Advances in Toll-like Receptor Signaling

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***Abstract*—The Toll-like receptor 4 (TLR4) was originally known as the lipopolysaccharide (LPS) signaling receptor but, as discoveries unfolded, the enormous amount of information generated helped the scientists to investigate immunoreceptor Toll gates. Considerable research led to a clear view of the bridging gates of innate and adaptive immunity known as TLRs. Since the discovery of the TLR in 1996 as a cellular receptor that captures molecules from invading pathogens, multiple molecules have been discovered to date. The lack of information on the intricate pathways downstream of TLRs has led to much research on this area at the molecular level. The purpose of this review article is to summarize the recent advances in our knowledge of cell membrane-located TLRs, as well as nucleic acid-sensing TLR signaling research.**

***Index Terms*— Innate immunity, NF-κB, PAMPs, TLR, TNF-α**

I. Introduction

Deciphering the signaling networks that underlie normal and disease processes remains a major challenge. Toll-like receptors (TLRs) are one such signaling pathways that sense invading microbial pathogens and play crucial roles in the activation of innate and adaptive immunity. However, excessive TLR activation can disrupt immune homeostasis and may be responsible for the development of autoimmune and inflammatory diseases. As such, the molecules and pathways that negatively regulate TLR signaling have been intensively investigated. Since some TLRs share ligands (many TLRs recognize similar pathogenic products), and heterodimerization between ligands is common [1], it is necessary to have a good knowledge of how excessive TLR activation disrupts immune homeostasis and effects the development of autoimmune and inflammatory diseases.

TLRs are found to be evolutionarily conserved from plants to mammals. *Toll* means "great" in English. In mid 1980's, Anderson *et al.* coined it for a protein that played a critical role in the early embryonic development of *Drosophila*. Later it was found that this protein was also essential for the host innate immunity against fungal infection in adult flies [2, 3]. In 1997, Medzhitov *et al.* were the first to report the cloning of a mammalian TLR homologue (identified as TLR4) of *Drosophila Toll* and termed it as "Toll-like receptor" [4]. Since then much research interest has been focused on the structure of TLRs. All TLRs share similar domain architecture comprising an extracellular leucine-rich domain which mediates the sensing of pathogens, a Toll-interleukin 1 receptor (TIR) domain which is required for downstream signal transduction and a single transmembrane helix [5].

To particularly emphasize on the number of TLRs, 13 of them have been discovered to date. Among them TLR1-9 are ubiquitously expressed in both human and mice. Human cells solely express TLR10, whereas mouse do not express TLR10 but do express TLR11, 12 and 13. Although various types of TLRs have common role in immunity, they basically differ in their ligand specificity, the usage of adaptor proteins, cell localization, and cellular responses (Fig. 1) [6]. However, TLRs are not the one and only receptors for invading pathogens, the system also possess non-TLRs such as retinoic-acid-inducible gene-I- (RIG-I-) like receptors (RLRs), nucleotide-binding domain and leucine-rich repeat containing gene family (alternatively named NOD-like receptors, NLRs), C-type lectin receptors (CLRs) and cytosolic DNA receptors (CDRs) [7, 8]. Recognizing the importance of TLRs in both physiological and pathological conditions, Beutler, Hoffman, and Steinman shared the 2011 Nobel Prize for their important discovery on the role of TLRs immune reactions [9]. In 1990, Beutler discovered that the protein encoded by *Toll* gene serves as a receptor on cells. However, Steinman identified the role of TLRs in dendritic cells (DCs), which is responsible for adaptive immune responses.

II. TLRs Located in the Cell Membrane and their Identified Ligands: An Overview

TLR1, 2, 4, 5, and 6 are located on the plasma membrane. Among them, TLR2 forms heterodimer with TLR6/TLR1 to recognize ligands such as peptidoglycan, lipoteichoic acid, lipoprotein from Gram-positive bacteria, lipoarabinomannan from mycobacterium, and zymosan from the yeast cell wall, diacylated mycoplasmal lipopeptide [10] and triacylated lipopeptides [11, 12]. To enhance the ligand induced cellular signaling processes, TLR2 is found to interact with CD14 (cluster of differentiation 14) [13]. Nevertheless, previous studies point out that the simultaneous expression of intra and

Manuscript received October 2, 2012.This work was supported by the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology (2012016803). This work was also partly supported by the Priority Research Centers Program (NRF 2012-0006687).

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extracellular domains of both TLRs (TLR1 and 2) are essential for executing the ligand induced cellular responses [13, 14]. For example, the response to Gram-positive bacteria (e.g. *Staphylococcus aureus*) is initiated by both TLR1 and 2 [15, 16]. TLR4, the first identified TLR mediates immune responses to Gram-negative bacteria (e.g. *Escherichia coli)* [17] by binding with the bacterial lipopolysaccharide (LPS) and its toxic moiety, lipid A [18]. In this scenario, TLR4 alone cannot confer response to LPS, hence it requires functionally interacting partners, such as MD-2 [10] (myeloid differentiation protein-2 or lymphocyte antigen 96), and CD14 for the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) to produce proinflammatory cytokines [19]. Though bacterial outer wall components like peptidoglycan and LPS are recognized by TLR1/2, 6 and 4, the recognition of bacterial flagellin is mediated by TLR5 from both Gram-positive and Gram-negative bacteria. The cellular responses to flagellin are same as peptidoglycans and LPS. TLR5 uses MyD88 (myeloid differentiation primary response gene 88) as adaptors to produce proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) [20, 21]. TLR5 is the only TLR which binds with flagellin (Table I). In this condition, it may require a special co-receptor for efficient signaling.

