
SIGIRR	single immunoglobulin IL-1 receptor-related molecule
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphisms
SOCS1	suppressor of cytokine signaling 1
STAT	signal transducer and activator of transcription
TAK	TGF-β-activated kinase
TANK	TRAF associated NF-κB activator
TBI	total body irradiation
TBK1	TANK binding kinase 1
TGF	transforming growth factor
Th cells	T helper cells
TIR	toll/IL-1 receptor
TIRAP	TIR domain–containing adaptor molecule
TLR	toll-like receptors
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain containing adaptor protein inducing IFN-β
TRIM	tripartite motif
25 OHD	25 hydroxy vitamin D

Introduction

The defense system of an animal is classified into specific adaptive immunity and non-specific innate immunity. The innate immunity has certain molecular receptors that recognize the pathogens invasion in the host. These molecular receptors are collectively called as pattern recognition receptors (PRRs) which are found in serum, on the cell surface, in endosomes and in the cytoplasm.[1] The PRRs are broadly classified into three categories, namely, NOD like receptors (NLR), RIG like receptors (RLR) and Toll-like receptors (TLRs). Each of these receptors recognizes different molecular patterns called as pathogen associated molecular patterns (PAMPs) which are expressed by the pathogens or the invading microbes, and danger associated molecular patterns (DAMPs) which are the components of damaged or apoptosed cells that act as endogenous stress signals. Figure 1 describes the receptors of the innate immune system. In addition, PRRs, particularly TLRs are the key molecules in discriminating between self and non-self-antigens.[2] Failure of TLRs to recognize the self-antigens result in many auto-immune disorders.

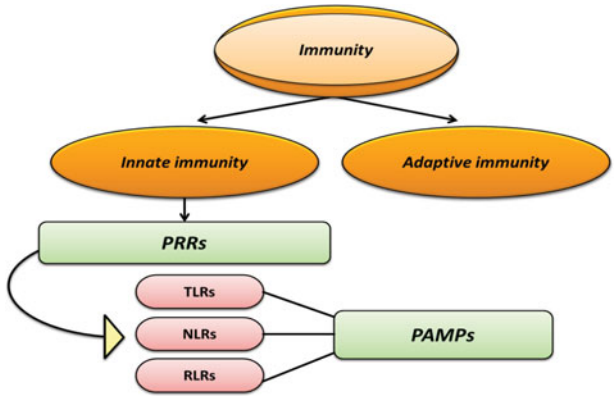

Figure 1. The molecules of innate immune system that are involved in the recognition and induction of immune response are called as pattern recognition receptors (PRRs). Toll Like Receptors (TLRs), NOD Like Receptors (NLRs) and RIG Like Receptors (RLRs) are the three types of PRRs associated with recognition of Pathogen Associated Molecular Patterns (PAMPs) expressed by the pathogens.

Among PRRs, NLRs are involved in regulation of inflammatory and apoptotic responses; RLRs are associated with intracellular recognition of RNA virus replication; and the unique elements TLRs are involved in alarming the immune system against extracellular or endosomal PAMPs like bacterial lipopolysaccharides, lipoteichoic acid, nucleic acids, etc.

The TLRs are germline-encoded transmembrane proteins derived from a toll gene family, which play a crucial role in the detection of many microbial patterns and activating the innate immune system.[3] The expressions of TLRs are different in different types of white blood cells.[4] They are expressed on dendritic cells (DCs), macrophages, natural killer (NK) cells, T and B lymphocytes and non-immune cells like epithelial cells, endothelial cells and fibroblasts. TLR ligands include PAMPs on infectious microorganisms, endogenous molecules and synthetic agonists.[5] There are at least 10 TLRs present in mammals each equipped with the unique ability to recognize different PAMPs. Signals transduced through the TLRs cause synthesis and secretion of pro-inflammatory cytokines and co-stimulatory molecules, which promote inflammatory responses that bring macrophages and neutrophils to sites of inflammation.

Significance of TLRs

The TLRs play a crucial role in both innate and adaptive immunity. Their ability to sense the endogenous DAMPS and exogenous PAMPs help them to generate ligand mediated signal transduction, which is finally involved with bringing out inflammatory response. There are increasing evidence in the recent days that suggests the

significance of TLRs and their ligands in many pathological conditions like inflammation, tumorigenesis and auto-immune disorders. Interestingly, they also have a significant role in immunotherapy and vaccination.

Significance of TLRs in ischemia and reperfusion injury

There is evidence that TLR4 promotes injury in liver, kidney, heart and brain.[6] Hence, as put forth by Chao,[7] down regulation of TLR2, TLR4, or MyD88 in ischemic injury reduce myocardial inflammation. TLR4 is also found to be responsible for the increased T cell response in burn injuries[8] and graft inflammation, sterile injury and alloimmune responses in tissue transplantation.[9]

Sepsis is a clinical syndrome characterized by severe infection with fever and leukocytosis. The up regulation of TLR2 and TLR4 on immune and non-immune cells during sepsis was found to be associated with tissue injury in organs.[10]

The role of TLRs in hypercholesterolemia-induced arterial injury has been put forward by many scientific studies.[11,12] While recently it has been revealed that TLR2 is markedly pro-atherogenic,[11] TLR3 is found to be involved in the protection of the integrity of the blood vessel wall.[12]

Significance of TLRs in tissue repair and regeneration

TLR response is critical in tissue injuries and subsequent tissue repair and regeneration, particularly in the liver and intestinal epithelium.[13] Further, TLR2 signaling also contributes significantly to wound healing. Epithelial TLRs detect microbial patterns and induce innate immune responses and thus help in the regulation of homeostasis.[6]

TLR4 and TLR5 are expressed in the basal layer of the corneal epithelial cells.[14] Whenever there is a breach in the squamous epithelium there is development of corneal inflammation and induction of keratitis through MyD88 dependent pathway by the functional TLR2, TLR4, and TLR9 which are also expressed in the corneal epithelium.

