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Review

Potential therapeutic targets for inflammation in toll-like receptor 4 (TLR4)-mediated signaling pathways

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ABSTRACT

Inflammation is set off when innate immune cells detect infection or tissue injury. Tight control of the severity, duration, and location of inflammation is an absolute requirement for an appropriate balance between clearance of injured tissue and pathogens versus damage to host cells. Impeding the risk associated with the imbalance in the inflammatory response requires precise identification of potential therapeutic targets involved in provoking the inflammation. Toll-like receptors (TLRs) primarily known for the pathogen recognition and subsequent immune responses are being investigated for their pathogenic role in various chronic diseases. A mammalian homologue of Drosophila Toll receptor 4 (TLR4) was shown to induce the expression of genes involved in inflammatory responses. Signaling pathways via TLR4 activate various transcription factors like Nuclear factor kappa-light-chain-enhancer (NF-κB), activator protein 1 (AP1), Signal Transducers and Activators of Transcription family of transcription factors (STAT1) and Interferon regulatory factors (IRF's), which are the key players regulating the inflammatory response. Inhibition of these targets and their upstream signaling molecules provides a potential therapeutic approach to treat inflammatory diseases. Here we review the therapeutic targets involved in TLR-4 signaling pathways that are critical for suppressing chronic inflammatory disorders.

Contents

1. Introduction

Inflammation is a host immune response to tissue injury or infection by recruiting regulatory immune cells. Despite its protective role in the host system against foreign pathogens, uncontrolled inflammatory response (for instance, sepsis) leads to tissue damage, dis-functioning of organ and even to mortality [1]. Thus, the establishment of a balanced inflammatory response is required for effective clearance of pathogen/injured tissue with minimum damage to the host. Macrophages are one of the vital innate immune cells and the first line of defense against any invasion which regulates the inflammation and tissue homeostasis [2,3,4].

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As one of the primary sources of proinflammatory cytokines, macrophages are critical role players in inflammatory diseases. Under the septic condition, recognition of LPS (endotoxin present in the cell wall of all gram-negative bacteria), bacterial RNA or DNA by TLR receptors activates the proinflammatory cytokine profiles in macrophages thus disrupting the homeostatic regulation of the immune system [5]. TLRs, which contain 10 members in human and 12 in mouse, contain adaptors such as myeloid differentiation protein (MyD88), toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP), TIR domain-containing adapter inducing IFN-Beta (TRIF) and translocation associated membrane protein 1 (TRAM) which are modulated in the inflammatory signaling upon activation with the stimuli. TIRAP mediates the recruitment of MyD88 in TLR1, TLR2, TLR4, and TLR6, whereas MyD88 is directly recruited by TLR5, TLR7, TLR9, and TLR11. TLR3 (which does not recruit MyD88) recruits TRIF upon stimulation, where TLR4 possess both MyD88 as well as TRIF mediated pathways [6]. The recruitment of adaptor protein is followed by a cascade of signaling pathway to activate NF-κB, AP-1, STAT-1, and IRFs, which mediates proinflammatory cytokine release.

Along with the proinflammatory signaling cascade, anti-inflammatory or regulatory molecules for inflammation are also activated after induction by various stimuli. For instance, higher expression of Vascular endothelial growth factor-C (VEGF-C), Nerve growth factor IB (Nur-77) as well as Selective androgen receptor modulators (SARM) in earlier phases of septic shock models point the induction of anti-inflammatory molecules along with the proinflammatory pathways. In this a way it maintains the homeostasis via forming a feedback loop to inhibit the uncontrolled inflammation [1,7]. In this article, we have highlighted on the recent advancements in discovering potential targets within the major TLR-4 mediated inflammatory pathways like NF-κB, AP-1, IRF3 and STAT1 in macrophages. Moreover this review also summarizes various known inhibitors and their molecular targets as listed in Table 1.

2. NF-κB and upstream targets

NF-κB is one of the major transcription factor which is indispensable for the classical inflammatory pathway in innate immune response. It triggers the genetic expression of inflammatory molecules upon the pathogen-associated molecular patterns (PAMPs) recognition in pattern recognition receptors (PRRs) [8,9]. Tremendous attention has been focused on NF-κB and associated signaling molecules which could serve as potential pharmaceutical targets leading to the subsequent interference in disease progression [10,11,12]. In spite of recent discovery and advancement in understanding of the signaling mechanisms, the precise mode of interaction of these molecules is still a debatable issue. Since interactions among diverse signaling components involved in this pathway rely on more than one of the upstream signals to regulate their downstream targets, it could be concluded that the transmission of signals requires a network rather than a linear array for the activation of NF-κB. Exposure of macrophage cells to LPS or inflammatory cytokines such as or IL-1β, viral infection or expression of particular viral gene products, UV irradiation, B or T cell activation, or other physiological and non-physiological stimuli are recognized by TLRs which leads to the activation of NF-κB target genes. Until now more than 20 ligands have been identified as TLR4 endogenous ligand that activates TLR4 pathway.

TLR4 utilizes four distinct adaptor proteins for signal propagation which include; MyD88, toll-interleukin 1 receptor (TIR) domain-containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing interferon-β (TRIF) and translocation associated membrane protein (TRAM) upon activation signaling by the PAMP ligation [13,14]. This results in triggering downstream signaling cascades in a bifurcated fashion with MyD88 and Mal/TIRAP on one hand and TRIF and TRAM on the other. In the study conducted by Takashima and group, they targeted TLR4 with a novel synthetic molecule, resatorvid. Resatorvid specifically target Cys747 in intracellular domain of TLR4 and inhibits its downstream signaling [15,16]. MyD88 death domain residues (Glu52 and Tyr58) interact with N-terminal death domain residue of IRAK4 leading to its activation downstream [17,18]. IRAK4, in turn, leads to the phosphorylation of Thr387 in the activation loop of IRAK1 [19]. The activated IRAKs dissociate from MyD88, and further interact with tumor necrosis-associated factor 6 (TRAF6), an E3 ubiquitin ligase. TRAF6 is a member of the tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family that consist of TRAF domains (TRAF-N and TRAF-C), which are responsible for interaction with TRAF proteins and other signaling molecules via their N-terminal ring finger and zinc finger domains [20]. The E2 ubiquitin-conjugating protein 13(UBC13) complex catalyze the synthesis of a Lys 63-linked polyubiquitin chain of TRAF6 and thereby induce TRAF6-mediated activation of transforming growth factor-beta–activated kinase 1 (TAK1) and TAK1-binding proteins, TAB1, and TAB2, at the membrane portion [21]. Shi and his group have shown that a ring protein, TRIM30α reduces NF-

Table 1
Therapeutic agents that target different molecules in TLR4 signaling pathways and their targeted diseases.