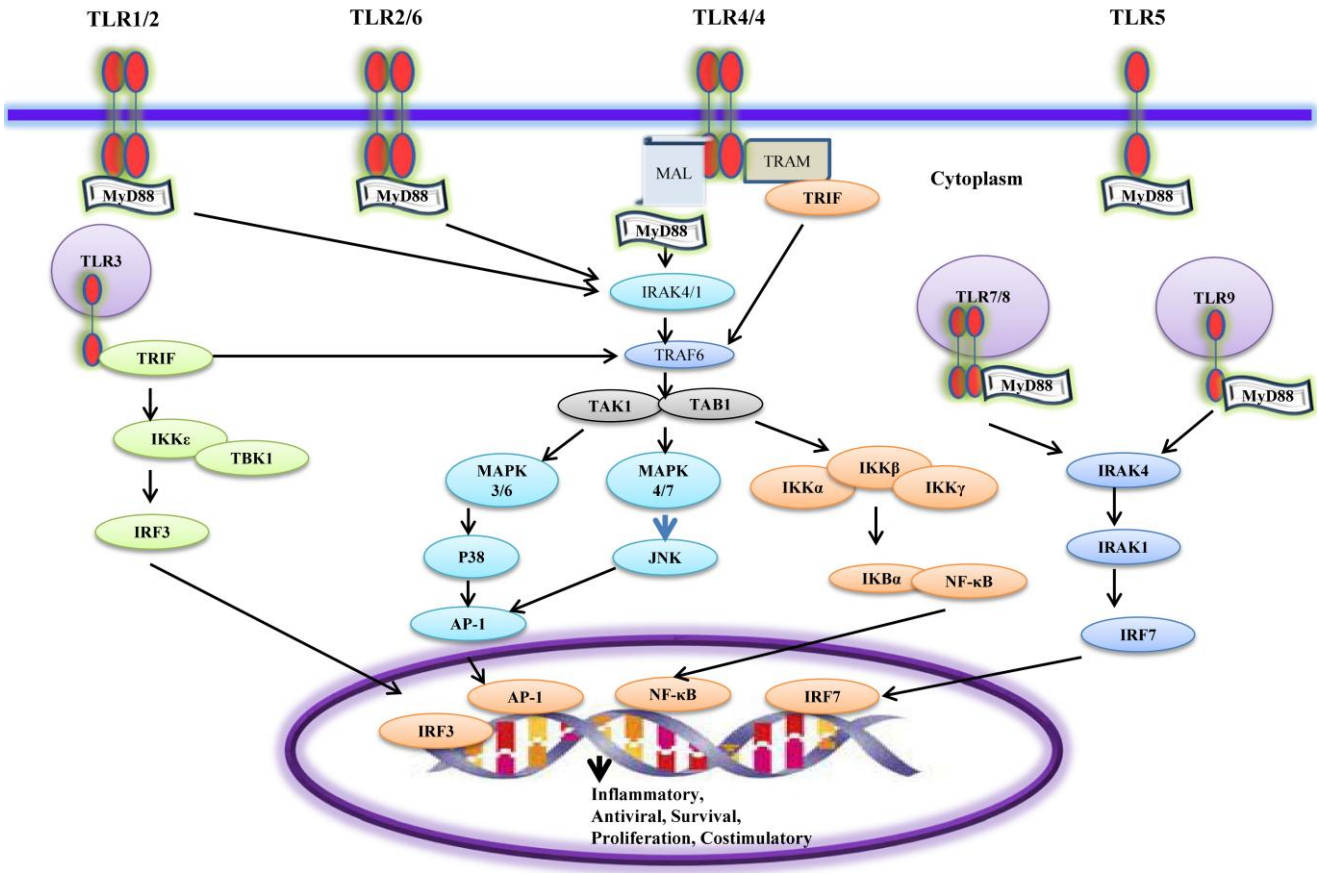

Fig. 1. Various TLRs and their locations. The cell membrane bound TLRs such as TLR1, 2, 4, and 5 use MyD88 as adaptor protein for signal transduction. TLR4 alone uses three or more adaptors apart from MyD88 to elicit antiviral responses. Endosome located TLRs also use MyD88 except, TLR3, it uses TRIF, the universal adaptor protein recruited by TLR3 and TLR4 for antiviral responses. All these pathways except TLR3 converge on TRAF6 (Tumor necrosis factor receptor associated factor 6), to activate NF-κB, which in turn translocate to nucleus for the upregulation of various genes involved in innate and adaptive immune responses. p38MAPK and JNK are also activated downstream of TLR4, 5, 1, 2, and 6 leading to the cellular response of proliferation and inflammation. TLR3 activates IRF3, which leads to antiviral genes upregulation. IRF7 is activated by TLR7, 8, and 9, the downstream signaling events trigger the production of antiviral immune responses. IRF, Interferon regulatory factor; IRAK, IL-1R-associated kinase; IKK, inhibitor of kappa light polypeptide gene enhancer in B-cells kinase; MyD88, Myeloid differentiation primary-response protein 88; MAPK, Mitogen activated protein kinase; TRAM, TRIF-related adaptor molecules, TIRAP, TIR domain containing adaptor protein; TRAF, TNF receptor-associated factor; TAB, TGF-activated kinase 1/MAP3K7 binding protein; TAK, TGF activated kinase.

TABLE I. Types of TLRs, their locations, damage-associated molecular pattern molecules (DAMPS), pathogen-associated molecular pattern molecules (PAMPS), adaptors, secretory products, and the diseases in which they have been implicated.

