Review

Nuclear Factor-κB: Activation and Regulation during Toll-Like Receptor Signaling

Ruaidhrí J. Carmody[1, 2] and Youhai H. Chen[1]

Toll-like receptors (TLRs) recognize distinct microbial components to initiate the innate and adaptive immune responses. TLR activation culminates in the expression of appropriate pro-inflammatory and immunomodulatory factors to meet pathogenic challenges. The transcription factor NF-κB is the master regulator of all TLR-induced responses and its activation is the pivotal event in TLR-mediated activation of the innate immune response. Many of the key molecular events required for TLR-induced NF-κB activation have been elucidated. However, much remain to be learned about the ability of TLRs to generate pathogen-specific responses using a limited number of transcription factors. This review will focus on our current understanding of NF-κB activation by TLRs and potential mechanisms for achieving a signal-specific response through NF-κB. *Cellular & Molecular Immunology*. 2007;4(1):31-41.

Key Words: NF-κB, toll-like receptor, signal transduction, innate immunity, transcription

Introduction

Toll-like receptors (TLRs) are key components of the innate immune defense and are critical in establishing adaptive immunity. Intense research into the biology of TLRs in recent years has revealed the ability of the innate immune system to recognize specific molecular patterns of microbes and to shape an immune response tailored to the invading microorganism. In addition to playing a key role in the innate immunity against various infectious agents, TLR signaling has also been implicated in the pathogenesis of a number of human diseases including sepsis, atherosclerosis, asthma and autoimmune disorders (1). These findings have led to the discovery of an expanded and diverse range of novel potential therapeutic targets. The function of TLRs as key initiators of the innate immune response relies on their ability to recognize microbial pathogens through molecules that are known as pathogen-associated molecular patterns (PAMPs). Recognition triggers a signaling cascade which culminates in the transcription of pro-inflammatory and immunomodulatory factors which initiate both innate and adaptive immunity (2). To date, at least 12 TLRs have been identified, some of which can recognize several structurally unrelated ligands and may be expressed on the cell surface or be intracellularly compartmentalized (Table 1). TLRs are expressed by various immune cells such as dendritic cells (DCs), macrophages, B cells and specific types of T cells as well as non-immune epithelial and fibroblast cells.

TLRs not only recognize pathogens but also elicit a specific transcriptional response tailored to the type of pathogen encountered. The ability to rapidly distinguish between Gram negative and Gram positive bacteria, between virus and fungi is critical for the optimal response of the innate immune system and the subsequent adaptive immune response. While pathogen recognition begins at the receptor level, it is the signaling components downstream of each receptor and the way they interact with each other that ultimately determines the specific transcriptional response. The transcription factor NF-κB was quickly recognized as the master regulator of TLR induced responses. All TLR signals converge on NF-κB and its activation is critical for TLR function. This review will focus on the activation of NF-κB by TLRs and how NF-κB may allow for pathogen-specific transcriptional responses.

NF-κB

Since its discovery some 20 years ago, the NF-κB family of proteins has become one of the most widely studied transcription factors (3). This is due in large part to its pleiotropic effects, its tightly regulated inducibility and its

[1]Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA;

[2]Corresponding to: Dr. Ruaidhrí J. Carmody, 713A Stellar Chance, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 422 Curie Blvd., Philadelphia, PA 19104, USA. Tel: +01-215-573-7389, Fax: +01-215-573-3434, E-mail: rcarmody@mail.med.upenn.edu

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Table 1. Recognition of pathogen associated molecular patterns (PAMPs) by TLRs

PAMP	Origin	TLR	Cellular compartment
Bacterium			
Lipopolysaccharide	Gram-negative bacteria	TLR4	Cell surface
Diacyl lipopeptides	Mycoplasma	TLR6/TLR2	Cell surface
Triacyl lipopeptides	Bacteria/Mycobacteria	TLR1/TLR2	Cell surface
Lipoteichoic acid	*Streptococcus B*	TLR6/TLR2	Cell surface
CpG DNA	Bacteria/Mycobacteria	TLR9	Intracellular
Flagellin	Bacteria	TLR5	Cell surface
Peptidoglycan	Gram-positive bacteria	TLR2	Cell surface
Porins	*Neisseria*	TLR2	Cell surface
Virus			
DNA	Virus	TLR9	Intracellular
dsRNA	Virus	TLR3	Intracellular/Cell surface
ssRNA	Virus	TLR7, TLR8	Intracellular
Hemagglutinin	Measles	TLR2	Cell surface
Parasite			
Zymosan	*Saccharomyces*	TLR6/2	Cell surface
Mannan	*Candida*	TLR4	Cell surface
Phospholipomannan	*Candida*	TLR2	Cell surface
Fungus			
Glycoinositolphospholipids	*Trypanosoma*	TLR4	Cell surface
Profilin-like molecule	*Toxoplams gondii*	TLR11	Cell surface
Hemozoin	*Plasmodium*	TLR9	Intracellular