S. no.	Target molecule	Inhibitor	Disease	Reference
1.	TLR4	Resatorvid	Sepsis	[15,16]
2.	MD-2	Eritoran (E5564)	Sepsis	[109]
3.	SKY	Fostamatinib	Rheumatoid arthritis, atherosclerosis	[110]
4.	IKK	BMS-345541, CHS828	Melanoma, solid tumors	[111,112]
5.	NF-κB	SN-50[a]	Ovarian cancer	[113]
6.	TAB2 & TAB3	TRIM30α	Sepsis	[22]
7.	TBK1 & RIP1	Resveratrol	Chronic inflammatory diseases	[29]
8.	Akt	Wortmannin	Sepsis	[114]
9.	p38	SB203580	Sepsis	[115]
10.	ERK	Chloroquine	Malaria and other inflammatory disorders	[116]
11.	MEK/AP-1	U0126	AP-1 mediated inflammatory disorders	[117]
12.	IRF3	BX795	Chronic obstructive pulmonary disease	[118]
13.	MNK1	CGP052088	Cancer	[119]
14	IRF5	KAP1/TRIM28	Inflammatory disease	[120]
15.	JNK	SP600125	Inflammatory arthritis	[121]
16.	MyD88	ST2825	Inflammatory and autoimmune diseases	[39]
17.	PKCδ	Rottlerin	Inflammatory disease	[72,122]
18.	Erk	Pheophytin	Sepsis	[123]
19.	PI3K	AS-605240	Rheumatoid arthritis Multiple sclerosis	[124,125]
20	JAK1/JAK2	Ruxolitinib	Rheumatoid arthritis Psoriasis Myelofibrosis	[126]

[a] A peptide of 41 amino acid residue.

κB expression and thus reduced endotoxin shock by degradation of TAB2 and TAB3 entities [22]. TAK1 activates IKK (IκB kinase), a multiprotein complex that often contains two catalytic constituents (IKKα and IKKβ) and a regulatory component (NEMO/IKKγ) which phosphorylates IκBα at sites Ser32 and Ser36 subsequently followed by 26S proteasome degradation [20]. Burke and his group investigated on role of IKK as therapeutics target and shown BMS-345,541 as selective inhibitor of the catalytic subunits of IKK. The inhibitor binds to an allosteric binding site of the IKK catalytic subunits leading to its conformational change and diminishing its kinase activity function [23]. NF-κB complex is bound with the inhibitory IκB proteins in the cytoplasm, which restricts its translocation to the nucleus. However, activation signals from the upstream signaling cascade converge on IKK, which steers in the phosphorylation and degradation of IκB and release of free NF-κB [24]. So the released NF-κB subunits (mostly p65 and p50) translocate into the nucleus through the canonical pathway and activate a range of inflammatory genes such as IL-6, IL-12p40, and TNFα [19] as shown in Fig. 1. A well-known inhibitor to inhibits the subcellular traffic of NF-κB is SN50 peptide encompassing nuclear localization sequence which blocks the NF-κB [25]. Thus, targeting NF-κB nuclear translocation can again provide a potential therapeutic target.

Contrary MyD88 independent pathway leads to activation of TLR4 in response to the particular stimulus which further activates intracellular TIR domain that conveys downstream signals by recruiting adaptors like TRIF and TRAM [8]. TRIF contains an N-terminal domain that includes the TIR domain necessary for interaction with TLRs and a C-terminal RIP homotypic interaction motif, which can activate cell death pathways. Receptor-interacting protein (RIP) kinases are the group of threonine/serine protein kinases with a relatively conserved kinase domain but distinct non-kinase regions determining the particular function of each RIP kinase. The RIP kinases are a family of seven members, all of which share a homologous kinase domain but have different functional domains [26]. The C-terminal death domain kinase of RIP1 associates with TRAF6 to potentiate the activation of the IKK complex [27]. Park et al. have shown that RIP1 activates PI3K-Akt by negatively regulating mammalian target of rapamycin (mTOR) transcription via