TLRs	**Location**	**Immune cell expression**	**Ligands (DAMPS)**	**Ligands (PAMPS)**	**Signal adaptor**	**Major secretory products**	**Disease indications**
TLR1 + TLR2	Cell surface	Monocytes, Macrophages, Dendritic cells, B cells	Heat Shock Proteins (HSP 60, HSP 70, Gp96), High mobility group proteins (HMGB1), Proteoglycans (Versican, Hyaluronic Acid fragments), Extracellular matrix	Triacylated lipoproteins, PAMP3, CSK4, Peptidoglycans, Lipopolysaccharide	TIRAP, MyD88, Mal	Inflammatory cytokines	Colon cancer, Candidiasis
TLR2 + TLR6	Cell surface	Monocytes, Macrophages, Dendritic cells, B cells	Same listed above	Diacylated lipoproteins	TIRAP, MyD88, Mal	Inflammatory cytokines	Colon cancer, Gastric cancer, Hepatocellular carcinoma
TLR3	Endosome	B cell, T cell, Natural killer cell, Dendritic cell	mRNA and tRNA	dsRNA, Poly IC, tRNA, siRNA	TRIF	Inflammatory cytokines, Interferon	Breast cancer, Colon cancer, Melanoma, Hepatocellular carcinoma, West Nile virus
TLR4	Cell surface	Monocytes, Macrophages, Dendritic cells, B cells	Heat Shock Proteins (HSP22/ 60/70/72, Gp96), High mobility group proteins (HMGB1), Proteoglycans (Versican, Heparin sulfate, Hyaluronic Acid fragments), Fibronectin, Tenascin-C, Ox-phospholipids, b-defensin 2, Amyloid-b, Ox-LDL, Extracellular matrix	Lipopolysaccharide, Viral envelop proteins	TRAM, TRIF, TIRAP, MyD88, Mal	Inflammatory cytokines, Interferon	Breast cancer, Colon cancer, Melanoma, Gastric cancer, Lung cancer, Hepatocellular carcinoma, Ovarian cancer, Sepsis, Experimental autoimmune encephalomyelitis, Atherosclerosis, COPD, Asthma
TLR5	Cell surface	Monocytes, Macrophages, Dendritic cells	n.d.	Flagellin	MyD88	Inflammatory cytokines	Gastric cancer, Cervical squamous cell carcinomas
TLR7	Endosome	Monocytes, Macrophages, Dendritic cells, B cells	ssRNA	ssRNA, Imidazoquinolines (R848), Guanosine analogs (Loxoribine)	MyD88	Inflammatory cytokines , Interferon	Chronic lymphocytic leukemia
TLR8	Cell surface	Monocytes, Macrophages Dendritic cells, Mast cells	ssRNA	ssRNA, Imidazoquinolines (R848)	MyD88	Inflammatory Cytokines, Interferon	Systemic lupus erythematous
TLR9	Endosome	Monocytes, Macrophages Dendritic cells, B and T cells	Chromatin IgG complex	CpG DNA, CpG ODNs	MyD88	Inflammatory Cytokines, Interferon	Breast cancer, Gastric cancer, Hepatocellular carcinoma, Cervical squamous cell carcinomas, Glioma, Prostate cancer, Malaria, Systemic lupus erythematous

III. TLRs LOCATED IN THE ENDOSOME AND THEIR IDENTIFIED LIGANDS: AN OVERVIEW

Not all TLRs are located on the cell membrane, however some TLRs are intracellular, i.e., they are located in endosomes. Such nucleic acid sensing TLRs include TLR3, 7, 8, and 9, that are primarily involved in the recognition of nucleic acids (Table I). dsRNA, a replication intermediate of most viruses, and poly (I: C), a synthetic analog of dsRNA are known ligands for TLR3 [22, 23]. Both ligands trigger MyD88-independent pathway using TICAM1 (Toll-interleukin 1 receptor domain-containing adaptor molecule-1; also termed TRIF) adaptor protein, thereby leading to the production of IFN-β (interferon beta), which plays a potent role in the maturation of DCs [24]. TLR7 expressed in plasmacytoid dendritic cells (pDCs) binds with small synthetic molecules such as loxoribine and imidazoquinoline compound R848 [25]. However, single-stranded RNA (ssRNA) from vesicular stomatitis virus and influenza viruses are the natural ligands for TLR7 [26-28]. Unlike TLR3, TLR7 uses MyD88 as adaptor protein, to induce the production of interferon alpha (IFN-α), TNF-α, and interleukin (IL)-12. In addition, guanosine and uridine rich ssRNA from human immunodeficiency virus (HIV)-1 are found to be identified by both TLR7 and 8 [25]. R848 is a synthetic imidazoquinoline with antiviral activity, and ssRNA [23; 19], a natural compound that induces TLR8 signaling. Both the ligands are identified by TLR8 in the endosomes leading to the secretion of cytokines [26]. TLR8 also binds with GU-rich ssRNA oligonucleotides derived from HIV-1 [25].

Previous studies have shown that measurable quantities of nucleic acids circulate both in healthy individuals and in patients with several pathological conditions. Moreover, increasing evidence suggests that these nucleic acids are preferentially released in circulation in the form of nucleosomes through apoptosis and necrosis [29]. DNA was originally believed to be non-damaging agent [30], however exposure to free DNA in the cell can lead to the activation of specific receptors that lead to autoimmune and inflammatory diseases. Such DNA sensing receptors include TLR9 and damage associated molecular patterns (DAMPs). Unmethylated CpG-ODN and single strand CpG ODN are known to bind with TLR9 [31]. Additionally, TLR9 not only differentiates bacterial and mammalian DNA, however, it also distinguishes different motifs of CpG DNA, for e.g., sequences of GTCGTT and GACGTT belonging to human and murine DNA respectively can also be identified [32]. Three classes of CpG-DNA such as CpGA, CpGB, and CpGC are identified. CpGA strongly activates pDCs [33] for the increased production of IFN-α, CpGB, strong B cell activator leads to the production of co-stimulatory and antigen presenting molecules [34], and CpGC activates both B cells and pDCs [35]. TLR9 in line with TLR7 and 8 also recruits MyD88 as the signaling protein for NF-κB activation [36].