emerging role in a wide range of human diseases. There are five NF-κB genes, *NFKB1, NFKB2, RELA, c-REL* and *RELB*, which in turn code for seven proteins: p105, p50, p100, p52, p65, c-Rel and RelB (Figure 1). p50 is generated from limited proteasomal processing of the p105 protein as is p52 from the p100 protein. The p50 and p52 proteins lack the transactivational domain found in the C-terminal region of the other NF-κB proteins. Their homodimers are considered repressors of transcription. All of the NF-κB proteins contain the so-called Rel homology domain (RHD) which comprises approximately 300 amino acids of the N-terminal region and is responsible for NF-κB dimerisation, DNA binding and interaction with the inhibitory IκB proteins (see below). Individual NF-κB proteins form hetero- and homo-dimers with other NF-κB subunits to produce 15 possible dimers. NF-κB DNA binding sites consist of 10 base pairs with the consensus sequence designated as 5'-GGGRNWYYCC-3', where R is a purine, N is any base, W is an adenine or thymine and Y is a pyrimidine. The number of NF-κB target genes is now in the hundreds (for an up-to-date list, see www.nf-kb.org), which include a large number of immuno-modulatory factors such as cytokines and chemokines. The significance of NF-κB in both the innate and adaptive immune systems has been highlighted through the generation of mice deficient in individual family members (4). Moreover, the relevance of NF-κB to human disease now extends beyond disorders of the immune system to include cancer and atherosclerosis (5, 6).

Core components of the NF-κB activation pathways

Although a diverse range of stimuli may activate NF-κB, almost all pathways converge on the same core components of the NF-κB activation apparatus, the IKK complex. The IKK complex represents a rapid response system for the induction of NF-κB, which in the uninduced state is maintained in the cytoplasm through interaction with the inhibitory IκB proteins. Activation of the IKK complex induces phosphorylation of the IκB proteins, leading to their K48-polyubiquitination and proteasomal degradation. The degradation of IκB protein allows for translocation of the liberated NF-κB dimers to the nucleus where they bind their cognate sites in DNA and activate gene transcription. Of note is the recent discovery that the retention of NF-κB in the cytoplasm by IκB proteins is not as passive as once believed (7). New findings have revealed a continuous nucleo-cytoplasmic shuttling of IκB/NF-κB complexes in unstimulated cells, with the majority of complexes present in the cytoplasm due to potent nuclear export signals in the IκB proteins overcoming the nuclear localization signals of the NF-κB subunits (8). The phosphorylation of IκB proteins is carried out by the IκB kinase (IKK) complex which is composed of four essential elements, IKKα, IKKβ, IKKγ/NEMO and the recently identified ELKS. IKKα and IKKβ are serine/threonine kinases which share high sequence