TLRs expressed in the epithelium of the intestine, lungs and urinary tract have been found to be involved in the perpetuation of mucosal inflammation leading to highly morbid conditions like inflammatory bowel disease or neonatal necrotizing enterocolitis (NEC); pneumonitis, pneumonia, or asthma; interstitial nephritis, cystitis, and urethritis, respectively.[15] In neonates, intestinal bacterial colonization starts after the first few days of birth. During this important immunomodulatory state, if TLRs are expressed, they will subsequently lead to interaction between intestinal epithelium and bacterial ligand; evoking inflammation in the developing intestine leading to NEC.[16]

Significance of TLRs in allergy and infection

Activation of TLRs has been shown to aggravate and ameliorate airway reactivity and inflammation as in case of asthma in animal models. TLR3, TLR4 and TLR9 ligands have been shown to exacerbate allergic airway inflammation in mice.[17] On the other hand, a study conducted by Nadeem et al.[18] suggests that treatment with TLR7 agonist activates TLR7 and enhances antioxidant network in the lung, thus providing protection against ROS-mediated airway reactivity and inflammation.

Deficiency of TLR4 expression is associated with increased susceptibility to Candida infection. Interestingly, as TLR2 is involved in the production of pro-inflammatory cytokines that recruits neutrophils to the site of infection, both TLR2 and TLR4 are found to be associated with the pathogenesis of Candidiasis.[19]

Significance of TLRs in auto-immune disorders

In recent studies, it has been indicated that endogenous TLR ligand-mediated signaling plays an important role in auto-immune disorders. Heijden et al.[20] detected the presence of bacterial DNA and peptidoglycans in the joints of patients with rheumatid arthritis (RA) and other arthritides, where they might enhance synovial inflammation through TLR ligand-mediated signaling. It has also been demonstrated that TLR9 and TLR7 are found to be involved in the perpetuation of systemic lupus erythematosus (SLE).[21] The danger signals released by the demyelinated nerve that are sensed by TLR9 evoke pathologic immune response against auto-antigens in multiple sclerosis.[22] The endogenous monosodium-urate monohydrate (MSU) crystals formed from uric acid released by damaged cells act as DAMP and trigger the TLR2 activation finally leading to cartilage degeneration.[23]

Significance of TLRs in tumorigenesis and tumor progression

It has been found that TLRs have both positive and negative roles in tumorigenesis. However, to date, TLRs have had the opposite effects on tumor progression. Though TLR ligands can suppress tumor growth, TLR agonists can promote the survival of malignant cells and increase their resistance to chemotherapy.[24] Li et al.[6] reported that tumor cells, which undergo apoptosis, release endogenous DAMPs that are associated with tumor progression. Further, Yu and Chen[25] explained the contribution of TLR signaling in tumor progression and

chemoresistance by indicating the expression of TLRs on tumor cells and tissues. Specifically, TLR4, TLR7, TLR8, and TLR9 signal-mediated chronic inflammations have pro-tumor effects on cancer cells. Rakoff-Nahoum and Medzhitov[26] reported that activation of TLR4 and its signal transduction through MyD88 adaptor protein orchestrates tumor formation and metastasis. They are also responsible for tumor immune escape, resistance to apoptosis and immunosuppression by tumor cells.[27] In contrast, stimulation of TLR3 and TLR5 signaling were reported to induce antitumor T-cell response.[28]

Significance of TLRs in cancer immunotherapy

The significance of TLRs was explained by Paulos et al. in total body irradiation (TBI) cancer immunotherapy.[29] One of the mechanisms by which TBI augments the activity of adoptively transferred T cells is through recognition of microbial LPS by TLR4 that activates the innate immune system in the radiation-injured gut.

Significance of TLRs in vaccines

TLRs act as natural adjuvants to vaccines that contain attenuated live or heat-killed viruses or bacteria. The role of TLRs is noteworthy in control of adaptive immune response through maturation of DCs, induction of cytokines and co-stimulatory proteins expressions, and reversal of tolerance. Therefore, as natural adjuvants in vaccines, they help DCs in better antigen presentation, subsequently leading to a good immune response.[30]

Mechanism of action of TLRs and the signaling pathways

Table 1 describes the various ligands of different TLRs and their role in innate immunity. The transmembrane TLR proteins detect the invading pathogens and binds to the microbial molecules. Following the formation of TLR and PAMP molecule complex, dimerization of TLRs induces a cascade of TLR signaling to trigger expression of various genes like cytokines, chemokines, monohistocompatibility complex (MHC) and co-stimulatory molecules which are involved in the host immune function.[1]

The extracytoplasmic leucine rich repeats (LRR) motifs of TLRs bind with the ligand while its intracytoplasmic TIR domain recruits specific adaptor molecules like MyD88, MAL, TRIF, TIRAP, and/or TRAM which leads to activation of NFκB and IRFs down signaling. There are two different TLR signaling pathways depending on the adaptor molecule recruited namely MyD88 dependent and MyD88 independent/ TRIF dependent pathway.[31]

MyD88 dependent pathway

MyD88 (Myeloid Differentiation Primary Response protein 88) is a protein coding gene that is associated with the TIR domain of TLRs in TLR signal transduction. This protein possesses TIR domain at its carboxy terminal while its amino terminal has a death domain. It is a universal adaptor molecule used by almost all TLRs except TLR3. The adaptor protein MyD88 which plays a crucial role in TLR signal transduction is a component of 'shared' signaling pathway that is induced by all TLRs as well as by the IL-1R (interleukin-1 receptor) family.[1] Figure 2 describes the MyD88 dependent pathway of TLR signaling.