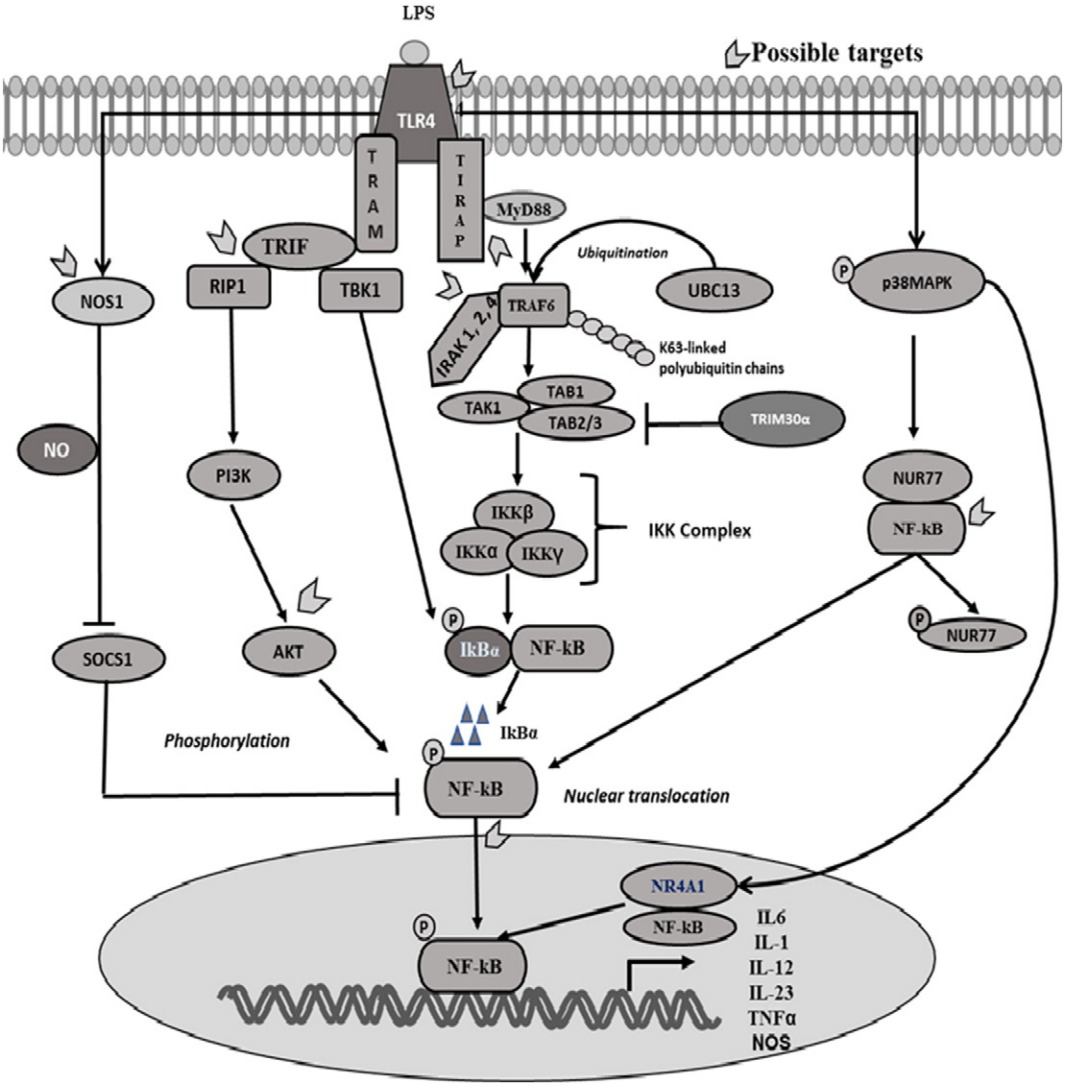

Fig. 1. Overview of TLR4-mediated NF-κB hyper inflammatory signaling pathway. LPS recognition is facilitated by TLR4 which leads to recruitment of various adaptor proteins like TRIF/TRAM and TIRAP/MyD88 receptor complex. The signaling bifurcates into MyD88-dependent and MyD88-independent pathways which converge to mediate the activation of pro-inflammatory cytokine. In MyD88-dependent pathway upon stimulation, IRAK-4, IRAK-1, and TRAF6 are recruited to the receptor. IRAK-4 then phosphorylates IRAK-1. Phosphorylated IRAK-1, together with TRAF6, dissociates from the receptor and then TRAF6 interacts with TAK1, TAB1, and TAB2, which induces the activation of TAK1. Activated TAK1 phosphorylates the IKK complex, consisting of IKKα, IKKβ, and NEMO/IKKγ, and thereby induces phosphorylation of IκBα followed by its degradation to release free NF-κB for nuclear translocation. On the other hand in MyD88-independent pathway, TBK1 along with TRIF and RIP1 form a signaling complex leading to degradation of IκBα and activation of PI3K/AKT pathway respectively. Moreover, p38MAPK phosphorylate NUR77, which in the bound state with NF-κB prevent its DNA binding ability. SOCS1 inhibits the NF-κB phosphorylation and thus its nuclear translocation. But NOS1 derived NO, leads to nitrosation of SOCS1 followed by degradation resulting in NF-κB phosphorylation and pro-inflammatory cytokines production.

an NF-κB-dependent mechanism [27,28]. Youn and his group have shown that TBK1 along with TRIF and RIP1 form a signaling complex (TBK1/TRIF/RIP1) and consequent downstream regulation of IκBa degradation [29].The same result has been found by another research group that MyD88 independent pathway activates a novel serine/threonine kinase TANK-binding-kinase 1(TBK1) which induces S36 phosphorylation of IκBa, followed by its degradation and releasing free NF-Kb [30]. Thus, targeting RIP1 and TBK1 could be potential therapeutics target for NF-κB pathway.

Other than the members of the classical pathway, there exist various signaling molecules which culminate at signal nodes downstream of TLR4 to mediate NF-κB hyperinflammatory responses. Upon activation of inflammatory pathways, negative regulatory genes are triggered to maintain the homeostasis. Among these, Nur77 is an orphan nuclear receptor belonging to the nuclear receptor subfamily four groups A member 1 (NR4A) family, which expresses immediately after the NF-κB mediated inflammatory stimuli [31]. Nur77's direct interaction with p65 inside the nucleus prevents its association with IκB as well as binding to the DNA and results in impeding the NF-κB downstream gene activation. However, the interaction of p38 with Nur77 phosphorylates both Nur77 and p38, which hampers the Nur77-p65 interaction and activation of p38 and its downstream pro-inflammatory signaling cascade [7], respectively. In short, p38 assisted phosphorylation of Nur77 results in its degradation [32,33] and opened the way for NF-κB functioning. Intriguingly, from the study of Li et al. [7], treatment with a chemical compound named n-pentyl 2-(3,5-dihydroxy-2-(1-nonanoyl)-phenyl)acetate (PDNPA) revoked the interaction between Nur77 and p38 by competing with p38 for its ligand binding domain, which stabilizes the anti-inflammatory caliber of Nur77. This poses Nur77 to be a novel target for inhibition NF-κB mediated signaling. On the other hand, proinflammatory mediators like nitric oxide (NO), Tumor necrosis factor (TNF-α) and Interleukin-1 (IL-1) released by the activated macrophage immediately upon the stimulations such as infection or tissue injury are also the essential modulators for inflammatory advancement [34]. Till date, numerous studies had shown that the inducible nitric oxide synthase (iNOS) or nNOS mediated release of NO proceeds the inflammatory phenotype of macrophages and thereby worsen the survival rate during sepsis [8,35,36]. The recent finding demonstrates that NOS1-derived NO regulates TLR4-mediated inflammatory gene transcription, as well as the intensity and duration of the resulting host immune response [36].

3. AP-1 and upstream targets

AP-1 is one of the other major transcription factors activated by the TLR mediated signaling pathways in macrophages. Ligation of TLRs with their stimulants elicits the signaling cascades, which activates innate effector response together with the initiation of control signals to adaptive immune response [37]. The TLR mediated signaling starts with the cytoplasmic recruitment of adaptor proteins such as TIRAP, MyD88, TRIF, TRAM and SARM via TIR-TIR interaction. MyD88 recruitment is necessary for all the TLR signaling cascade except TLR3, where TRIF mediated pathway is observed. On the other hand, TLR-4 mediates both MyD88 and TRIF dependent signaling. MyD88 and TRIF are recruited into the cytoplasmic domain of TLR-4 with the help of bridging adaptor proteins TIRAP and TRAM respectively [38]. A study by Loiarro and colleagues proved MyD88 as potential therapeutic target by targeting it with novel synthetic peptide-mimetic compound (ST2825). The compound inhibited MyD88 dimerization and interfere with IRAK recruitment, thus reducing AP1 activation [39]. MyD88 dependent pathway for the AP-1 activation is similar to that we explained in the NF-κB and upstream target section until the TAK1 activation (Fig. 2). TAK1 activates both IKK complex, which leads to the activation of NF-κB, and MAPK family like extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and protein 38 (p38), leads to AP-1 activation [40]. Eventually, MAPK leads to the activation and nuclear translocation of AP-1 subunits such as c-fos, c-jun, ETS domain-containing protein (ELK-2), and Activating transcription factor 2 (ATF-2) and it's binding to DNA responsive elements, which leads to the initiation of proinflammatory cytokine release in macrophages [41,42]. On the other hand, MyD88 independent pathway, which is TRIF mediated pathway. The TLR-4 stimulation activates the TRIF, and its subsequent binding to TRAF6 leads to the recruitment and activation of TAK1 [43]. Further, the TAK1 phosphorylates the MAPKs for AP-1 activation as explained in the MyD88 mediated pathway. However, the fifth adaptor protein named SARM is known to play a negative regulatory role of AP-1 mediated pathway [44] (Fig. 2). Although SARM is a cytosolic TIR-containing protein, it does not activate NF-κB, like the other adaptor proteins. Earlier, SARM was reported to be targeting and inhibiting the TRIF mediated activation of NF-κB. However, no effect was seen on MyD88 mediated pathway [45].