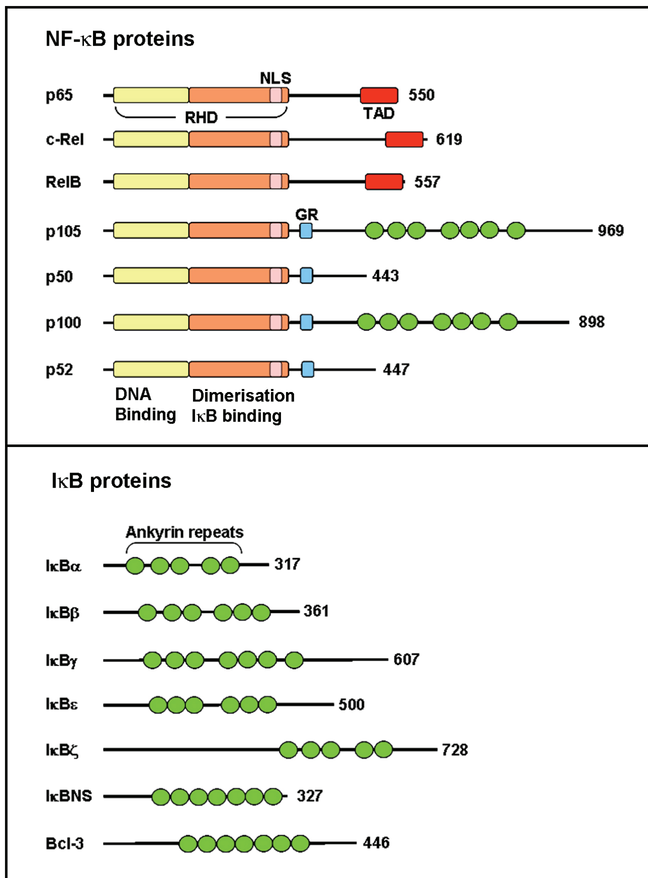

Figure 1. The NF-κB and IκB protein families. There are seven NF-κB proteins – p65, c-Rel, RelB, p105, p50, p100 and p52 – all of which share the Rel homology domain (RHD). The RHD is responsible for DNA binding, dimerisation and the binding of IκB proteins to NF-κB subunits. Only three NF-κB proteins contain transactivation domains (TAD) – p65, c-Rel and RelB – located in the C-termini. All NF-κB proteins contain a nuclear localization sequence which allows the translocation of NF-κB from the cytoplasm to the nucleus. A glycine rich region (GR) is present in the p105 and p100 proteins which signal their limited proteasomal processing to generate the p50 and p52 proteins, respectively. The transcriptional activity of NF-κB is regulated by the IκB proteins. The IκB proteins are characterized by the presence of a variable number of ankyrin repeats which mediate the interaction of IκB proteins with the RHD of NF-κB. IκBγ is the C-terminal region of p105, generated as an alternative splice product from the *NFκB1* gene. IκBα, IκBβ, IκBγ and IκBε reside primarily in the cytoplasm and function to prevent NF-κB translocation to the nucleus. Activation of NF-κB requires the proteasomal degradation of IκBα, IκBβ, IκBγ and IκBε to allow NF-κB translocation to the nucleus. NF-κB induces the expression of these IκB proteins which serves as an important negative feedback loop. IκBζ, IκBNS and Bcl-3 are predominantly nuclear in localization and are not degraded following NF-κB activation. The function of the nuclear IκB proteins appears to be in the regulation of NF-κB transcriptional activity, although the mechanism remains unknown.

homology (9). Phosphorylation of IKKα and IKKβ appears to be critical for their kinase activity and at least two essential phosphorylation sites, serines 177 and 181 of IKKβ corresponding to serines 176 and 180 of IKKα, have been identified in the kinase domain activation loop (10). Mutation of these serines to alanines abolishes the kinase activity and the NF-κB-inducing properties of both IKKα and IKKβ, while mutation to glutamate results in constitutive kinase activity and NF-κB activation (10). In addition, the N terminal region of both IKK kinases contains a stretch of serines which are highly phosphorylated during activation. The phosphorylation of these serines serves a negative regulatory function and appears to be IKK dependent (10). Recent studies have revealed that both IKKα and IKKβ are important in TLR-induced NF-κB activity (11) while IKKβ appears to be dispensable for the non-canonical or alternative pathway of NF-κB activation triggered by stimuli such as CD40L, lymphotoxin-β and BAFF, involving RelB/p52 heterodimers (12). Another conserved region in both IKKα and IKKβ that is essential for the interaction with IKKγ/NEMO has been termed the Nemo-binding domain (NBD) (13). Indeed, a cell permeable peptide encoding the NBD is sufficient to prevent NF-κB activation (13). This finding, along with other genetic studies (14) has clearly established IKKγ/NEMO as an essential component for the activation of NF-κB. The precise mechanism of IKKγ/NEMO function is unclear at present but it may serve to bring IKKα and IKKβ together so that they can phosphorylate each other (15). In addition to interacting with IKKα and IKKβ, IKKγ/NEMO also interacts with a number of proteins associated with NF-κB activation including RIP and A20 (see below) (16). Thus, IKKγ/NEMO may serve as a scaffold or platform for the interaction of modulators of NF-κB. Furthermore, recent studies have suggested IKKγ/NEMO phosphorylation, ubiquitination and sumolyation as important mechanisms in the regulation of IKK complex activity. The phosphorylation of IKKγ/NEMO has been reported to occur following TNF-α and IL-1 stimulation and appears to negatively regulate IKK activity. The phosphorylation occurs primarily on serine 376 and is IKKβ dependent, indicating that this may be an autoregulatory mechanism (17, 18).

