

BMB Reports – Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-16-059

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Article Type: Mini Review

Keywords: Glycogen synthase kinase 3 β (GSK3 β); Toll-like receptor (TLR);
Inflammatory cytokines; Type I interferons; IFNs

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Running Title: The role of GSK3 β in TLR-mediated responses

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ABSTRACT

Toll-like receptors (TLRs) play a critical role in the innate immune response against pathogens. Each TLR recognizes specific pathogen-associated molecular patterns and then activates adaptor protein MyD88 or TRIF-assembled signaling complex to produce immune mediators, including inflammatory cytokines and type I IFNs. Although the activation of TLR is important for host defense, its uncontrolled activation can damage to the host. During the past decade, numerous studies have demonstrated that GSK3 β is a key regulator of inflammatory cytokine production in MyD88-mediated TLR signaling via TLR2 and TLR4. Recently, GSK3 β has also been implicated in TRIF-dependent signaling pathway via TLR3. In this review, we describe current advances on the regulatory role of GSK3 β in immune responses associated with various TLRs. A better understating of the role of GSK3 β in TLR signaling might lead to more effective anti-inflammatory interventions.

INTRODUCTION

Innate immune system is the first line barrier of host defense during pathogen infection and is also critical for effective adaptive immune system. Toll-like receptors (TLRs), highly conserved type I transmembrane pattern recognition receptors (PRRs), are expressed on antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs). There are 12 members of TLR family in mammals, and each TLR recognizes highly conserved structural motifs, known as pathogen-associated molecular patterns (PAMPs) from various pathogens (1, 2) or danger-associated molecular patterns (DAMPs) that are endogenous molecules released from necrotic or dying cells. The engagement of TLRs by PAMPs activates multiple signaling pathways to induce specific immune mediators, including inflammatory cytokine and type I interferons (IFNs) to eliminate pathogens. Upon ligands binding to TLRs, TIR domain-containing adaptor proteins such as myeloid differentiation factor 88 (MyD88) and toll-interleukin 1 receptor (TIR) homology-domain-containing adaptor-inducing interferon- β (TRIF) are recruited to the TLR signaling complex. All TLRs except TLR3 activate the MyD88-dependent pathway (3, 4). TLR-MyD88 signaling complex recruits interleukin-1 receptor-associated kinase (IRAK) family, which results in activation of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (5). TRAF6, an E3 ubiquitin ligase, activates downstream transducer molecule transforming growth factor β -activated kinase 1 (TAK1) complex via lysine (K)-63 chain ubiquitination (6). TAK1 mediates mitogen-activated protein kinases (MAPKs) and I kappa B kinase (IKK) activation, which lead to the activation of activator protein 1 (AP1) and nuclear factor-kappa B (NF- κ B), respectively (4, 5, 6). TLR3 and TLR4 induce the TRIF-dependent pathway (3, 4). TLR-TRIF signaling complex recruits downstream adaptor molecules, including TRAF6, TRAF3, and receptor-interacting protein 1 (RIP1), which results in activation of IFN regulatory factor 3 (IRF3), AP1, and NF- κ B (4, 7,

8). Although these TLR signaling pathways are critical for host cell defense, uncontrolled or excessive activation of TLRs can also cause inflammatory host cell damage, such as autoimmune disease and chronic inflammation (1, 3). Thus, TLR signaling pathways must be tightly controlled.

Glycogen synthase kinase 3 (GSK3) was originally identified as an enzyme that regulates glycogen metabolism (9). GSK3 is a highly conserved and ubiquitously expressed serine/threonine kinase. Mammalian GSK3 has two isoforms, GSK3 α and GSK3 β , encoded by two distinct genes, *gsk3 α* and *gsk3 β* . Although both two isoforms are structurally similar, they are not functionally identical (10, 11). GSK3 is involved in various cellular functions, including embryonic development, cell differentiation, cell proliferation and cell death (12, 13, 14). GSK3 is constitutively active under basal conditions, whereas inhibited by phosphoinositide 3-kinase (PI3K)-Akt or MAPKs pathway through serine phosphorylation (Ser21 for GSK3 α and Ser9 for GSK3 β) (15). In addition, tyrosine phosphorylation of GSK3 (Tyr279 for GSK3 α and Tyr216 for GSK3 β) promotes its activity (16). During the past decade, numerous studies have demonstrated that the regulatory roles of GSK3 β in innate immune responses, especially TLR signaling pathways (17, 18, 19). Although GSK3 has been reported as a key mediator of MyD88-dependent cytokine production in TLR signaling against bacterial infection, little is known about the regulatory roles of GSK3 in viral infection. Recently, we demonstrated that GSK3 β plays a role in TLR3-mediated pro-inflammatory cytokine production by promoting the TRIF-assembled signaling complex (20).

