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The Yin and the Yang of STAT1 Downstream of TLR4 Endocytosis: STAT1 beyond Interferon Signaling

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Abstract

Lipopolysaccharide (LPS)–induced toll-like receptor 4 (TLR4) endocytosis has emerged as a key step for the production of interferon (IFN)-β, which activates the transcription of antiviral response genes through Janus kinase (JAK)/pTyr^{701} signal transducer and activator 1 (STAT1) signaling. TLR4 endocytosis also promotes proinflammatory cytokines production, at least in part through mediating a late-phase of nuclear factor (NF)-κB activation. However, NF-κB activation alone cannot explain the full spectrum of how TLR4 endosomal signaling conduits the production of proinflammatory cytokines. Our study identified STAT1 as a proinflammatory effector downstream of TLR4 endocytosis independent of IFN-β signaling or NF-κB activity. In human macrophages, TLR4 endocytosis activates noncanonical phosphorylation of STAT1 at Thr^{749} (pThr^{749}), which subsequently promotes the proinflammatory response rather than the IFN response. pThr749 STAT1 prolongs the half-life of interleukin (IL)-6 mRNA through activating the transcription of AT-rich interactive domain-containing protein 5A (ARID5A), which stabilizes IL-6 mRNA. Furthermore, pThr^{749} STAT1 promotes a late-phase of the transcription of IL-12. We demonstrated that pThr^{749} confers STAT1 with distinct gene-regulatory prosperities and facilitates STAT1 binding to a noncanonical DNA motif (5'...TTTGANNC...3') at the promoter regions of ARID5A and IL-12. Our results indicate that different phosphorylation of STAT1 confers distinct DNA binding and gene regulation downstream of TLR4 endocytosis where pTyr^{701} promotes the IFN response while pThr^{749} promotes the proinflammatory response. By unveiling an alternative activation of STAT1, our study adds another piece to the puzzle of how TLR4 endocytosis regulates the production of proinflammatory cytokines, which may help in developing biologics or diagnostics for proinflammatory diseases.

Commentary

Developing defense mechanisms by the host is fundamental to ensure its survival against various microbial pathogens. At the heart of the host defense against microbes is its ability to initiate an immune response to detect and eliminate potential microbial threats. However, in many cases the aberrant immune response is the cause of the host's clinical symptoms of infections rather than the microbe itself [1]. Therefore, understanding the mechanisms governing the initiation and the regulation of the host's immune response against various microbial encounters is critical for our understanding of the host-microbe interaction. Over three decades ago, Charles Janeway Jr. proposed a model of pathogen detection describing two characteristics of innate immune receptors: first, the ability to distinguish between self and non-self molecules, and second is the ability to promote adaptive immune response to the non-self microbial products [2]. Toll-like receptors (TLRs) were the first among many other innate immune receptors to fulfill Janeway's prediction. TLRs were discovered as the human homolog of *Drosophila* Toll protein and were subsequently identified for their ability to recognize conserved pathogens-associated molecular patterns (PAMPs) followed by driving an innate immune response and adaptive immunity [3]. TLR4 was the first member to be characterized, followed by the identification of bacterial lipopolysaccharide (LPS) as the microbial ligand activating the TLR4 [3-6]. TLRs are type I transmembrane proteins, which share conserved functional domains. The N-terminal extracellular domain consisting of leucine-rich repeats in a horseshoe-like structure for ligand recognition, a single transmembrane domain and an intracellular Toll-interleukin (IL)-1 receptor (TIR) domain for signaling transduction [7-9]. TLRs

can be classified according to their cellular localization into cell surface or intracellular TLRs. Cell surface TLRs are located at the plasma membrane and recognize microbial cell surface molecules such as TLR1, 2 and 6 (bacterial lipoprotein), TLR4 (LPS) and TLR5 (flagellin) [10-15]. On the other hand, intracellular TLRs localize in endosomes and detect nucleic acids such as TLR3 (double-stranded RNA), TLR 7 and 8 (single-stranded RNA) and TLR9 (unmethylated CpG containing single-stranded DNA) [16-22]. This compartmentalization of TLRs is fundamental for the specificity of their ligand recognition and the subsequent engagement of specific adaptor molecules that initiate signaling cascades and culminate in an appropriate immune response [23,24].

This paradigm is clear in the case of TLR4, which exploits different adaptors to induce distinct signaling pathways, thus expanding the repertoire of transcribed genes and potentiating the production of a wide array of immune mediators. Among these immune mediators is IL-6, which is a pleotropic cytokine with diverse effects on immune and non-immune cells, and affects the host homeostasis [25-27]. Thus, coordinated regulatory mechanisms exist to regulate TLR4 driven production of immune mediators, especially IL-6. At the plasma membrane, TLR4 recognizes LPS through a multi-receptor complex of LPS-binding protein (LBP), CD14 and MD2, which triggers TLR4 dimerization [28-33]. Dimerized TLR4 at the plasma membrane interact with a sorting adaptor, TIR Domain Containing Adaptor Protein (TIRAP), which recruits the signaling adaptor protein Myeloid differentiation primary response 88 (Myd88) [34-38]. Myd88 conduits TLR4 surface signaling as a part of a large oligomeric supra-molecular organizing center (SMOC) called Myddosome consisting of oligomers of TLR4, TIRAP, Myd88 and IL-1 receptor-associated kinases (IRAKs) [39-43]. The Myddosome through IκB kinases (IKK) and mitogen-activated protein kinases (MAPK) signaling activates nuclear factor-kappa B (NF-κB) and activator protein 1 (AP-1), respectively, culminating in the transcription of a multitude of proinflammatory cytokines such as tumor necrosis factor (TNF), IL-12 and IL-6 [44-47]. Although activating the transcription of proinflammatory cytokines is a key step for initiating the immune response, non-transcriptional regulation is critical for tailoring the immune response and prevent aberrant production of these cytokines. A clear example of the Myddosome-dependent non-transcriptional regulation is the regulation LPS stimulated IL-6 and IL-12, conceivably because of their important role in driving the proinflammatory response and shaping the adaptive immunity [25,48]. At the resting state, Regnase-1 prevents aberrant production of IL-6 and IL-12, but not TNF, by targeting their mRNA for degradation [49]. Upon LPS stimulation the IKK complex phosphorylates the DSGXXS motif of Regnase-1 resulting in its degradation and subsequently promotes IL-6 and IL-12 mRNA stability and production [50].