The polyubiquitination of IKKγ/NEMO occurs through K63-linked chains and does not target the protein for degradation but instead appears to serve as a post-translational modification signal rather like phosphorylation. So far this polyubiquitination has been observed in the TNF-α (19), Nod2 (20) and T cell receptor pathways (21). Although its precise role remains uncertain, polyubiquitination of IKKγ/NEMO appears to be required for NF-κB activation in the above pathways. Interestingly, different lysine residues of IKKγ/NEMO appear to be targeted in different pathways, suggesting multiple pathways towards IKKγ/NEMO modification and possibly a central role for polyubiquitin of IKKγ/NEMO in the activation of NF-κB. The sumolyation of IKKγ/NEMO has been identified as an important mechanism for the activation of NF-κB in response to genotoxic stress (22). Sumolyation involves the covalent

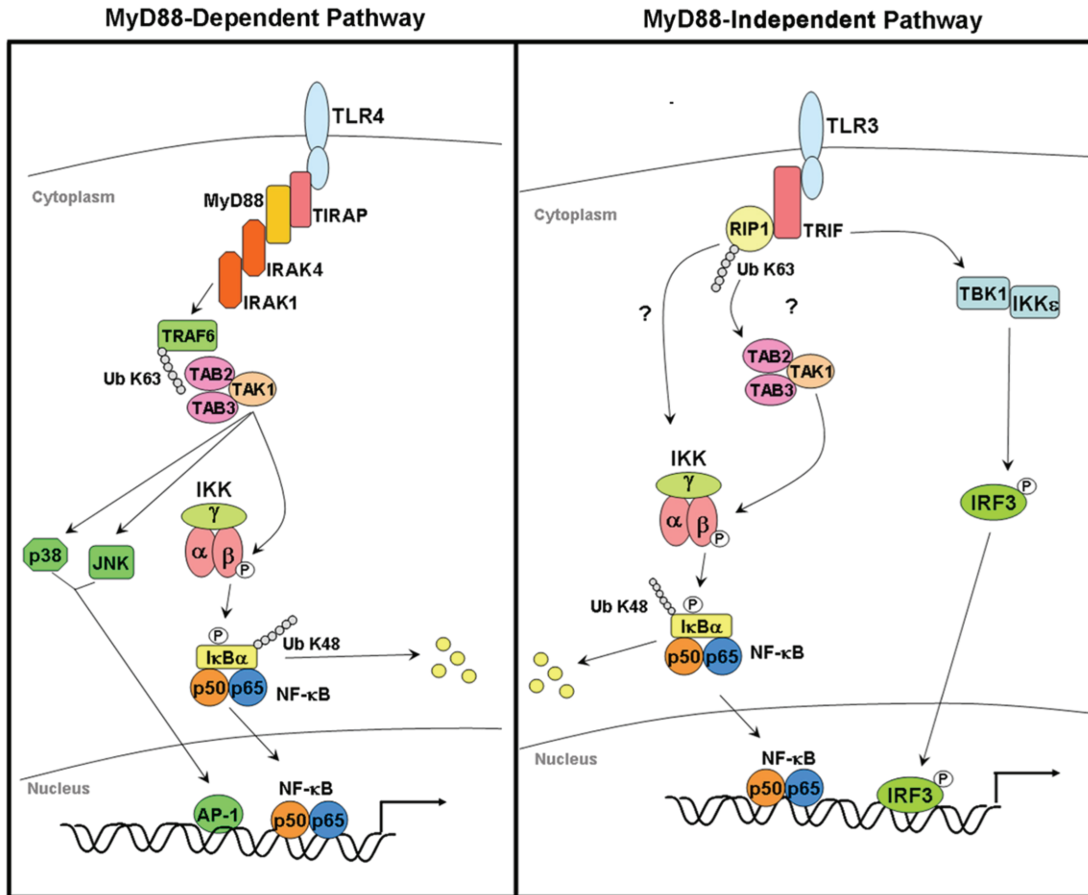

Figure 2. TLR activation of NF-κB. TLR activation of NF-κB occurs through MyD88-dependent and independent pathways. MyD88-dependent activation of NF-κB occurs following receptor induced association of MyD88 directly to the TIR domain of the TIR, or as in the case of TLR4, through the bridging adaptor TIRAP. This allows the formation of a complex which includes IRAK1, IRAK4 and TRAF6. K63-polyubiquitination of TRAF6 is critical for the binding of TAK1, TAB2 and TAB3. Activated TAK1 phosphorylates and activates IKKβ which in turn phosphorylates IκBα to signal for its K48-polyubiquitination and subsequent proteasomal degradation. TAK1 activity is also required for the activation of JNK and p38, leading to the activation of the AP-1 transcription factor. MyD88-independent activation of NF-κB requires the adaptor TRIF, which binds directly to TLR3 and uses the bridging adaptor TRAM to bind to the TLR4 receptor. TRIF recruits RIP1 which becomes K63-polyubiquitinated and requires TAK1, TAB2 and TAB3 to activate NF-κB, although the exact mechanism is unclear. In addition, TRIF activates the IKK related kinases IKKε and TBK1 which phosphorylate and activate the IRF3 transcription factor.

attachment of ubiquitin-related SUMO proteins to lysine residues in target proteins. In the case of NEMO, sumolyation induces its nuclear localisation and interaction with the DNA damage sensor ATM which then triggers IKKγ/NEMO mono-ubiquitination and nuclear export (23). The cytoplasmic IKKγ/NEMO and ATM complex then activates the IKK complex and NF-κB through an unknown mechanism. The ELKS component of the IKK complex is the most recently identified and appears to recruit IκBα to the IKK complex (24).

IκB proteins