In this review, we first look at current knowledge about the involvement of GSK3 in TLR-mediated immune responses in innate immune cells. In addition, we focus on the regulatory role of GSK3 β in TLR3-mediated immune response triggered by viral infection.

GSK3 β IN TLR4 RESPONSES

TLR4, the first reported mammalian TLR, is the most well characterized PRR that mainly recognizes lipopolysaccharide (LPS) on the cell surface from Gram-negative bacteria (21, 22). TLR4 mediates both MyD88- and TRIF-dependent signaling pathways to produce inflammatory cytokines and type I IFNs (Fig. 1). The engagement of TLR4 by LPS activates MyD88-dependent pathway through TIR domain containing adaptor protein (TIRAP), and then recruits downstream IRAK family and TRAF6 (4, 5). TRAF6 interacts with TAK1, TAK1-binding protein 1 (TAB1) and TAB2, and catalyzes K63-linked ubiquitination, resulting in the phosphorylation and activation of TAK1 (6). Activated TAK1 phosphorylates canonical IKK complex and MAPK, and thereby activates transcription factors NF- κ B, CREB and AP1, which lead to the regulation of pro- and anti-inflammatory cytokines production (Fig. 1). Phosphoinositide 3-kinase (PI3K) is also involved in TLR4 signaling pathways (23). LPS stimulation activates PI3K-Akt pathway to regulate inflammatory genes (24).

Several groups have reported that activity of GSK3 β regulates pro- and anti-inflammatory cytokine production in TLR4 responses. Martin *et al.* initially demonstrated the regulatory roles of GSK3 β which is involved in diverse TLR-mediated inflammatory cytokine production (25). LPS stimulation leads to the phosphorylation and inactivation of GSK3 β through the PI3K-Akt-dependent pathway. In GSK3-inactivated human monocytes using pharmacological inhibitors or GSK3 β -deficient mouse embryonic fibroblast (MEFs), the levels of pro-inflammatory cytokines, such as IL-1 β , IL-6, IL-12 and TNF- α , were reduced, while the levels of anti-inflammatory cytokine IL-10 were enhanced. Moreover, GSK3 β modulated the transcriptional activity of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) to interact with nuclear coactivator CREB-binding protein

(CBP). In addition, a recent publication has reported that the ability of GSK3 β to control pro- and anti- inflammatory cytokine production is also regulated by mammalian target of rapamycin complex 1 (mTORC1) pathway in LPS-stimulated human monocytes (26). Studies by Nandan, *et al.* and Paul *et al.* further indicated that GSK3 β could also regulate IL-10 production via CREB against visceral leishmaniasis (VL) in murine macrophages and human macrophages (27, 28).

Apart from the above, it has been reported that GSK3 β was involved in various signaling mechanisms for cytokine production. For example, GSK3 β differentially regulated IL-1 β production and anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra) production, the endogenous IL-1 β inhibitor. Inhibition of GSK3 β activity or siRNA knockdown of GSK3 β augmented LPS-induced IL-1Ra production through ras-related C3 botulinum toxin substrate 1 (Rac1)-ERK axis in human monocytes (29). GSK3 β was also required for the synergistic action of IFN- γ on LPS-induced IL-6 production in murine macrophages (30). It has been reported that the priming of macrophages by IFN- γ , a macrophage activating factor, potentiates TLR-induced cytokine production. GSK3 β interacted with signaling transducer and activator of transcription 3 (STAT3) and positively regulated the activation of IFN- γ -induced STAT3. Moreover, recent studies demonstrated that GSK3 β was required for the ubiquitination and stabilization of apoptosis signal-regulating kinase (ASK1), which is an important mediator in TLR-mediated inflammatory cytokine production (31). These data suggest that GSK3 β as a potent mediator of inflammatory cytokine production in TLR4 signaling pathway.