IV. EXPRESSION OF VARIOUS TLRs IN DIFFERENT CELLS

Several TLRs have been reported in B cells (Table I) [37]. Cognasse *et al.* [38] have reported secretion of IL-6 by TLR9 expressing memory B cells. Mansson *et al.* [39] also reported secretion of IL-6 *via* activation of B cells with IL-2, IL-10, recombinant CD40 ligand, and TLR2, 7, and 9 ligands. Recently Agarwal and Gupta [40] reported extensively about the 24 cytokines and chemokines secretion in response to TLR ligands, like Pam3CSK4 (TLR1/2), Imiquimod (TLR7), and CpG-ODN (TLR9) on B cells. The majority of the cytokines and chemokines were produced by CD19+CD27+ memory B cells, and in general TLR2 showed stronger signal than TLR7 and 9 to induce their production. Using immortalized skin-derived (HMEC-1) and primary dermal endothelial cells (HDMEC), [41], TLR expressions were screened. They found that endothelial cells express TLR7 (for HDMEC) and TLR8 (for HMEC-1) under resting conditions but can express all 10 TLRs in proinflammatory conditions. During various TLR-specific ligand stimulation (Pam_3Cys, lipoteichoic acid (LTA), poly(I:C), LPS, flagellin, imiquimod, and CpG DNA), the authors found that endothelial cells not only express most of the 10 TLRs in resting state but also express all 10 receptors of this family under proinflammatory conditions. B cells, pDCs, monocytes, and macrophage lineage express TLR9 mRNA or protein [36, 42-45]. Moreover, it is found that human peripheral blood mononuclear cells (PBMCs) express TLR9 [46]. DCs are the most potent and powerful antigen presenting cells in the immune system. Based on the presence of surface markers, DCs are divided as myeloid dendritic cells (mDCs) and pDCs. Both the DCs express TLR1/2/6, and its activation leads to DC maturation and secretion of various cytokines such as IL-6, IL-8, IL-10, IL-12 and TNF-α [43, 45, 47].

V. INTRACELLULAR PATTERN RECOGNITION RECEPTORS

Among pattern recognition receptors (PRRs), NLRs represent a new class of intracellular PRRs. NOD1 and NOD2, members of the NLR family, sense the presence of bacterial peptidoglycan derivatives (Table II). NOD1 in specific, binds with meso DAP (diaminopimelic acid) muramyl peptides, which are found in peptidoglycan of most gram-negative bacteria and certain gram positive bacteria. On the other hand, NOD2 binds with muramyl dipeptide produced by all bacteria [48]. The downstream signaling of NOD1 and NOD2 are quite similar in triggering proinflammatory pathways of NF-κB, p38, JNK, and ERK [49]. *In vivo* NOD deficient mice have demonstrated the activation of NOD1 and NOD2 against pathogens such as *Helicobacter pylori, Listeria monocytogenes, Staphylococcus aureus, and Legionella pneumophila* leading to cytokine and chemokine production [50-53]. In contrast to these reports, Oliveira *et al.* [54] reported that NOD1 and NOD2 actions are species specific,

TABLE II. Intracellular pattern recognition receptors.

Receptor	Types of receptors	Subfamilies	Ligands
NLR	NLRA, NLRB, NLRC, NLRP	CIITA, NAIPS, NOD1, NLRC4, NOD2, NLRC3, NLRC5, NLRXI, NLRP1, NLRP2,9,11, 4, NLRP10	n.d.
RIG1	AIM2, DAI, DDX41, IFI16, LRRFIP1, MDA-5, RIG-I, RNA pol III	n.d.	Vaccinia, Mouse cytomegalovirus *F. tularensis*, *L.monocytogenes*, AT-rich BDNA, Human cytomegalovirus, Herpes simplex 1, *S. pneumoniae*, Herpes simplex, dsDNA sequence-independent 70>>50 bp, Vesicular stomatitis, *L. monocytogenes*, dsDNA, dsRNA, GC rich Z-DNA, Picornavirus, Encephalomyocarditis, Rabies, Sendai, Dengue, Rotavirus, Murine hepatitis, Murine norovirus I, Poly IC, Newcastle disease, Sendai, Influenza, Japanese encephalitis, Measles, Rabies, Hepatitis C, Dengue, Adenovirus, Epstein Barr, *L. pneumophila*
C-Type lectin receptors	TYPE I, TYPE II, SOLUBLE	DEC 205, MMR, DECTIN I, DECTIN II, MINCLE, DC-SIGN, DNGR 1, MBL	Curdlan AL, Hkca, Hksc, Zymosan
Cytosolic DNA receptors	DAI, RIG1, LRRFIPI, AIM2	n.d.	Poly (dA:dT) Naked, Poly (dA:dT) LyoVec, Poly(dG:dC) Naked, Poly(dG:dC) LyoVec
Inflammosomes	IL-1β, IL-18, NLRPI inflammosome, NLRC4 inflammosome, NLRP3 inflammosome, AIM2 inflammosome	n.d.	Alum crystals, ATP, CPPD crystals, Hemozoin, MSU crystals, Nano sio2, Nigericin, Poly (dA:dT) Naked Poly (dA:dT) LyoVec

for example, they have no role in controlling the immune response elicited by *Brucella abortus.* RIG-I and melanoma-differentiation associated protein-5 (MDA5), belong to RNA helicases, which detect viral pathogen associated molecular patterns (PAMPs). RIG-I recognizes viral RNAs from the Flaviviridae, Orthomyxoviridae, Paramyxoviridae, and Rhabdoviridae families, whereas MDA-5 is involved in response to Picornaviridae [55]. The ligand for RIG-I is 5'triphosphate moiety present on the single or double stranded RNA [56, 57]. Recent studies point out that MDA-5 is critical for protection against Theiler's virus-induced demyelinating disease.