Stimulation of MyD88 molecule causes association of TIR domains of MyD88 and TLR, which is followed by interaction of death domains of both MyD88 and IRAK-4 (IL-1R-associated kinase). The recruitment of IRAK-4 by MyD88 leads to the formation of a complex called Myddosome complex.[32] During the formation of this complex IRAK-4 activates IRAK-1 which is then autophosphorylated at several sites. Now, the phosphorylated IRAK-1 associates with the RING-domain E3 ubiquitin ligase TRAF-6 (TNF receptor-associated factor 6) to activate either AP-1 or TAK-1/TAB (TGF-β- activated kinase) complex through K-63 linked polyubiquitination of TAK-1 and TRAF-6 itself. AP-1 is activated through activation of MAP (Mitogen-activated protein) kinase.[5] Activation of the TAK-1/TAB complex, enhances the activity of the IκB kinase (IKK) complex, which induces phosphorylation and subsequent degradation of IκB[33] finally leading to nuclear translocation of transcription factor NF-κB. Consequently, it induces the transcription of inflammatory cytokines.

MyD88 independent pathway

MyD88 independent pathway is also known as the TIR domain containing adaptor inducing IFN-β (TRIF) dependent pathway. This pathway is considered specific to only few TLRs. Although this pathway activates NF-κB in delayed phase, it is not sufficient to induce cytokine expression.[34] TLR4 and TLR3 are the two TLRs that require this pathway for activation of NF-κB and MAP kinases.[35] When these TLRs are stimulated by their ligands it leads to recruitment of either TRIF or TRAM molecule and activation of IRF-3 or IRF-7 followed by delayed phase NF-κB activation.[36] Activation of IRF leads to production of IFN-β which in turn activates STAT1 that induces several IFN-inducible genes.[37]

Table 1. Ligands of different TLRs and their role in innate immunity.

S. no	TLR	Species	Ligand	Role in innate immunity	Ref
1	TLR1	1. Human	1. Triacyl lipopeptides (Pam3CSK4)	1. Induce IL-6 production in human tonsillar B cells	101
		2. HeLa cell line	2. Soluble factors	2. Induction of the IL-8 promoter	102
		3. Mice	3. Osp A	3. Production of TNF-α and IL-6	103
		4. Human embryonic kidney 293 (HEK293) cell line	4. Porin PorB	4. Induce TLR1 signaling after binding to TLR2	104
2	TLR2	1. Mice	1. Lipoarabinomannan	1. Activation of NF-KB-driven luciferase activity in lung inflammation 2. Stimulate NFκB, induce apoptosis in THP-1 monocytice cells	105
		2. Human	2. Bacterial lipoprotein	3. Enhance antigen uptake and simultaneously increases cytokine production 4. IL-8 production, DC maturation, cell activation, induce NFκB activation	106
		3. HEK293 cell line	3. Porin	5. Cell activation, induce NFκB activation 6. Induction of the IL-8 promoter	104
		4. HEK293 cell line	4. Peptidoglycan	7. Increased gene expression and secretion of proinflammatory cytokines (IL-6) and chemokines (IL-8) 8. Activate macrophages and produce inflammatory responses	107
		5. HEK293 cell line	5. Lipoteichoic acid	9. NFκB activation and cytokine production	107
		6. HeLa cell line	6. A phenol-soluble modulin	10. Cytokine IL-10, IL-6 and IL-12) secretion in DCs and macrophages to induce immunological tolerance through secretion og TGF- β	102
		7. Human keratinocytes	7. Phospholipomannan	11. induce cardiomyocyte inflammation, activate NF-κB, and up-regulate the expression of pro-inflammatory cytokines in human monocytes in a CD14-dependent fashion	108
		8.		12. Activates the innate immune response MyD88 and NF-κB-dependent pathway, act as an adjuvant promoting Ag-specific T cell responses	
			8. Glycosylphosphatidyl inositol anchor	13. Induction of proinflammatory cytokine IL-6 in monocytic cells and surface expression of CD150	109
		9. TLR2-ve cell lines	9. Glycolipids		
		10. mice	10. Zymosans		110
					111
		11. NFκB-luciferase knock-in mice, human	11. HSP70		
					112,113
		12. Mouse alveolar macrophage cell line	12. Hyaluronan		
		13. Human			114
			13. Hemagglutinin		
					115

(*Continued on next page*)

Table 1. (Continued)