TLR4 also utilizes TRIF-dependent pathway through TRIF-related adaptor molecule (TRAM) to produce IFN- β . Studies by Wang *et al.* indicated that GSK3 β negatively regulates

LPS-induced IFN- β production in vitro and in vivo (32). In both wild-type and MyD88-deficient macrophages, GSK3 β phosphorylation was induced after LPS stimulation. While pharmacological inactivation or siRNA knockdown of GSK3 β resulted in higher levels of IFN- β , forced expression of kinase-dead GSK3 β resulted in lower levels of IFN- β in response to LPS. Interestingly, GSK3 β modulated LPS-induced nuclear levels of c-Jun, but not NF- κ B or IRF3, to regulate IFN- β production. Together, these studies suggest that GSK3 β is necessary for TLR4-mediated inflammatory response.

GSK3 AND TLR2 SIGNALING

TLR2 is expressed on cell surface and forms heterodimers with TLR1 or TLR6. In general, TLR2 recognizes bacterial cell wall components, including peptidoglycan (PGN), lipoteichoic acid (LTA) and lipoprotein, and TIRAP and MyD88 were participated in signal transduction to produce pro- and anti-inflammatory cytokines (33, 34, 35).

Studies by Martin *et al.* showed that the involvement of GSK3 in TLR2-mediated cytokine production (25). Pharmacological inhibition of GSK3 resulted in higher levels of IL-1 β and IL-12p40 production, whereas lower levels of IL-10 production, in response to LTA. Subsequently, it was demonstrated that the regulatory roles of GSK3 β in IFN- γ and TLR2-mediated IL-10 production in human macrophages (36). IFN- γ enhanced TLR2-mediated GSK3 activity through PI3K-Akt pathway, and GSK3 suppressed IL-10 production by regulating transcriptional activity and expression of AP1. Zhang *et al.* investigated that the regulatory role of GSK3 β in *Francisella tularensis* infection in murine macrophages related to TLR2 signaling pathway (37). PI3K-Akt-GSK3 β axis differentially modulated pro- and anti-inflammatory cytokine production through TLR2-mediated NF- κ B and CREB activity in

response to *F. tularensis* live vaccine strain (LSV) stimulation. Similarly, GSK3 β regulated TLR2-mediated inflammatory response in murine microglial cells against *Staphylococcus aureus* infection (38). Inhibition of GSK3 β by pharmacological inhibitors reduced TNF- α production and inducible NO synthase (iNOS) synthesis through regulating NF- κ B activity. Interestingly, recent studies showed that GSK3 α and β isoforms differentially regulated *S. aureus*-induced IL12-p40 expression in endothelial cells (39). In stimulation of PGN from *S. aureus*, both isoforms of GSK3 were phosphorylated through TLR2-PI3K-Akt pathway. The levels of IL-12p40 were elevated in GSK3 α knockdown, while reduced in GSK3 β knockdown. Together, these studies suggest that GSK3 is required for TLR2-dependent immune response.

GSK3 β IN TLR3 SIGNALING

TLR3 recognizes the double-stranded RNA (dsRNA), an intermediate produced during the replication of many RNA viruses (40, 41, 42). TLR3 activation triggers signaling pathways through the sole adaptor TRIF to produce type I IFN and pro-inflammatory cytokines (Fig. 2). It has been reported that the TLR3-mediated IFN- β production was impaired in the TRIF knockout mice, suggesting that TRIF is essential for TLR3-mediated signaling pathway (43). The TLR3-TRIF signaling complex recruits downstream adaptor molecules, including TRAF3, TRAF6 and RIP1, which lead to the activation of transcription factors, such as IRF3, AP1 and NF- κ B (Fig. 2). It has been reported that TRAF3 is a critical linker between TRIF and tank-binding kinase 1 (TBK1)-IKK ϵ complex (7). It has been known that TBK1 directly phosphorylates IRF3, which enhances its dimerization and nuclear translocation, resulting in the production of IFN- β (42, 44, 45). RIP1 and TRAF6 are also recruited to TRIF and further interacts with downstream TAK1, which subsequently activates AP1 and NF- κ B, through

MAPKs and IKK α -IKK β complex, respectively (46, 47). While many studies have suggested that the regulatory roles of GSK3 β in TLR signaling, it is poorly understood how GSK3 β regulates TLR3 signaling.