Later, according to the report of Peng et al. [44], SARM is involved in both TRIF as well as MyD88 mediated AP-1 inhibition via hampering MAPK phosphorylation under the LPS stimulation in human macrophages, however, function of SARM in the mouse may differ. Furthermore in their report, the regulatory role of N-terminal polybasic motif and its significance on SARM function in humans has been clearly described. N-terminal deletion restricted nuclear localization of SARM, resulted in its augmented inhibitory effect on AP-1 in human macrophage [44], which suggest SARM as a potential target to activate in inflammatory diseases in both cases of MyD88 independent and dependent activation of NF-κB and AP-1. As upstream machinery, MAPKs (including ERK, p38, and JNK) play a crucial role, and therefore a vital target for therapy, in AP-1 mediated inflammation in macrophages. Kinases such as MAPK, MAPK Kinase (MAPKK) and MAPK Kinase Kinase (MAPKKK) readily activate ERK, JNK, and p38 in monocytes under the LPS stimulation. The LPS induced activation of tyrosine kinase phosphorylates the TEY motif of ERK in the raf-1 dependent manner [46]. Further, the inhibition of ERK with its specific inhibitors showed significant downregulation of proinflammatory cytokines and prostaglandin E2, which indicated the role of ERK pathway, is independent of the other two MAPKs [47].

Similarly, JNK pathway is also activated rapidly with the endotoxin stimuli. MAPKKKs, MKK4, and Mkk7 are the direct activators of JNK, and the activated JNK phosphorylate the N-terminus of c-jun and other transcription factors (including ATF-1 and ELK-2), which regulate proinflammatory gene regulation [48]. Likewise, p38, which contains TGY motif in its kinase domain, is reported to be activated by various MAPK molecules like TAK1, ASK1, MKK3, MKK6 and so on. p38, in turn, activates certain transcription factors namely ATF-2, Elk-1, CHOP, MEF2C and Sap1a [46], as well as a series of downstream kinases which regulate the function of transcription factors such as cAMP response element-binding protein (CREB) and ATF-1 [49]. Also, use of the inhibitory p38 molecule, such as SB203580, inhibited the p38 mediated proinflammatory cytokine response, however, the mRNA accumulation of respective cytokine was not interrupted [50], further confirming the role of p38 in their translation and activation of downstream AP-1 (Fig. 2).

4. STAT-1 and upstream targets

Signal transducer and activators of transcription (STAT) are a family of transcription factors that mediate antiviral functions of the immune system through interferon signaling. Interferon-dependent signaling involves three categories of interferons: Type I interferon (IFNα/β) produced mostly by all stimulated cell types including macrophages [51], Type II interferon (IFNγ) produced predominantly by cells of the immune system, however, receptor for IFNγ (IFNGR1/2) are widely expressed in variety of cell types including macrophages [52] that are capable of responding to IFNγ [53] and Type III interferons (IFNλ) [54] composed of three members IFNλ 1–3 (IL29, IL-28A, and IL28B) produced mainly by epithelial cells and other cell types of immune system

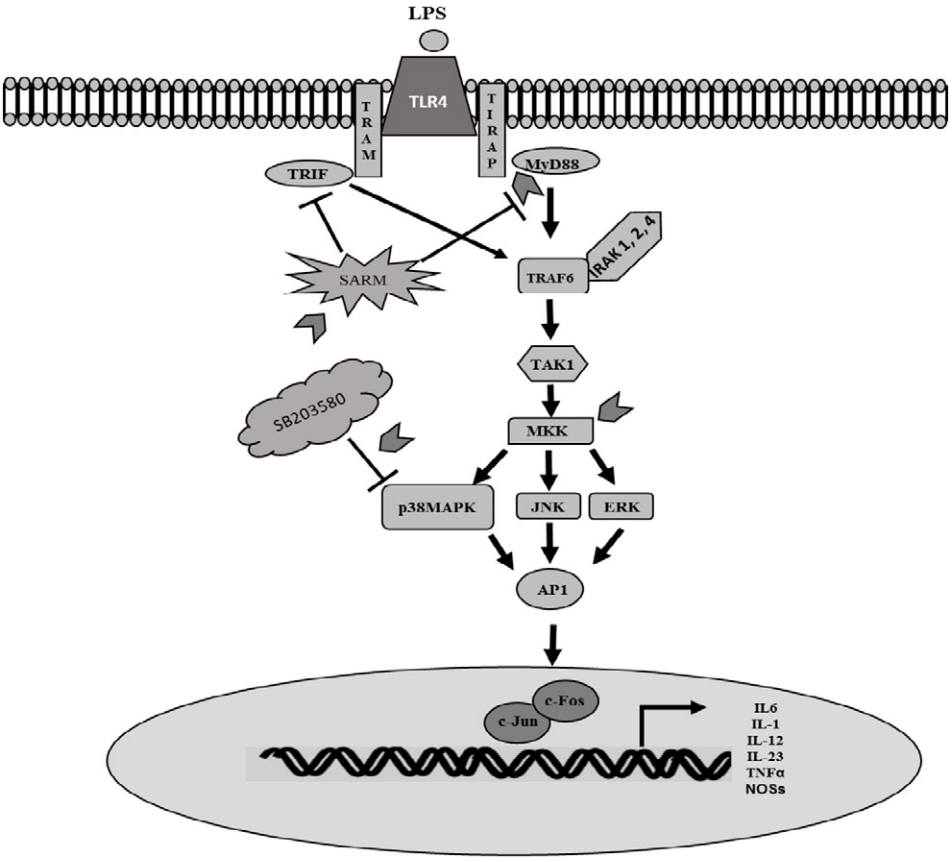

Fig. 2. TLR-4 mediated intracellular AP-1 inflammatory signaling pathway. Stimulation of extracellular TLR4 triggers and recruits intracellular adaptor proteins TIRAP and TRAM. Each of these adaptors activates MyD88 and TRIF, respectively, where both of these can be responsible for the activation of TRAF6/IRAK complex. In turn, phosphorylation of TAK1 leads to MKK activation, which leads AP-1 transcription factor to bind to DNA and elicit proinflammatory cytokine gene expression. Arrows indicate the possible therapeutic targets.

including macrophages and dendritic cells [55,56,57]. The tissue-specific expression of IFNλ is primarily due to the presence of IL-28A receptor subunit IL-28Rα on epithelial cells of gastrointestinal, reproductive tracts and other immune cells [58]. Studies on alveolar macrophages of COPD patients revealed interesting facts about macrophage priming that up-regulates TLR4 mediated signaling and STAT1 activation in response to IFNγ. Also, inhibition of STAT1 repressed the IFNγ mediated TLR4 expression in alveolar macrophages. Thereby proposing inhibition of IFNγ signaling using JAK and STAT1 inhibitors as a therapeutic target in COPD patients [59]. STAT1 and 2 belong to the 7 membered STAT family [60]. All interferon types function primarily through activation of STAT1 and STAT2 transcription factors. Receptors of IFN I and III, known as IFNAR and IFNλR are associated with kinases JAK1 and Tyk2 [61]. Further, STAT1 and STAT2 are phosphorylated at Y660 and Y701 respectively leading to activation of both STAT1 and STAT2 and further resulting in STAT1/2 heterodimer formation. These heterodimers then bind to p48 (DNA binding protein), thereby forming a transcriptional complex interferon-stimulated gene factor 3 (ISGF3). ISGF3 binds to IFN-stimulated response elements (ISREs) at the promoter site of IFNα/β inducible genes. However, studies also suggest that STAT1/2 heterodimer alone binds to GAS in the target genes to stimulate the gene expression without an involvement of p48 [62].