S. no	TLR	Species	Ligand	Role in innate immunity	Ref
3	TLR3	1.	1. dsRNA 2. Polyi:C	induces type I interferon (IFN), inflammatory cytokine/chemokine production and dendritic cell (DC) maturation, activation of NK cells and CTLs	116
4	TLR4	1. Mouse macrophages	1. Lipopolysaccharide	1. Formation of a complex between PI 3-kinase and MyD88, induction of IL-1β, TNF-α and macrophage inflammatory protein (MIP)-2, induce proinflammatory cytokine production through NF-KB signaling 2. Functional maturation of mouse macrophages, induction of TNF-α by Human Monocyte Cultures 3. Production of major proinflammatory cytokines such as IL-1, IL-6, or TNF-α; activate DC and promote the development of Th1-like responses	117
		2. Mouse, human		4. Activate macrophages and produce inflammatory responses	
			2. Mannan	5. Induce NF-κB activation, induce PGE_2, IL-1β, and TNF-α production, by macrophages	118
		3. Human PBMCs		6. Induce proinflammatory cytokine production through NF-KB signaling	
			3. Envelope F protein	7. Activates the innate immune response by driving NF-κB-mediated cytokine expression 8. Activate macrophages through MyD88 and TRAF6	
		4. Mouse macrophages 5. Ba/F3 cell line	4. Glycosylphosphatidyl inositol	9. Activate NF-κB, and up-regulate the expression of pro-inflammatory cytokines in human monocytes in a CD14-dependent fashion	117
			5. Flavolipin	10. Low molecular weight HA elicits pro-inflammatory responses by modulating the TLR4 or by activating NF-kB; high molecular weight HA manifests an anti-inflammatory effect via CD receptors and by inhibiting NF-kB activation	
		6. Mouse		11. Enhancement of dendritic cell maturation and alloreactive T-cell responses	119
			6. Taxol	12. Upregulate mRNA expressions of MCP-1, TNF- a, induction of proinflammatory cytokines, accelerate podocyte injury	
		7. HEK293 cell line			120
			7. Fusion protein		
		8. Murine macrophage RAW264.7 cells			121
		9. Human	8. HSP60		122
			9. HSP70		
		10.			112
			10. Oligosaccharides of hyaluronic acid (HA)		123
		11. Mice			
			11. Polysaccharide fragments of heparan sulfate		
		12. Mouse podocytes	12. Fibrinogen		124
					125

5	TLR5	1. Human	1. Flagellin	1. Induce secretion of cytokines, chemokines and co-stimulatory molecules from T cells	97
6	TLR6	1. Human	1. Lipoproteins	1. Stimulate NFκB, induce apoptosis in THP-1 monocytice cells	106
7	TLR7	1. Human airway and lung epithelial cells	1. ssRNA	1. Induce production of inflammatory cytokines	126
		2. Mice	2. Imidazoquinolines	2. Synthesis of IFNα, inflammatory cytokine production by macrophages, proliferation of splenocytes or maturation of DCs	127
				3. Produce type I IFNs and other cytokines, maturation of pDCs, stimulate T-cells	
		3.		4. Reduces plasma virus concentration in chronic hepatitis C infection	
			3. Bropirimine		128
		4. Human			
			4. Guanosine analog Isatoribine		129
8	TLR8	1. Human airway and lung epithelial cells	1. ssRNA	1. Induce production of inflammatory cytokines	126
9	TLR9	1. Human, murine	1. Unmethylated CpG DNA	1. Induce production of inflammatory cytokines	130
		2. Human lymphoid tissue	2. Chromatin-IgG complexes	2. Links innate and adaptive immune systems in the development of systemic autoimmune disease	131
10	TLR10		No known ligand		132

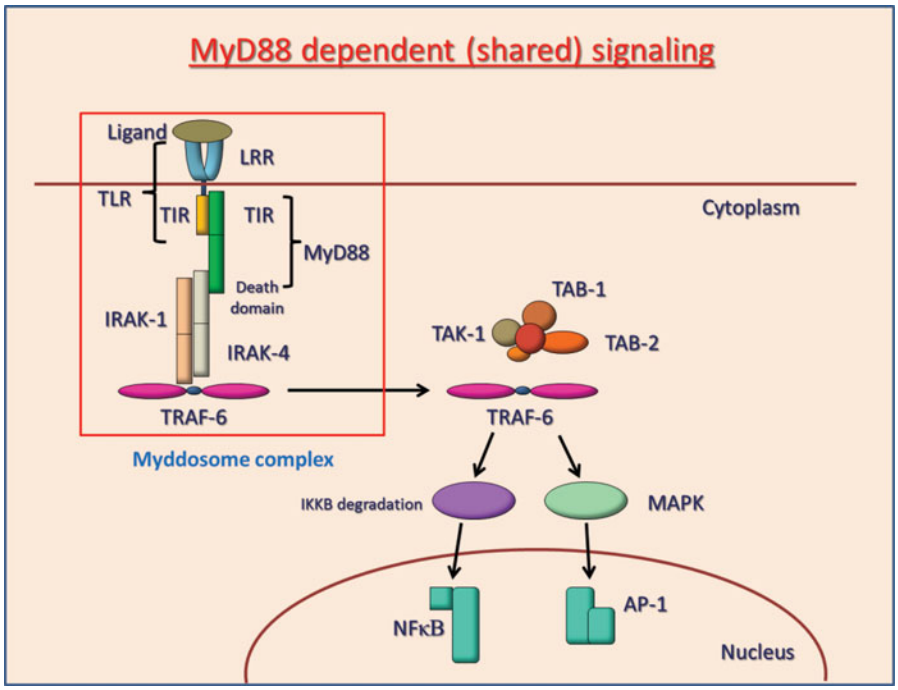

Figure 2. MyD88 dependent pathway of TLR signaling. This pathway is called as 'shared' signaling pathway is induced by all TLRs as well as by the IL-1R family. Stimulation of MyD88 molecule by ligand associated TLR primes interaction MyD88 and IRAK-4. Following the formation of Myddosome complex, IRAK-4 activates IRAK-1. After auto-phosphorylation IRAK-1 associates TRAF-6 to activate either AP-1 or TAK-1/TAB complex through polyubiquitination of TAK-1 and TRAF-6 itself. AP-1 is activated through activation of MAP kinase. Activation of TAK-1/TAB complex causes degradation of IκB finally leading to stimulation of NF-κB. This signal transduction causes synthesis and secretion of pro inflammatory cytokines and co-stimulatory molecules required to elicit appropriate immune response.

Figure 3 describes the MyD88 independent pathway of TLR signaling.