Retinoic acid-inducible gene 1-like receptor (RLR)-mediated signaling pathway shares a number of components in common with TLR-mediated signaling pathways (48, 49). Studies by Lei *et al.* showed that GSK3 β positively regulated RLR-mediated IFN- β induction at the TBK1 level (50). GSK3 β but not GSK3 α modulated virus-triggered TBK1 auto-phosphorylation and self-association, which lead to the IRF3 activation and IFN- β induction independently of the kinase activity of GSK3 β . Consistently, our previous studies demonstrated that GSK3 β interacted with TRAF3 and positively regulated TLR3-mediated IFN- β production (20). The expression levels of IFN- β were reduced in GSK3 β knockdown murine macrophages or *Gsk3b*^{-/-} MEFs in response to poly I:C stimulation. Moreover, the poly I:C-induced IRF3 phosphorylation, nuclear translocation and dimerization, and TBK1 phosphorylation were reduced in *Gsk3b*^{-/-} MEFs compared with control cells. In contrast, neither GSK3 inhibition with SB216763 or knockdown of GSK3 α affected IFN- β expression in murine macrophages. These two studies suggest that GSK3 is required for both TLR3- and RLR-induced antiviral response, and the effects of GSK3 β on IFN- β production is independent of its kinase activity.

Although the mechanisms of TLR3 signaling leading to type I IFNs have been well identified, the mechanisms of inflammatory cytokine production were largely unknown. Recently, we demonstrated that GSK3 β had a positive role in regulating the pro-inflammatory cytokine production in TLR3 signaling (20). Knockdown of GSK3 β but not GSK3 α or inhibition of its kinase activity significantly reduced the poly I:C-induced pro-inflammatory cytokine production in murine macrophages. GSK3 β positively regulated poly

I:C-induced ERK and p38 phosphorylation, and thereby enhanced c-Fos expression. Interestingly, GSK3 β interacted with TRAF6 but not with RIP1, and underwent K63-linked ubiquitination at K183 by TRAF6. Moreover, GSK3 β ubiquitination potentiated the formation of the TRIF-mediated signaling complex. These results suggested that GSK3 β control TRAF6-TAK1-MAPK axis to regulate pro-inflammatory cytokine production, while TRAF3-TBK1-IRF3 axis to regulate IFN- β production. Altogether, these studies suggest that GSK3 β acts as a key regulator in TLR3-mediated antiviral responses.

CONCLUDING REMARKS

During the past decades, numerous studies have indicated a role for GSK3 β in TLR-mediated inflammatory response. In general, inhibition of GSK3 β differentially controls pro- and anti-inflammatory cytokine production by regulating CREB activity in MyD88-mediated TLR signaling pathways such as TLR2 and TLR4. In addition, GSK3 kinase activity is required for its role in inflammatory cytokine production. Whereas most studies of GSK3 β function have focused on its role in MyD88-mediated signaling pathways during bacterial infection, recent studies have been demonstrated that the roles of GSK3 β in TRIF-mediated antiviral responses. It has been reported that GSK3 β negatively regulates TLR4-mediated IFN- β production through transcription factor c-Jun. In contrast, our recent study has demonstrated that GSK3 β interacts with TRAF3 and acts as a positive regulator in TLR3-mediated IFN- β production through TRAF3-TBK1-IRF3 axis.

Interestingly, we also demonstrated that GSK3 β interacts with TRAF6 and its K63-linked ubiquitination by TRAF6 positively regulates TLR3-mediated pro-inflammatory cytokine production through TRAF6-TAK1-MAPK axis. Although further studies are needed to clarify the details about the regulatory mechanisms of TLRs signaling by GSK3 β , it is important that

GSK3 β has an ability to selectively regulate both MyD88- and TRIF-dependent pathway, suggesting that GSK3 β has a potential therapeutic target to treat several inflammatory states.

ACKNOWLEDGEMENTS

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2015R1A6A3A01020489) (to R.K.) and by the Ministry of Science, ICT and Future Planning (No. 2013R1A2A1A05005153; No. 2012R1A5A1048236) (to S.Y.L.).

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FIGURE LEGENDS

Fig. 1. GSK3 β in TLR4 responses. GSK3 β transcriptionally regulates pro- and anti-inflammatory cytokines and IFN- β production in TLR4 signaling pathway. In basal condition, GSK3 β is constitutively active. GSK3 β inhibits the binding of CREB to CBP, whereas promotes the binding of NF- κ B to CBP, leading to reduced anti-inflammatory cytokine production and enhanced pro-inflammatory production, respectively. GSK3 β also inhibits AP1, such as c-Jun and ATF2, leading to reduced IFN- β production. Upon increased PI3K activation, GSK3 β is phosphorylated and inactivated, leading to enhanced anti-inflammatory cytokine and IFN- β production and reduced pro-inflammatory cytokine production.