However, binding of IFN Type II to its receptor IFNγR causes its downstream association with kinases JAK1 and JAK2 that further phosphorylate STAT1 alone, forming STAT1 homodimer which translocate to the nucleus to reprogram target gene expression. STAT1 activation also depends on IFN Type II (IFNγ) mediated signaling [63]. Activated JAKs phosphorylate IFNγR1 at tyrosine Y440, which creates the binding site for STAT1 through its Src-homology 2 (SH2) domain [64]. Furthermore, JAK 1 and 2 phosphorylate STAT1 at tyrosine 701 [65], following which STAT1 forms homodimer via reciprocal interaction between phosphotyrosine of one and SH2 domain of other and further translocate to nucleus through GTPase-dependent Ran/TC4 mechanism [66]. Subsequently, phosphorylated STAT1 homodimer drives the expression of IFNγ responsive genes by binding to their gamma activated sequence elements (GAS) [67,68]. As shown in Fig. 3, binding of IFN Type II (IFNγ) to IFNγR receptor, results in auto and trans-phosphorylation of JAK1 and JAK2, which targets phosphorylation and activation of PI3K signaling pathway that subsequently activate kinase Akt causing phosphorylation and dimerization of STAT1 that further migrates to nucleus and drives the expression of interferon-stimulated genes. Interestingly, studies on STAT1 −/− mice that fail to trigger response to IFNα/β or IFNγ provide evidence for the significant role of STAT1 in IFN dependent signaling and subsequent inflammatory response [69].

Recently in vivo studies have demonstrated that STAT1 rapidly undergoes phosphorylation at Ser727 upon LPS challenge through TLR4 induction in a model of LPS hypersensitivity [70]. The study showed that membrane-bound TLRs: TLR2 and TLR4 induced Ser727 phosphorylation of STAT1 via MyD88 and TRIF dependent signaling but independent of interferon receptor complex (Fig. 3). This suggests a cross-talk between TLR's and JAK/STAT signaling pathways. STAT1 Tyr701 phosphorylation occurs downstream of type I IFN signaling that leads to STAT1 and STAT2 heterodimer. However, TLR4 downstream STAT1 homodimerization occurs via Ser727 phosphorylation. Moreover, Ser727 phosphorylation of STAT1 in IRF3/IRF7 −/− macrophages, shows deficient production of IFN type 1. Also IFNAR1 deficient macrophages shows post TLR4 stimulation with LPS suggesting that TLR4 activation is independent of IFN type I pathway. Moreover, blocking of Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), a downstream target of TLR4 signaling using its known inhibitor LY294002 blocked TLR4 induced STAT1 tyrosine phosphorylation, which further prevents STAT1 homodimer formation and therefore downstream inflammatory

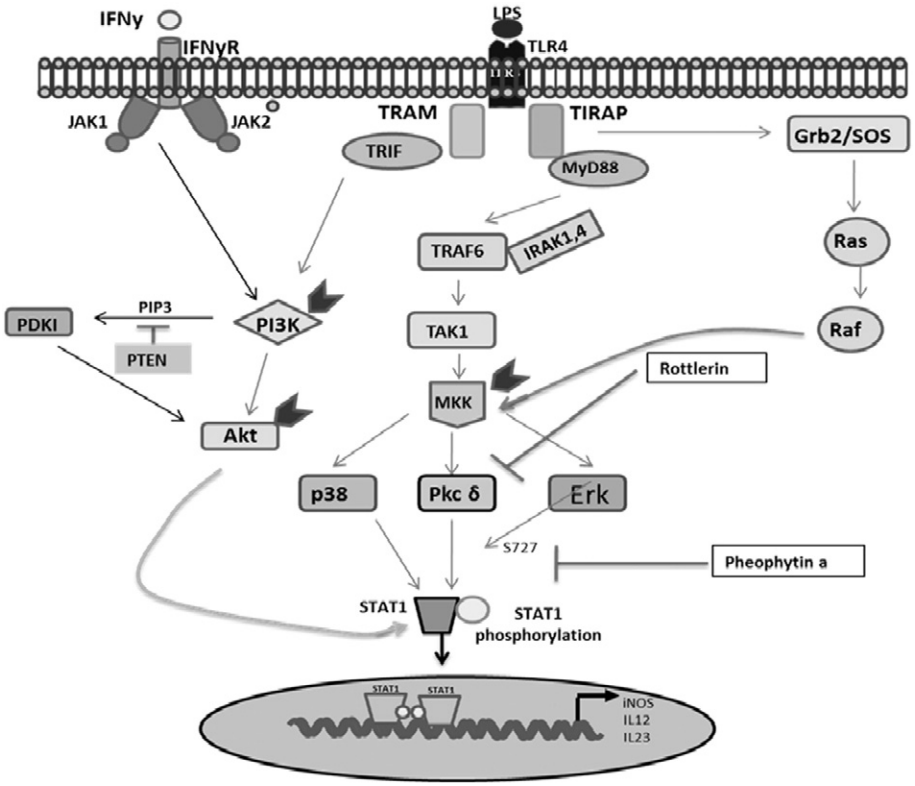

Fig. 3. TLR-4 mediated intracellular STAT1 inflammatory signaling pathway. LPS triggered TLR4 stimulates adapter molecules TRIF and TRAM, which then initiates the cascade of activation of downstream MAP kinases including p38, pkc, and Erk which phosphorylate STAT1 aiding its nuclear translocation and inflammatory response. Activation of TLR4 by LPS or interferon activates intracellular STAT1 signaling pathway. Receptors for IFNγ associate with kinases JAK1 and Tyk2, which activate PI3K signaling pathway causing downstream activation of Akt which then phosphorylate STAT1 causing its homodimerization and nuclear translocation. Thereby evoking the downstream inflammatory response. Arrows indicate the possible therapeutic targets.

signaling. Since MAP Kinases are important regulators of STAT1 signaling, significant focus has been made on inhibitors that target different MAP kinases. p38 MAP kinase inhibitor SB203580 blocked the serine-727 phosphorylation of STAT1, thereby preventing STAT1 mediated downstream signaling [71]. A polyphenol based rottlerin inhibitor of protein kinase c-delta demolished the Ser727 STAT1 phosphorylation [72]. Downstream, MAP kinase also induces Ser727 phosphorylation of STAT1, leading to its further homodimerization and nuclear translocation [73]. These findings strongly suggest that downstream of TLR4, MAP kinases p38 and pKC-δ appear to be indispensable for inducing Ser727 STAT1 phosphorylation in macrophages.

Adaptor proteins MyD88 and TIRAP mediate signaling via engagement of TLR4 receptor. The role of MyD88 has been investigated by studies on MyD88 deficient mice, which upon LPS induction induced rapid STAT1 Ser727 phosphorylation, indicating that additional signaling component TRIF might be involved in STAT1 serine phosphorylation independent of MyD88 via TLR4 [74]. Cross talk between STAT1 and PI3K/Akt signaling has been well studied. Exposure of HBMEC (Human brain microvascular endothelial cells) to HIV-1 mediated phosphorylation of PDK1 (Phosphoinositide-dependent kinase-1) at Ser241, Akt at Thr308 and STAT1 at Ser727 also enhanced the ISRE/GAS promoter activity [75]. These results suggested that activation of STAT1 could occur through a distinct mechanism mediated via P13K and PDK1 dependent signaling pathway. Since PI3K activation also occurs downstream of TLR4 signaling, it allows implications for the TLR4 dependent activation of PI3K which further activates Akt that targets STAT1 for phosphorylation triggering its homodimerization and nuclear translocation. This provides a potential of PI3K, PDK and Akt as therapeutic targets during STAT1 signaling. Moreover, Erk MAP kinase is known to phosphorylate STAT1 at Ser727 directly through the TLR4 pathway [76,77]. Recent studies have shown that pheophytin downregulates the transcriptional activity of inflammatory mediators such as NOS2 and COX2 by blocking Erk and STAT1 pathway [78].