The association of activated TLR4 with CD14 promotes its endocytosis, which is clathrin- and dynamin-dependent [51,52]. From endosomes, TLR4 dimers interacts with another sorting adaptor called TRIF-related adaptor molecule (TRAM), which seeds the formation of another SMOC, the Triffsome that initiate TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling. TRIF mediates the activation of IKK-related kinase ε (IKKε) and TANK-binding kinase (TBK1), which phosphorylate and activates the transcriptional regulator interferon regulatory factor 3 (IRF3) and the subsequent expression of genes encoding type I interferons (IFNs) [53]. Binding of type I IFNs to their receptor (IFNAR) on the same cell (autocrine) or adjacent cells (paracrine) activates Janus kinase 1 (JAK1) and tyrosine kinase 2 (Tyk2), which in turn promotes Tyr^{701} phosphorylation of signal transducer and activator 1 (STAT1), a key step for its transcriptional activity [54]. This phosphorylation event results in the binding of STAT1 with STAT2 and IRF9 to form the heterotrimer called IFN-stimulated gene factor 3 (ISGF3) complex, which binds to IFN-stimulated response element (ISRE) sites and initiates the transcription of multiple IFN-stimulated genes important for antiviral response [55]. Moreover, TRIF–mediated type I IFNs production promotes Caspase-11–dependent NLRP3 inflammasome activation followed by cell death and the release of IL-1β and IL-18 [56]. Although type I IFNs are fundamental for initiating the antiviral immune response, their contribution to host defense against bacterial pathogens is elusive, with increasing evidence showing that they are dispensable for the production of proinflammatory cytokines [57-60]. Although TRIF signaling promotes proinflammatory cytokine production through sustaining a late-phase activation of NF-κB [61,62], several observations have challenged this idea and showed that neither TRIF deficiency nor interfering with TLR4 endocytosis affects the kinetics of NF-κB activation [63-65]. Thus, it remains unclear how TLR4 signaling from endosomes promotes the production of proinflammatory cytokines.

In contrast to IFN-$pTyr^{701}$ STAT1, STAT1 deficiency results in diminished production of IL-6 and enhanced survival in response to bacterial infections [66,67], indicating that the role of STAT1 extends beyond that of its Tyr^{701} phosphorylation in the context of shaping the proinflammatory response. Our group has identified AT-rich interactive domain-containing protein 5a (Arid5a) as a post-transcriptional stabilizer of IL-6 mRNA through counteracting the Regnase-1 effect [68]. Notably, Arid5a/Regnas-1 regulation extends beyond IL-6 to

other immune modulatory molecules such as OX40 [69]. Deficiency of Arid5a phenocopied that of TRIF and STAT1 in enhancing mice survival in murine endotoxic shock model [70], denoting a potential signaling pathway connecting these molecules. In this regard, we found that TLR4 endocytosis promotes the formation of a noncanonical TBK1/IKKβ kinase complex, which in turn mediates a noncanonical STAT1 phosphorylation at Thr^{749}. Intriguingly, $pThr^{749}$ STAT1 augments the TRIF-dependent macrophage proinflammatory cytokine production through distinct mechanisms independently of its $pTyr^{701}$ or the NF-kB activity. Of note, phosphorylation of Thr^{749} did not affect STAT1 nuclear translocation. Instead, it facilitated STAT1 binding to a noncanonical DNA motif (5'-TTTGANNC-3') at the promoter regions of ARID5A and IL-12 resulting in augmented production of IL-12 and IL-6 through augmenting their transcription and mRNA stabilization, respectively [71]. Collectively, our study highlights the importance of the spatiotemporal regulation of TLR4 signaling and its impact on mediating differential phosphorylation of STAT1 resulting in altering its DNA binding specificity and transcriptional outcome. Thus, our study provides a potential mechanistic explanation of how TLR4 signaling from endosomes promotes proinflammatory cytokines production independent of NF-κB activation. It requires future research for better understanding of the TLR4 proinflammatory endosomal signaling and to answer whether the balance between $pTyr^{701}$ and $pThr^{749}$ STAT1 dictates the fate of the macrophage immune response towards antiviral or proinflammatory, respectively; what is the *in vivo* biological effects of the $pThr^{749}$ STAT1 on the host's immune response; and, what are the molecular mechanisms regulating this phosphorylation.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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