Fig. 2. GSK3 β in TLR3 signaling. GSK3 β positively regulates both pro-inflammatory cytokines and IFN- β production in TLR3 signaling pathway. Upon TLR3 activation, GSK3 β interacts with TRAF6-TAK1 and undergoes K63-linked ubiquitination, and further promotes AP1, such as c-Fos, leading to pro-inflammatory cytokine production. GSK3 β interacts with TRAF3-TBK1 complex and enhances TBK1 phosphorylation, and thereby IRF3 activation, leading to IFN- β production.

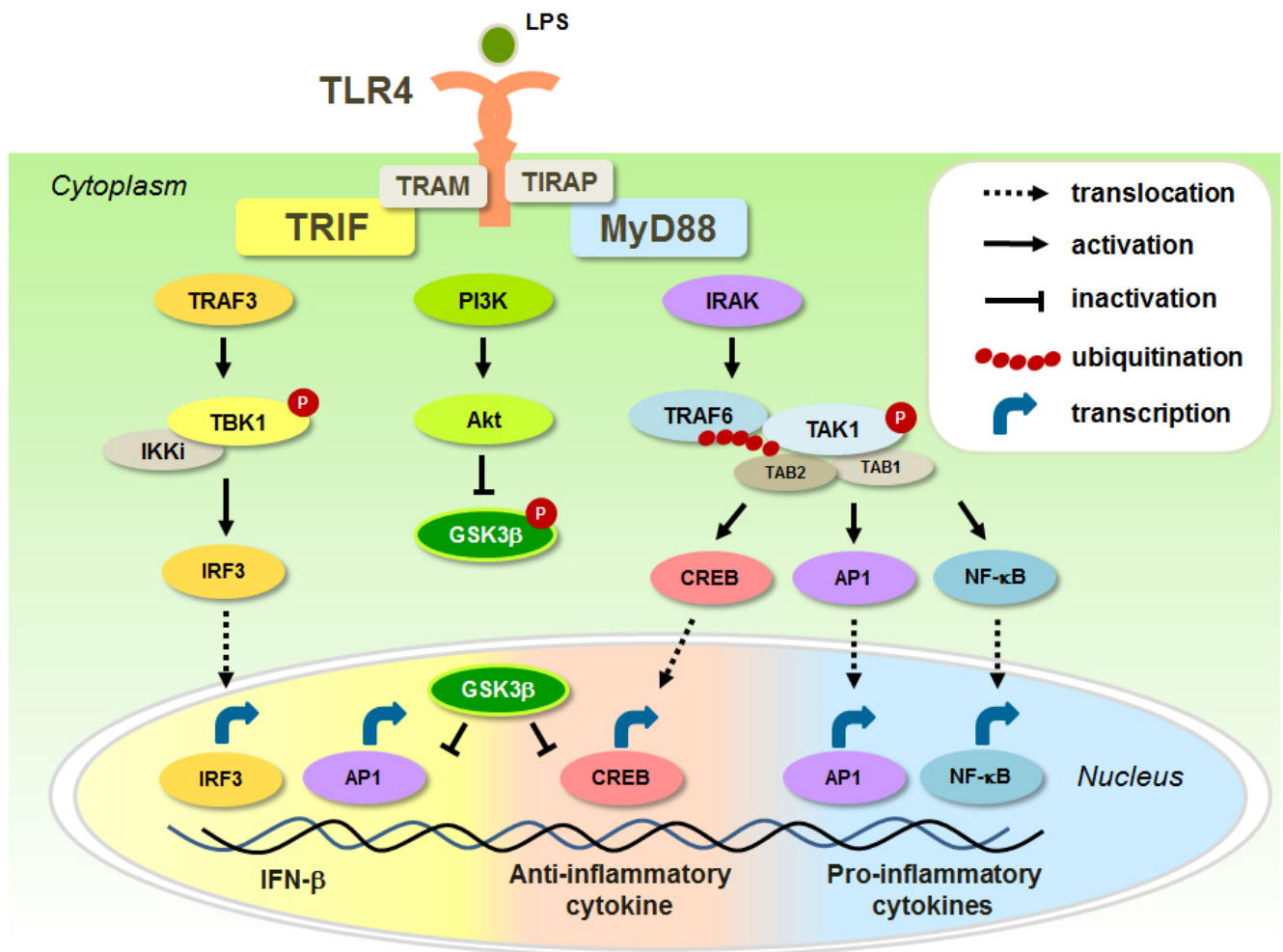


Fig. 1

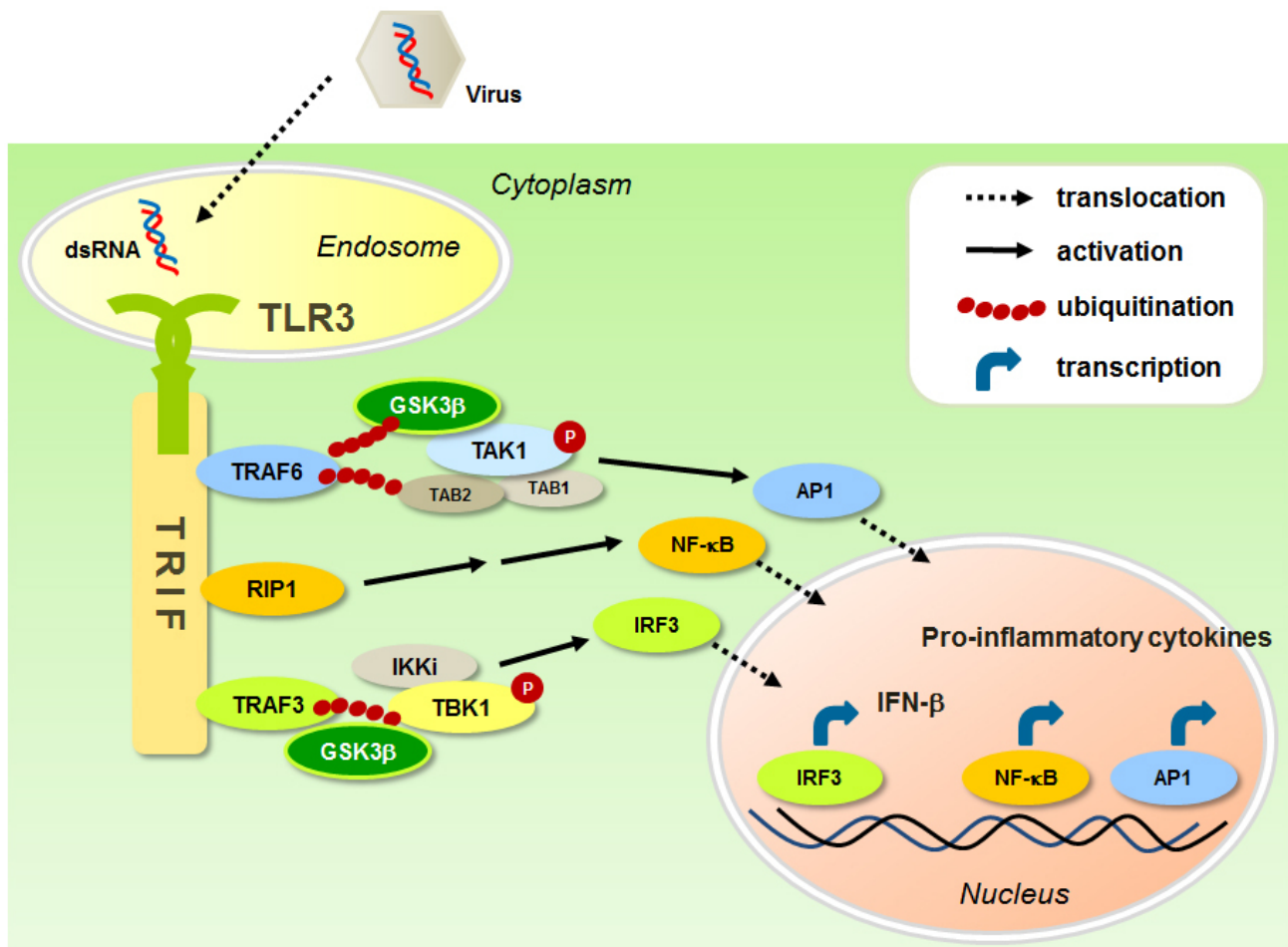


Fig. 2