Stimulation of biliary epithelial cell (cholangiocytes) with LPS activates NRas oncogene with the involvement of EGFR (Epidermal growth factor receptor). Recent reports suggest that inflammatory pathway triggered by LPS and TLR4 promotes cleavage of EGFR (Epidermal growth factor receptor) ligand AREG (Amphiregulin). Post cleavage, AREG promotes EGFR phosphorylation and recruitment of adaptor protein Grb2 (Growth factor receptor-bound protein 2) along with Guanine nucleotide exchange factor SOS1 (Son of sevenless homologue 1). It then stimulates the signaling through GTP-bound N-Ras (neuroblastoma-rafs oncogene) which further activates MAP kinase kinase, promoting the expression of pro-inflammatory cytokines via activation of STAT1 [79,80]. This study suggests a novel mechanism of STAT1 activation via TLR4 involving EGFR and downstream MAP kinase, indicating the therapeutic relevance of downstream MAP kinases in inflammatory diseases.

STAT1 mediated signaling pathway enhances the expression of pro-inflammatory genes such as iNOS which result in more NO production and subsequent amplification of the inflammatory response. Being a key regulatory pathway, STAT1 signaling receives further regulation by SOCS and protein tyrosine phosphatases such as SHP1 and SHP2 by dephosphorylating the IFN receptor and associated JAKs [81]. Since protein tyrosine kinases are crucial for activation of STAT1 pathway, proteins that negatively regulate kinase activity have become the focus of investigators. SOCS proteins downregulate STAT1 signaling by various mechanisms like direct binding and inhibition of JAKs tyrosine kinase activity [82], through ubiquitin-mediated proteasomal degradation of STATs [83] and by impeding the binding of STAT1 to phosphotyrosine

binding site of JAK2 [84]. In addition to SOCS, specific protein inhibitors of activated STATs (PIAS) disrupt the STAT1 mediated gene transcription by inhibiting the STAT1-DNA binding activity [85].

In addition to JAK/STAT1 pathway, IFNγ also activates other signal transduction pathways such as PI3K/Akt and p38 MAP kinase, which cross talk with each other to amplify the inflammatory response [71]. Studies report that p38 MAP kinase is required for STAT1 serine phosphorylation (Ser727) and transcriptional activation induced by interferons [73]. Activation of cytosolic phospholipase A2 (cPLA2) is essential for IFNα and IFNγ signaling. p38 MAP kinase activates cPLA2 which is indispensable for the formation of functional ISGF3 in response to IFN signaling. Another report suggests that p38α is required for ISRE and GAS driven gene transcription in response to Type I interferon (IFNα/β) but it does not modulate type II interferon-dependent gene transcription. IFN stimulation activates p38 and induced rapid ser727 phosphorylation, which was disrupted in the presence of p38 inhibitor but not by Erk1/2 inhibitor, indicating that p38 is actively involved in activation of STAT1. Interestingly, from the same report, the use of active mutant of MKK6 (mitogen-activated protein kinase kinase 6), an upstream target of p38 enhanced the STAT1 homodimer formation, contrary to its inhibition in presence of p38 inhibitor, suggesting strongly that p38 has a significant role in STAT1 phosphorylation and downstream transcriptional control of IFN inducible genes. Summarizing the known mechanism of STAT1 signaling upon LPS or interferon challenge suggests that modulation of STAT1 activity is a critical factor for fine-tuning the intensity of inflammatory response. It is, therefore, important to understand the STAT1 signaling pathway as a therapeutic target in various pathological conditions leading to chronic inflammatory disorders.

5. IRFs and upstream targets

Interferon regulatory factors (IRFs) are the family of transcription factors that commonly encompasses nine members in the mammalian system: IRF1, IRF2, IRF3, IRF4, IRF5, IRF6, IRF7, IRF8, and IRF9 [73,86]. Each IRF contains well-conserved novel helix-turn-helix DNA-binding motif and induces antiviral immunity. IRFs have identified primarily in the research of the type I IFN system and had been shown to have functionally diverse roles in the regulation of the innate and adaptive immune responses. IRFs are thought to be responsible for virus or TLR ligand–induced induction of type I interferon. Antiviral pattern recognition receptor TLR4 activates IRFs when they get a stimulus of LPS [87,86]. Upon binding of an individual ligand, TLR4 signals through MyD88-independent and MyD88-dependent pathway to activate IRFs. As depicted in Fig. 4, in MyD88 independent pathway LPS stimulation activates TLR4, which further activates intracellular TIR domain that conveys downstream signals by recruiting adaptors like TRIF and TRAM [8]. TLR4 activate the adaptor protein TRIF [88,89]. TRIF contains an N-terminal domain that includes the TIR domain necessary for interaction with TLRs and a C-terminal RIP homotypic interaction motif, which can activate cell death pathways. TRIF binds TBK1 also known as NAK through NAK-associated protein 1 (NAP1) and TRAF3. Two IKK-related kinases, TANK-binding kinase 1 (TBK1) and inducible IKK (IKKε), are involved in phosphorylation and activation of IRF3 [90,91]. TBK1/IKKε carries out C-terminal phosphorylation of IRF3 and IRF7 on serine/threonine residue. TBK1 belongs to the family of IκB kinase (IKK) and is expressed ubiquitously. Phosphorylated IRFs forms homodimers or heterodimers and get translocated to the nucleus and associates with the co-activator CBP/p300 [90].