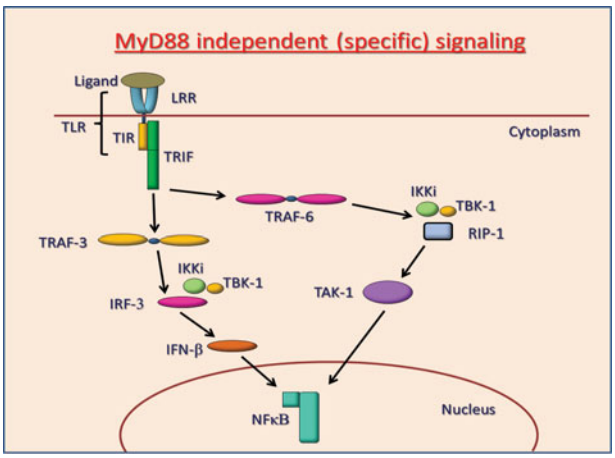

Figure 3. MyD88 independent pathway of TLR signaling. This pathway is also called as TRIF dependent pathway as it is specific to few TLRs (TLR4 and TLR3). Signals transduced through this pathway activate NF-κB in delayed phase. When these TLRs are stimulated by their ligands it leads to recruitment of either TRIF or TRAM molecule. Interaction of TRIF and TRAF-6 leads to ubiquitination of RIP-1 by IKKi /TBK1. RIP-1 activates TAK-I complex which is followed by activation of NF-κB and MAPK. On the other hand, interaction of TRIF and TRAF-3 recruits TBK1 and IKKi which causes IRF3 phosphorylation. This is followed by dimerization of IRF3 followed by binding of IRF3 to IFNβ promoter which induces the expression of inflammatory genes.

TRIF molecule interacts with TRAF-6 and recruits receptor-interacting protein 1 (RIP-1) which is ubiquitinated by IKKi /TBK1. RIP-1 activates TAK-I complex, which is followed by activation of NF-κB and MAPK.[5] Contrarily, TRAF-3 recruits TBK1 and IKKi, which cause IRF3 phosphorylation.[36] IRF3 forms a dimer and is translocated into the nucleus. Pellino 1 binds to DEAF-1 and influences binding of IRF3 to IFNβ promoter. Thus IRF3 induces the expression of type I IFN genes.[38]

Regulation of signaling pathways

The microbial components induce the TLRs to convey signals to produce inflammatory cytokines like TNF-α, IL-6, and IL-12 against the invaders. The excess production of these cytokines lead to grave systemic disorders like tumor growth and autoimmune disorders, and extreme conditions may lead to mortality of the host. Hence, regulation of the TLR signaling pathway and a balance between the signaling pathways is necessary to keep the host system safe. However, apart from PAMPs, TLR signaling is influenced by cross-talk of other molecules also that may positively or negatively impose an effect on the signal transduction. Thus, it is important to consider the TLR signaling in the context of its regulation by other molecules.

Many molecules are involved in modulating the TLR pathways to induce appropriate response. Among them

LPS, SOCS1, IRAK1, NFκB, and SIGIRR are considered important.

LPS is an endotoxin released by the Gram negative bacteria. The phenomenon where exposure to minute amounts of LPS leads to a transient unresponsive state or a severely reduced response to subsequent exposures is called endotoxin or LPS tolerance.[39] One of the reasons for the endotoxin tolerance (ET) is a drastic reduction in TNFα production due to exposure of the immune cells to suboptimal levels of endotoxins. One more way of developing ET may be through downregulation of inflammatory genes like TNFα, IL-6, IL-12, IL-1β, CCL3, CCL4, and CXCL10. The failure of signaling mechanism in ET is associated with decreased TLR4–MyD88 complex formation, impairment of IRAK-1 activity and defects in the activation of MAPKs and NF-κB.[40]

SOCS1 belongs to the SOCS family of proteins and this molecule is a negative regulator of JAK-STAT and cytokine signaling pathways.[41] These are the critical regulators of TLR signaling. Either blockade of IRAK-1 phosphorylation or degradation of TIRAP is the way by which SOCS1 executes its negative regulatory mechanism on the TLR signaling. The LPS induced expression of SOCS1 in macrophages down regulates the signaling pathway by inhibiting NF-κB and STAT1 activation in macrophages. Deficiency of SOCS1 renders the host hypersensitive to LPS-induced endotoxin shock and offers increased production of inflammatory cytokines.[42]

IRAK-M, a member of the IRAK family of serine/threonine kinases, is found in monocyte/macrophages, and lacks kinase activity as key residues are absent in its putative kinase domains.[43] Deficiency of IRAK-M allows increased production of inflammatory cytokines in response to TLR ligands. The molecular mechanism behind the inhibitory activity of IRAK-M seems to be drawn out through blocking the formation of the IRAK-1/TRAF6 complex by preventing dissociation of IRAK-1/IRAK-4 from MyD88 either by inhibiting their phosphorylation or by stabilizing the TLR/MyD88/IRAK-4 complex.[44]

NF-κB is the master regulator of all TLR signaling mechanisms and its activation is the critical event in TLR-mediated activation of the innate immune response. While the rapid activation of this molecule is pivotal for the desired immunological response, the ability of the system to switch off this molecule after the immune response is also given an equal importance in order to prevent the potential damages by over expression of the pro-inflammatory genes. This transcription factor existing as homo- and heterodimers regulates the expression of several inflammatory molecules like TNFα, IL-1β, and IL-16. p65/p50 NF-kB heterodimer of NF-κB, involved in the signal transduction, translocates into the nucleus and binds to specific gene promoters to initiate transcription. During ET, there is reduced production of p65/p50 NF-kB heterodimer. The p50 NF-κB homodimer which lacks the transactivation domain is over produced and exerts an inhibitory effect on the expression of inflammatory genes by competitively binding to specific promoters.[45]