Certain CLRs and RLRs recognize PAMPs of oncogenic infectious agents. PAMPs of *Mycobacterium tuberculosis*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Candida albicans* and HIV1 are detected by MRC1 (CD206, CLEC13D, mannose receptor). PAMPs of *Candida* spp, HIV-1 are recognized by CD207 (CLEC4K, langerin), in specifically PAMPs of HIV-1 are recognized by LY75 (CD205, CLEC13B, DEC-205), CLEC1B (CLEC-2), and CLEC4A (DCIR). PAMPs of *Mycobacterium* spp, *Schistosoma mansoni*, *C. albicans*, HCV, HIV-1, cytomegalovirus binds with CD209 (CLEC4L, DC-SIGN). Each bacterium has some specific binding capacity to CLRs, for example, PAMPs of *Mycobacterium* species binds with CLEC7A (Dectin-1). *M. tuberculosis*, *C. albicans*, *Paracoccidioides brasiliensis* and *Histoplasma capsulatum* are sensed by CLEC6A (CLEC4N, Dectin-2). PAMPs of *M. tuberculosis* and *C. albicans* bind with CLEC4E (Mincle) [8, 58, 59]. All PRRs are known to activate inflammosomes, a multiprotein complex, whose activation leads to caspase 1 activation [60]. NLRP1, NLRP3, NLRC4 (NOD like receptor family) and AIM2 are the four inflammosomes identified to date. The expression of various TLR-induced genes in response to various PAMPS and DAMPS are summarized in Table III.

VI. SUPPRESSION OF TLR SIGNALING BY CROSSTALK

TLRs are activated by bacteria, virus and other pathogens, sometimes the stimulation of one agent can inhibit other agent responses, causing the susceptibility to the main agent [61]. In order to better understand the mechanisms underlying innate immune responses, these kinds of studies have to be carefully designed during polymicrobial infections. Physiological crosscheck mechanisms and regulatory points serve as good invigilators; however sometimes the system can be overlooked thus leading to lethal immune related diseases. In a previous study, mice infected with virus showed increased susceptibility to bacterial infection and died because of the decreased production of a gene encoding p40 subunit of interleukin 12 (IL-12b) upon activation of TLRs [61]. There is active crosstalk between G proteins and TLRs *via* secreted factors that can inhibit TLR signaling to escape host response. Pasteurella multocida toxin (PMT) from Gram-negative *Pasteurella multocida* activates heterotrimeric G proteins Gαq, Gα13, and Gαi that effectively suppress LPS-stimulated IL-

12p40 production and leads to impairment in T cell-activating ability of LPS-treated monocytes [62].

The 14-3-3 proteins are a family of ubiquitously expressed, acidic proteins consisting of seven known mammalian isoforms (β, γ, ε, σ, ζ, τ, and η), which are expressed in all mammalian cells [62-64]. This protein binds to a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors and play vital roles in cell survival, proliferation, growth, differentiation, and intracellular signaling. This protein has been implicated in many diseases such as cancer and neurological diseases. Recently, 14-3-3 proteins have been implicated in the modulation of TLR signaling whereby the stimulation of RAW cells with LPS induces PKCε phosphorylation associated with 14-3-3β in a MyD88-dependent manner [65]. A proteomics approach by Butt *et al.* [66] identified 14-3-3ε and 14-3-3σ proteins as new members of the TLR signaling complex. Working towards the functional characterization of 14-3-3ε and 14-3-3σ in TLR signaling, both of these proteins were shown to impair TLR2, 3, 4, 7/8, and 9 ligand-induced IL-6, TNF-α, and IFN-β production [66].

Five TIRAP peptides, TR3, TR5, TR6, TR9, and TR11 inhibited LPS-induced cytokine mRNA expression and MAPK activation. The macrophage responses towards inflammatory stimuli are kept under tight control in normal conditions; for which the cells need to have an internal balance in the form of intracellular feedback loops. One such effective feedback mechanism, which forms the axis of the inflammatory response, is the p38-STAT3 signaling proteins [67].

SOCS1 acts as a negative regulator of TLR signaling. SOCS molecules bind to several tyrosine-phosphorylated proteins, including MAL (TLR signaling) and IRS1/2 (insulin signaling) [68]. Subsequent to phosphorylation, MAL interacts with SOCS1, resulting in polyubiquitination and subsequent degradation. BCAP acts as a linking adaptor protein for B-cell receptor (BCR) and CD19, and is involved in the activation of PI3K [69, 70]. BCAP-mediated TLR signaling limits the production of proinflammatory cytokines IL-6, IL-12, and TNF-α, which is crucial for regulating immune responses. E3 Ubiquitin protein ligase CBL-B and SYK (Spleen tyrosine kinase) are involved in the degradation of MyD88 and TRIF, thus leading to the negative regulation of TLR signaling [71] (Fig. 2).

Over expression of A20 (also known as TNFAIP3; tumor necrosis factor alpha-induced protein 3) abrogates TLR4 induced NF-κB activation by deubiquitinating TRAF6 [72, 73]. TRAF family member-associated NF-κB activator (TANK) acts as a negative regulator of TLR and BCR signaling by inhibiting TRAF6 activation. Ubiquitination of TRAF6 is upregulated in TANK-/- macrophages during TLR and BCR activation suggesting that TANK inhibits TLR signaling downstream [74]. PIN1, RBCK1, CUL1 and RAUL