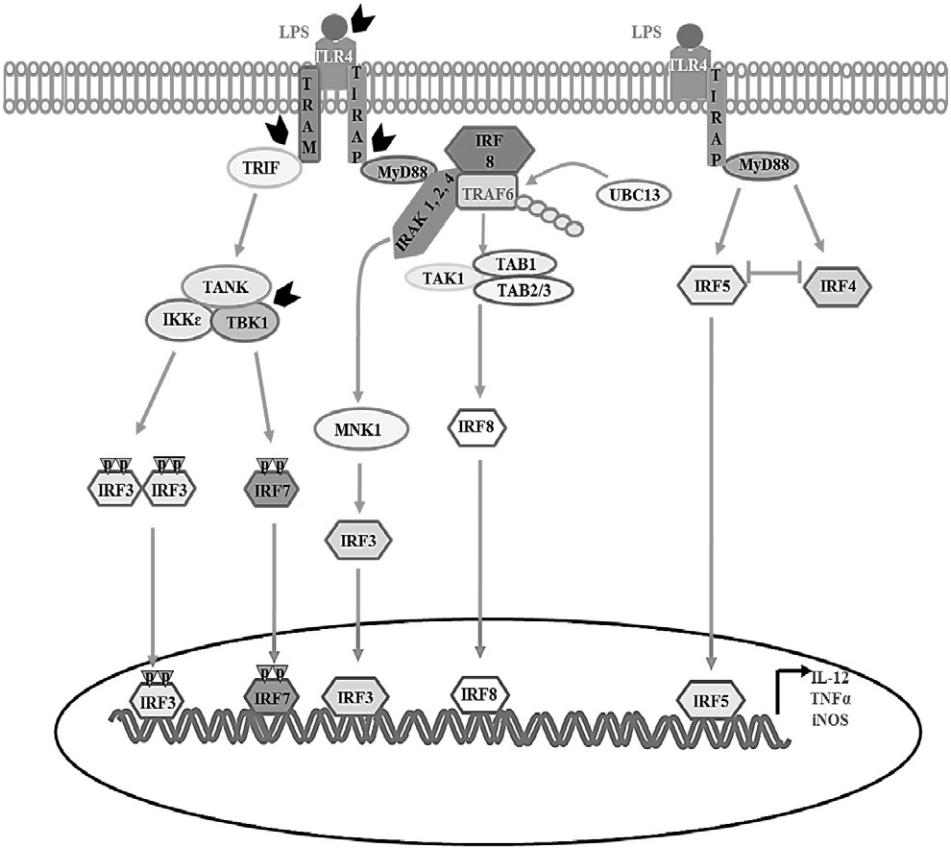

Fig. 4. TLR4 mediated intracellular IRF inflammatory signaling pathway. Activated TLR4 stimulates intracellular TIR domain that recruits adapter molecules TRIF and TRAM. TRIF binds TBK1/IKKε which then initiates the cascade of activation and dimerization of IRF3 and IRF7 in the nucleus. Through MyD88 pathway IRF5, IRF3 and IRF8 get activated. MyD88 is recruited to the TLR4 after activation, which activates IRAK1 and IRAK4 kinase, which associate with TRAF6. TRAF6 inhibit proteasomal degradation of IRF5. IRF8 also binds to TRAF6 and regulates its ubiquitination. TRAF6 ubiquitination enhanced by IRF-8 is important for MAP kinase activation. These IRFs have binding site ISRE on various proinflammatory genes leading to their expression. IRF4 also get activated on LPS stimulation but it regulates the expression of IRF5.

Without any stimulus IRF3 constitutively shuttles in and out of the nucleus, but phosphorylated IRF3 in association with CBP/p300 is retained within the nucleus. Inside the nucleus IRFs dimers binds to interferon-stimulated response element (ISRE) consensus motifs present in the promoters region of IFNβ and other genes (CXCL10, CCL5, ISG15, IFIT1, Arginase II, TNFα, IL-6, etc.) leading to activation of innate immune defense mechanism [92,93,94,95]. Genes activated by IRFs do autocrine and paracrine signaling that amplify the downstream targets of IFN-stimulated genes (ISGs). Secreted IFNβ triggers expression of IRF7 via the Jak-STAT signaling pathway [96]. Thus, newly synthesized IRF7 is thought to participate in the induction of IFNα. Given that TRAF6 also binds TRIF, synthesized IRF7 might be recruited to the TRAF6-TRIF complex where it is phosphorylated by TRIF-associated TBK1 and IKKε [43]. Further, studies will be needed to clarify this point. IKKε is playing a role in the development of inflammatory diseases, so IKKε could be considered as a therapeutic target. IKKε and TBK1 have overlapping functions in inflammatory signaling pathways, hence inhibiting activity of both kinases may be a better therapeutic approach. After TLR4 stimulation with LPS, IRF4 expression levels are also increased. IRF4 may act as a negative-feedback regulator of inflammatory pathway because it is known to inhibit activation of IRF5 by competing for interaction with MyD88 adaptor [97]. Binding of IRF4 to MyD88 increased competition for IRF5 to bind on it leading to the reduction of IRF5 activation and nuclear translocation. IRF4 and IRF5 share the same binding site on MyD88, which is distinct from that for IRF7 binding sites. IRF4 inhibits IRF5 dependent proinflammatory genes activated through TLR4 and controlled the expression of M2 macrophage markers that are anti-inflammatory. This pathway can be used for development of a proper therapeutic strategy for inflammatory diseases [98].

IRF5, transcription factor have diverse functions, such as the activation of genes encoding inflammatory cytokines such as tumor necrosis factor (TNF), IL-12, IL6, and IL-23 [99]. Human IRF5 is expressed as distinct splice variants depending upon the type of cells. According to cells, IRF5 shows specific expression, cellular localization, regulation and functions [98]. Unlike IRF3 and IRF7, IRF5 act as a master transcription factor downstream of the MyD88 signaling pathway in the activation of proinflammatory cytokines genes. MyD88-IRF5 pathway is activated in macrophages with stimulation of TLR4 by LPS. MyD88 is recruited to the TLRs leading to activation of IRAK1 and IRAK4 kinase, and association with TRAF6. TRAF6 is a K63-specific ubiquitin ligase that inhibits proteasomal degradation of IRF5 [100], and regulates signal transduction [101]. TRAF6-mediated K63-linked ubiquitination is essential for nuclear translocation of IRF5 and target gene regulation. Activation of IRF5 by MyD88 involves the formation of a complex consisting of IRAK4, IRAK1, and TRAF6 [102]. Within the complex, IRF5 undergoes K63-linked ubiquitination on lysines at positions 410 and 411, which are located in the TRAF6 binding motif of IRF5. TRAF6-dependent ubiquitination of IRF5 is a critical event in the regulation of IRF5 function [103]. Association of MyD88 with IRAK1 activates IRF5 by phosphorylation. Phosphorylated IRF5 translocate to the nucleus and binds at ISRE motifs, predominantly conserved in promoter region of genes encoding for inflammatory cytokines. In vivo study in IRF5-deficient mice showed that the induction of IL-6, TNFα, and IL-12p40 was partially defective when stimulated with LPS [97]. But the loss of IRF5 does not affect the viability of macrophages after LPS stimulation. Therefore, IRF5 specifically plays a role in the induction of proinflammatory cytokines through TLR4 pathway. Therefore, IRF5 can be a potential target for therapeutic interventions aimed at controlling inflammatory response. Modulation of IRAK4 kinase activity can be a therapeutic approach for the treating inflammatory diseases through TLR4.