Membrane-bound proteins harboring the TIR domain, SIGIRR (single immunoglobulin IL-1 receptor-related molecule) and T1/ST2 have also been shown to be involved in negative regulation of TLR signaling.[46] In both SIGIRR- and T1/ST2-deficient mice, the LPS-induced inflammatory response was enhanced.[47] A RING finger protein, TRIM30-α and intracellular tyrosine phosphatases like SH2-containing protein tyrosine phosphatase 1 (SHP1) also negatively regulate TLR-mediated production of pro-inflammatory cytokines.[48] Glucocorticoids negatively regulate TLR signaling cascade by inhibiting the activation of p38 MAP kinase, IRF3-dependent genes and TBK-1.[49] An adaptor molecule of the TLR signaling pathway, SARM, targets TRIF molecule, inhibits NF-κB and IRF3 and thus TLR3 and TLR4 signaling.[50]

Some processes can be either positive or negative regulators of TLR signaling. For example, while K48-linked ubiquitination of IkB mediated degradation of TLRs inhibits the TLR responses; the K63-linked ubiquination of TRAF6 activates the NF-κB pathway.[33] Thus, several molecules are postulated to modulate TLR signaling pathways. The combination of these negative regulators may finely coordinate the TLR signaling pathway to limit exaggerated innate immune responses which causes harmful disorders.

Factors affecting the expression of TLRs

Although the quantification of the expressed mRNA of a gene helps to identify and diagnose any ailments in an animal, there are several factors which swiftly alter the expression of a gene leading to improper diagnosis of a condition.[51] They include external factors (temperature, humidity and ventilation), internal factors (oxygen availability, nutrition, inflammation, tissue repair), genetic factors (non-coding RNA, mutation, SNP, CNV) and animal factors (age, sex, species, breed, stress, immune status). Figure 4 describes the factors affecting the expression of TLRs.

Genetic factors

The transcription of genes depends on the integrity of the DNA in which two copies of each gene are encoded. More the intact number of copies of genes that are available for transcription, more will be the level of expression.[52] However, DNA mutations are the potential causes for alterations in gene expression and associated defective

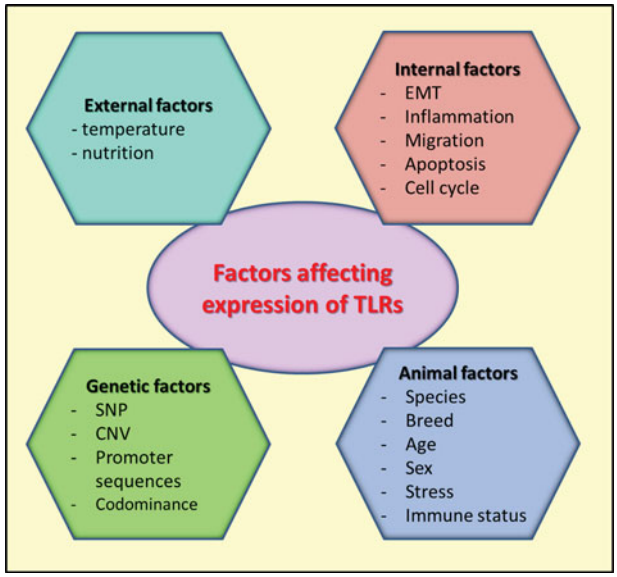

Figure 4. The **factors affecting the expression of TLRs** Expression of TLRs by the host immune system is a critical step in detection and culmination of infection. However, various factors govern the magnitude of their expression. They are broadly grouped into external (temperature, nutrition), internal (EMT, inflammation, migration, apoptosis, cell cycle), genetic (SNP, CNV, promoter sequences, codominance) and animal (species, breed, age, sex, stress, immune status) factors. Based upon prevalence and regulation of these factors, the expression of TLRs varies considerably.

cellular functions. Sometimes due to duplication or deletion of the stretches of DNA, the copy number variants (CNV) may increase or decrease, which are ultimately associated with disease status as the transcription to translation encode abnormal or defective proteins. Further, the quantification of RNA transcripts is usually correlated to the CNV in the genome.[53]

The genetic markers like single nucleotide polymorphisms (SNP) cause structural and functional defects of an encoded protein due to change in a single amino acid sequence of a gene, which helps in diagnosing any disease condition.[54]

Promoter sequences are the natural regulators of gene expression. The proximity of a promoter to the consensus sequence advances its transcription rate. Any mutation or epigenetic modifications in this region alter the function of the promoter region and also the RNA transcription rate.[55] It is not unusual in the TLR family of proteins, and therefore, even heterozygous coding variants can influence the outcome of the expression.[56]

Internal factors

The important cellular events like inflammation, normal cell cycle, apoptosis also lead to significant shifts in gene expression profiles.

Though inflammation is aimed at terminating the danger signal and eventually restoring tissue and organ homeostasis, the chronic inflammation can lead to systemic disorders and other conditions. Since the inflammatory response is dependent on rapid activation of gene expression through various molecules and transcription factors, expression profiles of normal and inflamed cells differ hugely.[57]

During tissue repair and wound healing (sometimes in oncogenesis too) the damaged cells alter their physical and chemical properties. This complex process is called epithelial-mesenchymal transition (EMT) which is exactly the reverse of a process that occurs during normal tissue development. It drastically changes the entire gene expression of a cell. Thus the TLR RNA expression profiles of such cells vary greatly from normal cells as TLR2 is involved in wound healing.[58]

Migration is another cellular process that is closely related to EMT. But it also occurs in cells that are not undergoing EMT. Embryonic cells, healing cells, tumor cells and even the immune cells migrate either locally or distally. As the cytoskeletal components are reorganized in such cells to allow for movement, the genes regulating cell–cell attachment are also turned down. This affects the gene expression profiles of the cells.[59]