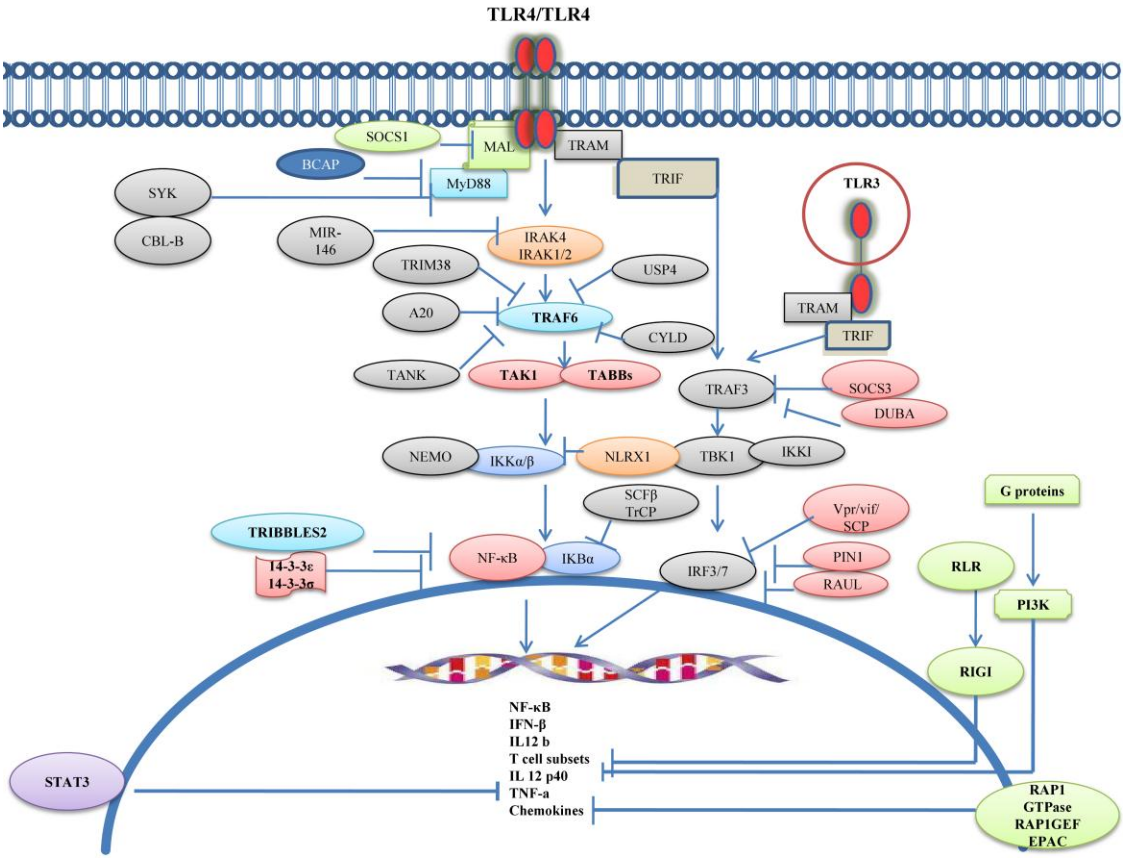

Fig. 2. TLR signaling inhibitory points. Over activation of TLRs are fatal, physiological system has naturally produced crosscheck points in the form of negative regulatory proteins to prevent over-activation. For example PI3K, Rap1 GTPase, Rap1GEF, Epac1, BCAP Tribbles 2 perform negative regulatory role

to produce anti-inflammatory responses. BCAP, B-Cell adaptor for PI3K; CYLD ,Cylindromatosis; GEF, rho/rac guanine nucleotide exchange factor; RLRs, RIG-I-like receptors; miR-146, microRNA-146; NLRs; NOD like receptors; NLRX1 Nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family member X1; PIN1, peptidyl-prolyl isomerase, DUBA, deubiquitinating enzyme A; RAP1EPAC, replication associated protein and exchange protein directly activated by cAMP1; RIG1, Retinoic Acid Inducible Gene 1; RAUL , replication and transcription activator (RTA)-associated ubiquitin ligase; SYK; Spleen Tyrosine Kinase, CBL-B Casitas B-lineage lymphoma; SCF Skp1–Cul1–F-box;mir-146, SOCS3, Suppressor of Cytokine Signaling 3; TANK, TRAF-family member-associated NF-κB activator; TRIM, Tripartite-motif Containing; USP4, Ubiquitin specific peptidase 4.

belong to cellular ubiquitin ligases which are involved in the degradation of IRF3. Several cellular ubiquitin ligases are involved in the degradation of IRF-3, including Pin1, RBCK1, Cul-1, and RAUL [75-77]. HECT domain ubiquitin (Ub) E3 ligase, RAUL, limited type I IFN production by directly catalyzing lysine 48-linked polyubiquitination of both IRF7 and IRF3 followed by proteasome-dependent degradation [78]. Tripartite motif containing (TRIM) protein (comprises of more than 70 members) contains a ring domain, B-box domain and coiled coil region [79] which are involved in various biological and physiological processes. A recent study showed that mouse TRIM30α interacts with TAK1 and promotes the degradation of TAB2 and TAB3, resulting in the inhibition of TRAF6 ubiquitylation consequently leading to the inhibition of NF-κB activity [80].

TABLE III. Gene expressions of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9.

Gene signatures	**Genes**
Gene signature specific to pathogens, such as bacteria, viruses, and fungi	*Ccl2 (Mcp-1), Cd14, Fos, Hras1, Il10, Il12a, Il1b, Il6, Irak1, Hmgb1, Hspa1a (Hsp701a), Jun, Lta (Tnfb), Ly86, Ly96, Nfkbia (Ikba/Mad3), Pglyrp1, Ptgs2 (Cox2), Rela, Ripk2, Tlr2, Tlr4, Tlr6, Tnfrsf1a, Ticam1 (Trif), Eif2ak2 (Prkr), Ifnb1, Ifng, Il12a, Il6, Irf3, Rela, Tbk1, Tlr3, Tlr7, Tlr8, Tnf, Ticam1 (Trif), Clec4e, Hras1, Hspa1a (Hsp701a), Tlr2, Tirap*
Genes expressed by the activation of MyD88-dependent and -independent pathways	*Tollip, Irf3, Map3k7 (Tak1), Nr2c2, PeIi1, Tbk1, Ticam2, Tlr3, Tlr4, Traf6, Ticam1 (Trif), Irf3, Map3k7 (Tak1), Nr2c2, Peli1, Tbk1, Ticam2, Tlr3, Tlr4, Traf6, Irak1, Irak2, Map3k7 (Tak1), Myd88, Nr2c2, Tirap, Tlr1, Tlr2, Tlr4, Tlr5, Tlr6, Tlr7, Tlr8, Tlr9, Traf6*
Gene signatures specific to the NFκB pathway, JNK/p38 pathway, JAK/STAT pathway, interferon regulatory factor (IRF) pathway, and cytokine-mediated signaling pathway	*Btk, Casp8, Chuk (Ikka), Fadd, Agfg1, Ikbkb, Il1r1, Il10, Il1b, Irak1, Irak2, Irf3, Ly96, Map3k1, Map3k7, Nfkb1, Nfkb2, Nfkbia (Ikba/Mad3), Nfkbib, Nfkbil1, Nfrkb, Ppara, Rel, Rela, Tnf, Tnfaip3, Tnfrsf1a, Tradd, Ube2n, Ube2v1, Elk1, Fos, Il1B, Jun, Map2k3 (Mek3), Map2k4 (Jnkk1), Map3k1 (Mekk), Map3k7, Mapk8 (Jnk1), Mapk8ip3, Mapk9, Tnf, Ccl2 (Mcp-1), Csf2 (GM-CSF), Ifng, Il12a, Il2, Il6, Cxcl10 (Inp10), Ifnb1, Ifng, Irf1, Irf3, Tbk1, Ccl2 (Mcp-1), Cebpb, Csf3 (GCSF), Il1A, Il1b, Il1r1, Il6, Il6ra, Irak1, Irak2, Rela, Tnf, Tnfrsf1a*
Genes involved in adaptive immunity	*Cd80, Cd86, Hspd1, Ifng, Il10, Il12a, Il1b, Il2, Map3k7, Traf6*
Adaptors and TLR interacting proteins	*Btk, Cd14, Hmgb1, Hras1, Hspa1a (Hsp70 1a), Hspd1, Ly86 (MD-1), Ly96 (MD-2), Mapk8ip3, Myd88, Peli1, Pglyrp1, Ripk2, Ticam1 (Trif), Ticam2 (Tram), Tirap, Tollip*
Effectors	*Casp8 (Flice), Eif2ak2 (Prkr), Fadd, Irak1, Irak2, Map3k7 (Tak1), Nr2c2, Ppara, Traf6, Ube2n, Ube2v1*