IRF8 transcription factor is expressed specifically in the cells of hematopoietic origin and is induced by IFNγ and LPS at the protein and mRNA level in macrophages. IRF8 protein that is expressed downstream of TLR4 signaling depends on Notch-Recombining binding protein suppressor of hairless (Notch-RBP-J) Notch-RBP-J signaling pathway. RBP-J regulates translation of IRF8 by modifying TLR4 signaling pathway that depends on IRAK2. Activated IRAK2 recruits different kinases MKK3/6, ERK, p38, mitogen-activated protein kinase-activated protein kinase 2 (MK2) and MAP kinase-interacting serine/threonine-protein kinase 1(MNK1) and various factors (TRAF6, eIF4E, and TTP) that are required for cytokines mRNA stabilization and translation initiation [104]. So, TLR signaling are integrated at the level of IRF8 protein synthesis that induces proinflammatory macrophage polarization [105]. RBP-J the key nuclear transducer in Notch signaling that amplifies TLR4-induced expression of crucial mediators of activated proinflammatory macrophage and thus induces innate immune responses against *Listeria monocytogenes*. Upon TLR4 stimulation, IRF8 binds to TRAF6 and regulates its ubiquitination. TRAF6 ubiquitination enhanced by IRF8 is critical for MAP kinase activation. It has been studied that the activation of ERK and JNK kinases is significantly impaired in macrophages from IRF8-deficient mice [106]. IRF8 can be targeted as a potential therapeutic molecule to regulate immune responses. IRF-8 is essential for IL-12 and iNOS gene activation [105]. After activation of macrophages, IRF8 is recruited to the proximal promoter of IL12b and iNOS. The iNOS promoter contains a novel ISRE where IRF8 plays an activating role [107]. After binding of IRF8 to its target gene promoters, RNA polymerase II is recruited for transcription initiation [108]. Association of TRAF6 with IRF8 and IRAKs pathways opens possibilities as potential therapeutic strategies to combat inflammation. As discussed previously activated TBK1 along with IKKε directly phosphorylate IFN regulatory factors 3 and 7 (IRF3 and IRF7) and hence are potential direct targets for inflammation. TBK1 kinase inhibition can lead to the downregulation of expression of TBK1 inducible proinflammatory genes such as TNFα, IL6, IP-10, IFNβ [109]. The summarized TLR4 mediated signaling pathways representing the cross talk among different pathways is shown in Fig. 5.

6. Conclusion

There is a high potential for targeting NF-κB, AP-1, IRF3 and STAT1 pathways and their upstream targets for the treatment of inflammatory diseases against endotoxin shock. In instances where inflammatory response becomes chronic or dysregulated, NF-κB is an obvious target for new types of treatment. Inhibition of IKK's phosphorylation, proteasomal degradation of IKB and blocking NF-κB nuclear translocation has emerged as a potential target to silence NF-κB-mediated hyper immune response. But inhibition of proteasome activity could potentially cause other side effects too. Also, it may be not feasible to block the NF-κB pathway for prolonged periods since NF-κB plays a crucial role in maintaining host immune response. However, short-term treatment with specific inhibitors of IKK activity might reduce possible side effects. Advancement in in-silico drug designing has produced many potent inhibitory drugs for AP-1 and upstream molecules. Regardless of the advances in high-throughput screening techniques and ligand-based molecular modeling which have identified numerous compounds capable of inhibiting AP-1 transcription activators, only one selective AP-1 inhibitor T-5224 has been investigated in phase II human clinical trials. So, there is an urgent need to yield more potent and specific AP-1 inhibitors as a sustainable therapeutic strategy for use in the human clinical conditions.

In this review, we had summarized various treatments down-regulating one of these transcriptional factors which may offer further opportunity to better counteract the inflammatory diseases. Downstream of TLR4 pathway, mitogen-activated protein kinase kinase (MKK), targets another MAP kinase in the pathway for phosphorylation and subsequent activation, including p38, PKcδ, and Erk, making MKK a potential target in STAT1 signaling pathway. STAT1 activation also initiates from IFNγ receptor amplifying viral mediated response through activation of PI3K and Akt kinase, which directly cause STAT1 phosphorylation and thereby its nuclear translocation and transcription of target genes. Here we review PI3K and Akt as important therapeutic targets in STAT1 signaling, thereby regulating the inflammatory response triggered IFNγR. Thus, the delineation of specific roles for

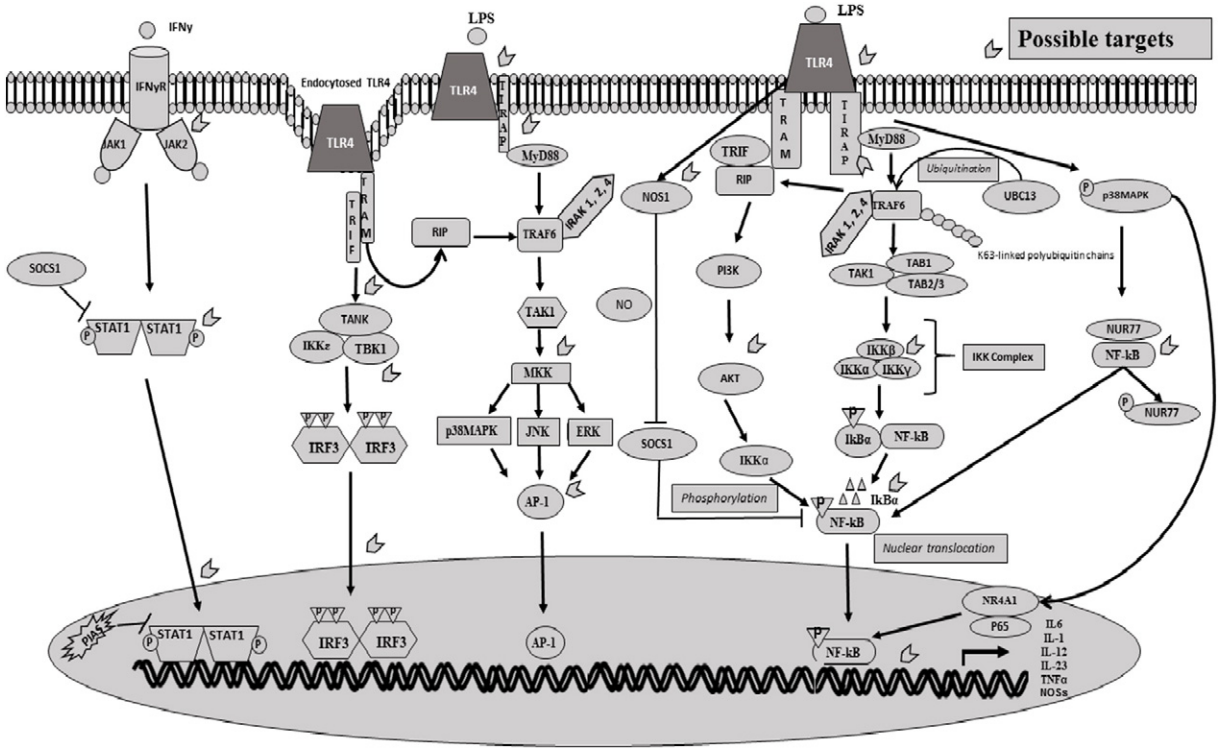

Fig. 5. Summarized TLR4 mediated signaling. Pathogen recognition initiates a signaling cascade. The signaling mechanism for NF-κB and AP1 is same upto TAK1 phosphorylation and then bifurcates. Also MyD88 independent pathway activates RIP which plays role in activation of both NF-κB and AP1.·Thus, there exist cross talk among pathways leading to complicating the signaling mechanism.

particular cytokines and the development of cytokine-directed therapeutics should become areas of intense investigation through major signaling pathways including NF-κB, AP1, IRFs, and STAT1, to treat chronic inflammatory diseases.

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