Apoptosis is the programmed cell death induced by a wide range of environmental and cellular stimuli. Since the transcriptions of various genes are involved in every apoptotic pathway, the resulting different gene expression profiles also differ from the normal cell.[60]

Cell cycle is a critical process which signifies the importance of development, senescence and apoptosis of a cell. This process regulates the genes that are associated with shut down of cell division. Because of its cyclical nature, the expression levels of genes fluctuate at regular intervals.[61]

External factors

Exposure of individuals with similar genotypes to different environmental conditions like diet, light, temperature, etc., alters the expressivity of a gene either negatively or positively. Various environmental factors act as precursors of mutation or modification of DNA, thus affecting the normal functions of cell cycle.[62]

Temperature plays an important role in TLR expression. A study in catla fish has observed induction of various TLRs and NOD receptor gene expressions when exposed to extreme hot and cold temperatures.[63] In contrary, Yan et al.[64] put forward that fever range temperature (39.5°C) actually increases the expression of TLRs in DCs. The higher expression of TLR8 and TLR10 in the heat stress has been proven to indicate that these

two genes may act as the immunological markers of heat stress in goats.[65]

The nutritional status of an animal was found to be more important in controlling TLR gene expression rather than temperature. Improving the nutrition during heat stress condition was proved to be highly beneficial to maintain the immune status against heat shock proteins in goats.[65,66]

Animal factors

Animal factors like species, breed, age, sex, stress, and immune status of the individual also affect the expression of genes. The TLRs comprise a family of evolutionary conserved pattern recognition molecules that have an essential role in mammalian innate immune defense. Recent observations suggest that several TLR orthologues are expressed differently in mice and humans. This species variation includes the expression of TLR transcripts in different cell types and different transcription regulation on cellular activation. Apparently some TLR genes have been placed into a completely different regulatory context during evolution.[67] To exemplificate the species dependent expression, the LPS stimulation increased TLR4 expression in human monocytes and neutrophils,[68] while the same did not cause an increased TLR4 expression in murine macrophages.[69] Breeds of the same species too will influence the expression of the TLRs. The relative genetic disparity between *Bos indicus* and *Bos taurus* is well established and this genetic distance would explain some of the TLR2 polymorphisms observed between these two subspecies. Consequently, there will be different selective pressures acting on the TLR and other immune related genes in these subspecies, which would result in sites being differentially fixed in their TLR genes.[70]

Penetrance of a gene is the proportion of individuals carrying a particular variant of a gene, and it is known to depend on the age of an animal. Age-related changes in immunity lead to poor adaptive immune responses and render them more susceptible to infections than the young, as age is accompanied by changes in expression and function of several TLRs that are part of the innate immune system. While the expression of TLR1 and TLR2 were positively correlated to age, the expression of TLR4 and TL8 were analyzed to negatively correlate. Interestingly, the expression of TLRs, particularly TLR2, TLR5, TLR7, and those involved in viral response were found to be negatively associated with serum 25OHD levels. But the serum 25OHD levels decrease with an advancement in age and thus increasing the expression of those particular TLRs.[71] Similar to this, Boehmer et al.[72] also found that there was age related decrease in the expression of TLR4 and TLR4 mediated expression of MAPK. Further, Renshaw et al.[73] also established the age related decrease in all the TLRs in the splenic and peritoneal cells. In contrary, Stewart et al.[74] reported that there was no age-wise difference in the expression of TLR4 and also established the positive relation of TLR expression to the intensity of physical activity.

There are reports that underline the enhanced quality of female's reproductive health due to high innate immune response in their reproductive tract that influence the early perception of pathogenic entry and generation of inflammatory responses.[75,76] The difference in the immune responses is by the virtue of the actions of endocrine hormones in both the sexes. While the androgens were revealed to suppress the immunity against bacterial endotoxin,[77] the estrogens were indicated to promote the disease resistance against bacterial infections through increased expression of TLR4 and CD14 on macrophages.[76,78] The enhanced immune response in females is accredited to all the TLRs. Especially, TLR2 is found to increase extraordinarily in both diestrus and estrus phase, signifying its sex related expression pattern.[75]

There are various kinds of stress and most of the types can alter the expression of TLR genes. To name a few, hypoxic stress up-regulates the expression of TLR4 in macrophages via hypoxia-inducible factor.[79] Hyperglycaemic stress induces expression of TLR in human monocytes.[80] Oxidative stress is the over accumulation of carbon monoxide in the cells, and it differentially inhibits TLR signaling pathways by regulating ROS-induced trafficking of TLRs to lipid rafts.[81] In addition, exercise stress, both acute aerobic and chronic is also shown to decrease the monocyte cell-surface expression of TLRs. Further, chronic exercise stress leads to decreased expression of both inflammatory cytokine production and the cell-surface TLR4 on monocytes.[82] All these stress factors may contribute to immune depression and higher susceptibility to infection.

Surprisingly, during pregnancy, at the maternal-fetal interface the TLRs are expressed not only in the immune cells, but also in non-immune cells, such as trophoblasts and decidual cells in order to eliminate 'infectious non-self' (bacteria, virus, etc.) and ensure tolerance to 'non-infectious self' (mother, placenta and fetus). However, the pattern of expression varies according to the stage of pregnancy.