VII. RECENT FINDINGS ON TLR SIGNALING

Recent studies have shown that the activation of TLRs not only leads to cellular defense mechanisms but also the upregulation of DNA repair genes and increased functional DNA repair [81]. Nuclear factor of kappa light polypeptide gene enhancer in B cells 2 [NF-κB2 (p100)) was identified as a *Tribbles 2 (Trib2)* binding partner regulating the TLR5 signaling pathway that leads to the inhibition of NF-κB activity. The altered expression of *Trib2* may play a role in inflammatory bowel diseases. Chevrier *et al.* [82] made some outstanding findings by selecting only the genes induced by viral infection by combining transcriptional profiling, genetic and small-molecule perturbations, and phosphoproteomics. With this approach, they uncovered 35 signaling regulators, including 16 known regulators, involved in TLR signaling. In particular, they found that polo-like kinases (PLK) 2 and 4 are essential components of antiviral pathways *in vitro* and *in vivo* and activate a signaling branch that involves a dozen proteins, including *Tnfaip2*, a gene associated with autoimmune diseases [82].

Several reports have shown the consequences of excessive activation of TLRs. Chronic exposure to TLR ligands further complicates the situation, leading to atherosclerosis [83] and tumor metastasis [84, 85]. Hence, it is necessary to control the excess activation of TLRs. Naturally, cells possess regulatory checks in the form of negative regulatory proteins. One such regulatory loop is PI3K (phosphoinositide 3-kinase), a family of serine/threonine kinases, that phosphorylates PIP2 and promotes cell survival, proliferation, and protein synthesis [86]. In connection with this finding, Troutman *et al.* [87] showed that BCAP (B-cell adaptor for PI3K) is a unique TIR domain-containing adaptor molecule that forms a critical link between TLRs and the activation of PI3K. BCAP may engage MyD88 *via* its TIR domain and potentially reduce the availability of MyD88 for the activation of NF-κB, thereby affecting the canonical pathway of TLR signaling. BCAP also mediates the activation of PI3K downstream of TLRs, and this has been demonstrated to have regulatory effects on the outcome of TLR signaling, including limited cytokine secretion and inflammation. These findings clearly show that the dysregulation of the complex PI3K pathway leads to diseases such as cancer, inflammation, and inflammatory bowel diseases [88]. TLR4 signaling promotes an epithelial–mesenchymal transition in human hepatocellular carcinoma induced by LPS [89]: TLR signaling contributes to germinal center antibody responses, and TLR4 has an essential role in early skin wound healing. Some interesting studies have even

pointed out that nickel induces human TLR4 activation but mouse TLR4 cannot generate this response [90].

VIII. INBUILT THERAPEUTIC MOLECULES FOR TLRS

Since TLR has certain properties to be considered as a therapeutic agent, proven by an extensive literature bank in this field, it is necessary to find the "bad guys" or pathogenic molecules downstream of TLR signaling, so that they can be targeted either in a positive or negative way as required. Chemoattractant receptors (CKRs) secreted by TLRs orchestrate the migration of leukocytes to infected tissues. Although there have been reports on the roles of TLRs in leukocytosis, many studies have observed a paradoxical role of TLRs in suppressing leukocytosis in infected tissues during sepsis [91]. Recently, Yi *et al.* [92] also observed the same phenomenon in monocytes treated with LPS. They showed that LPS induced rapid p38 MAPK activation, global redistribution of activated Rap1 (Ras-proximate-1 or Ras-related protein 1) and Epac1 (exchange proteins activated by cyclic AMP) and disruption of intracellular gradient. However, this could be overcome by simultaneously stimulating TLRs during natural infection to ameliorate the temporary inhibition of chemotaxis. In addition, the tethered TLR-TIR domain dimers inhibit TLR signaling. A study by Fekonja *et al.* [93] showed that this tethering is done by the immunosuppressive virulent factors TCPs (toxin-coregulated pilus), which are secreted by *Brucella melitensis* and strongly inhibit TLR signaling. Furthermore, the addition of an artificial strongly coiled dimerization domain conferred potent inhibition over a broad range of TLRs and IL-1R. Elucidating the structure of TCP molecules will be important for designing drugs in the therapeutic suppression of TLR activation (Fig. 3).

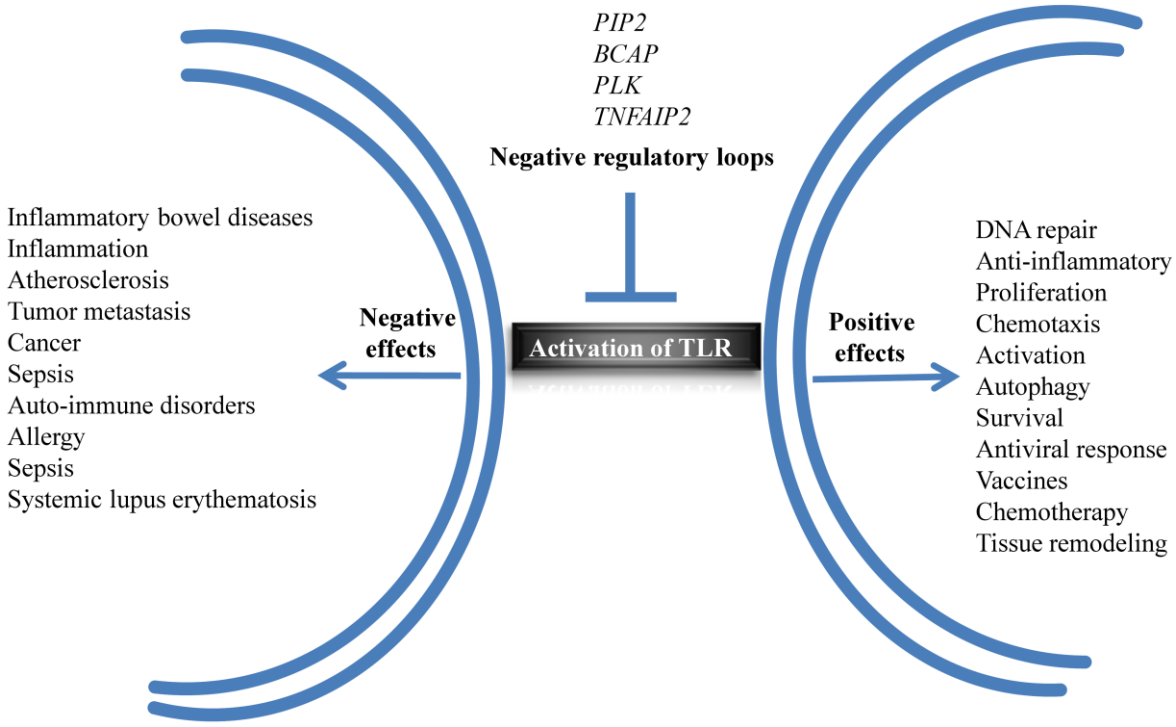

Fig. 3. Roles of TLRs as double-edged swords in inflammation and anti-inflammation. TLR activation has both positive and negative effects. TLRs are constantly monitored by physiological negative regulatory loops like PIP2, BCAP, PLKs, TNFAIP2 and many others. Over activation of TLRs leads to various diseases such as sepsis and inflammatory bowel diseases. On the other hand, positive effects of TLRs are essential in bridging the connection of innate and adaptive arms of immune responses.

IX. *IN SILICO* APPROACHES OF TLR SIGNALING

In an extensive *in silico* approach by Li *et al.* constraint-based modeling methods of human TLR signaling using kinases, phosphatases, and proteins identified at least 8 novel inhibition targets [94]. These targets were located with IL-1, NF-κB and ROS (Reactive Oxygen Species) pathways. The eight negative mediators are VAV1, RAC1, gp91-p22, p62-PKCz, Ajuba mediated IRAK1-PKCz binding, PKCz phosphorylation, MyD88 dimerization and TRAF6/IRAK1 ubiquitination. In addition, the authors have confirmed the experimental evidence for the role of VAV1 and PKCz isozyme in ROS production pathway and NF-κB pathway respectively *via* literature survey [95]. *In silico* approaches also reveal the role of TLR4 in colorectal cancer. Microarray data from Oncomine and Gene Expression Omnibus were used for heat map samples and showed an increased expression of TLR4 in colon adenoma. Especially the up regulation of *COX-2, β-catenin*, and *EGFR* positively correlated with the TLR expression.

In our lab, we have extensively studied about the structural studies of TLRs by taking advantage of computational facilities (sequence analysis and molecular modeling studies) to identify the following functions in TLR signaling pathways: (i) Prediction of TLR ectodomain using available TLR crystal structures as a template and identification of its possible ligand-binding region [96]. (ii) Structural basis identification of positive and negative regulators in TLR signaling [97, 98]. (iii) Identification of the interaction between the TIR domain and its adaptor molecules, which provides structural insights into the mechanism responsible for TLR mediated downstream activation or inhibition [99]. (iv) Comparative analysis of species-specific activation in TLRs [100] and (v) Evolutionary relationships of TLR downstream molecules such as IRAKs [101].

Currently *in silico* approaches are used in several studies especially in phylogenetic comparison to find the conservation of TLRs through evolution [102]. However, during *in silico* approaches it is necessary to upload data in public databases (example innateDB, ImmGen, Macrophages.com) that share data and impose rules to establish a common standard methodology. The findings of *in silico* methods have to be extrapolated *in vitro* and *in vivo* conditions for further verification, and to find the exact role of the above mentioned proteins in TLR activation and design appropriate drug therapies.

X. Directions for Future Research

With the compendium of information present in microarray data, it should not be an arduous task to delineate the myriad of information generated about the signaling, crosstalk, and overactivation of TLRs. Indeed, this information could serve as a good starting point for the identification of relevant signaling components in diverse biological systems, which could be followed with studies of perturbations, kinetics, ligand dependence, and signaling mechanisms during TLR activation. Consolidating various high throughput experimental information on TLRs, setting up open standard ways of data analysis and dynamic studies by systems biological approaches would be helpful in generating the integrated map on TLR signaling for the development of therapeutic drugs in the near future.

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