As TLRs are the key molecules of the immune system, the immune status of animals is a major regulator of their expression. Infiltrating eosinophils during allergic diseases such as bronchial asthma and atopic dermatitis provide a potential ligand system for TLR7 and TLR8, thus increasing their expression.[83] Likewise, high glucose (15 mmol/l) exhibited by diabetic patients significantly

induces increased time- and dose-dependent expression of TLR2 and TLR4 in human monocytes.[80] Similar results of time and dose-dependent expression patterns of TLR2 and TLR4 were also established for type I diabetes[84] and type II diabetes in human monocytes.[85] During inflammatory conditions of the liver, even though the TLR3 is up-regulated, infection by HBV may down-regulate the signaling and expression of TLR3 and TLR2/4.[86] In addition, TLR4 was found to be up regulated during intestinal inflammation,[87] surgical stress[121] and obstructive jaundice.[88] The role of TLR-4 has also been established in vascular organ maladies such as intestinal colitis,[15] myocardial inflammation,[89] injured kidney[124] and alcoholic liver.[90]

Function of TLRs

Expression in different cells

The immune system is able to recognize and impart the effector response against pathogenic molecules because of the presence of immune receptors called TLRs. These TLRs are widely distributed and are expressed on the membranes of leukocytes including DCs, macrophages, NK cells, cells of the adaptive immunity like T and B lymphocytes, and also in the non-immune cells like epithelial cells, endothelial cells and fibroblasts.[91]

Recognition of self and non-self antigens

TLRs are the bona fide PRRs that respond to specific molecules derived from bacteria and viruses, leaving the self-antigens apart.[31] The localization of TLR7, TLR8 and TLR9 in endosomal compartments are reported to discriminate between self and non-self, as host DNA and RNA seldom enter into endosomal compartments.[92] When the TLRs are triggered by microbial molecules, they induce the expression of costimulatory molecules that are necessary for the activation of naive T cells which are expressed in complex with MHC molecules. The costimulatory molecules flag the cells carrying microbial molecules as non-self antigens and activate the antigen-specific T cells, ensuring the immune response only against non-self-antigens.[93]

Detection of invading pathogens

The evidence for microbial perception by TLRs emerges from the models of infection in TLR-deficient mice. The knock-out mice for TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, and TLR11, when exposed to various pathogens showed decreased cell survivability, hyporesponsiveness against intruding pathogens, decrease in the proinflammatory cytokines numbers (TNF-a, IL-6, IL-1),[34] decreased proliferation of splenic B cells, decreased DC maturation, and decreased resistance to infection.[94]

Bridging innate and adaptive immunity (present on DCs)

The TLRs can be called as 'bridging molecules', since they are present on DCs that couple adaptive immunity and innate immunity. The TLRs detect the pathogens and convey them to the antigen presenting cells (APCs) which lethally damage the microbe by phagocytosis finally leading to destruction of the invading foreign particle. Intriguingly, the presence of a full set of TLRs on immature DCs helps in their maturation process, and hence a better antigen presentation capacity of DCs is accredited to TLRs.[30] However, stress conditions like increased atmospheric temperature acts to shift the adaptive immune function from cell mediated to humoral immunity, which ultimately weakens the immune function of the animals.[95]

Development of antigen specific immunity

The expression of TLRs on specialized APCs like DCs plays an important role in the initiation of the adaptive immune responses[1] as the recognition of microbial peptides leads to induction of genes that encode inflammatory cytokines and costimulatory molecules which along with phagocytosis-mediated antigen presentation, engineer the development of antigen-specific acquired immunity. In vaccine induced immunity, TLRs are the key molecules involved in the generation of antigen-specific acquired immunity against the pathogenic peptide as the ligands bind to TLRs either in the form of adjuvant or invasive moieties.[96]

Cytokine production, proliferation and survival

Since the TLRs are expressed on T cells, they modulate the activation of T cells. They induce expression, proliferation and survival of IFNγ on effector Th1 and Th2 cells; TNF-α, IL-6, IFN-α from DCs; type I IFN from monocyte-derived and plasmacytoid DCs.[36] The TLR5 ligand, the flagellin is outlined to induce cytokine secretion upon its interaction with TLR5 on T cells.[97] The production of cytokines is kept under check by SHP-1, a negative regulator of TLR-mediated production of proinflammatory cytokines, through production of type I interferons.[48] The expression of IL-12 induced by TLRs dictates the differentiation of activated T cells into T helper1 (Th) effector cells.[31]

Apoptosis of infected cells

TLR signaling induces apoptosis of microbial infected cells as the infected cell shut off its protein synthesis. The activation and survival of neutrophils depend on TLR4 activation of monocytes.[98] As TLR signaling pathway shares certain molecules like FADD, DREDD, TRIF, TRAF6, TAK, and IKK kinases with the apoptotic TNF pathway, it can be hypothesized that TLRs possess the ability to crosstalk with death signaling.[99] Apoptosis by TLR signaling limits the infection and down regulates the DC-mediated immune responses in sepsis.[100]

Release of interferons by viral infected cells

Apart from recognition of invading pathogens, the TLR also recognizes the viral nucleic acid and induces interferon production in the infected cells that are essential for defense against viruses.[48]

Conclusion

The identification of TLRs as the components of the innate immune system was a significant breakthrough in understanding the uptake and processing of pathogen patterns by the immune system. There are at least 10 TLRs present in mammals and each TLR is equipped with the unique ability to recognize different PAMPs. These TLRs are found to be significantly involved in allergy, cancer, immune therapy and vaccine stabilization in multiple ways. They are not only restricted for recognition of pathogens, but also these receptors have stretched their effectiveness to antigen presentation, apoptosis, production of interferons by the virus infected cells and so on. As they have multifaceted functions, their expression patterns also vary greatly according to physiological or pathological conditions prevailing in the host system. Though there are many advances made in explaining the structure, function and signaling pathways of these TLRs, there are many wide areas like contradictory effects of different TLRs upon the same function, the molecular basis and biochemical variation in the regulation of their signaling and variation in factors affecting the expression of these receptors that are still left open for future research efforts to be turned in to update the knowledge on these complex processes and functions.

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Declaration of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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