Although most information regarding the activation of NF-κB by the IKK complex has been determined through analysis of IκBα, there are a number of IκB proteins which share sequence and structural homologies (Figure 1). These IκB proteins contain repeated sequences approximately 30 amino acids long, termed ankyrin repeats, which are essential for binding to NF-κB (Figure 1). Interestingly, these IκB proteins exhibit variable affinities for NF-κB subunits. IκBα and IκBβ preferentially bind c-Rel and p65 containing dimers (25-27), while IκBε only binds c-Rel and p65 complexes (28). The C-terminal portion of p105 is also transcribed as a distinct transcript termed IκBγ (29) and has been demonstrated to inhibit c-Rel, p65/p50 and p50 homodimers, while alternative spliced forms are specific for p50 dimers (30). Only the C-terminal regions of p100 and p105 are effective at binding RelB, and are critical in inhibiting the activities of RelB/p52 dimers (31). Three IκB proteins, IκBζ, IκBNS and Bcl-3, have been found to be

predominantly nuclear, and appear to regulate nuclear NF-κB activities. These nuclear IκB proteins also appear to have different preferences for NF-κB dimmers: Bcl-3 binds only p50 and p52 homodimers (32, 33), IκBζ binds both p50 homodimers and p65/p50 dimers (34), while IκBNS appears to show little subunit preference (35). Significantly, the nuclear IκB proteins are not degraded following IKK activation and may even be induced by certain stimuli. The function of these nuclear IκB proteins appears to be the modulation of gene transcription by NF-κB in a subunit and promoter specific manner (36). The role of the cytoplasmic IκB proteins is not limited to conferring inducibility on NF-κB activity, as they also shape the extended NF-κB response following stimulation since they are target genes of NF-κB (37). The kinetics of IκB degradation and *de novo* synthesis confer an oscillatory nature on NF-κB mediated gene expression which shapes the strength and duration of the transcriptional response to a given stimulus (38-40).

TLR signaling

TLRs are type I membrane glycoproteins and share a cytoplasmic signaling domain termed the Toll-like/ interleukin-1 receptor homology domain or TIR domain. Ligation of TLRs by microbial components induces a signaling cascade leading to the appropriate transcriptional response. TLR ligand binding induces receptor dimerisation and a conformational change which recruits TIR domain containing adaptor proteins to the receptor TIR domain. Four signaling adaptor proteins have been identified to date; MyD88, TIRAP, TRAM, TRIF while a fifth, SARM, has recently been shown to negatively regulate TLR signal transduction (41). TIRAP acts as a bridging adaptor between TLR4, TLR1/2, TLR2/6 and MyD88. TRIF is associated only with TLR4 and TLR3, binding directly to TLR3 and using the bridging adaptor TRAM to bind to TLR4. TRIF is the only adaptor to activate IRF3 to mediate type I interferon induction, through the IKK related kinases IKKε and TBK1 (42). Adaptor usage by individual TLRs has allowed the definition of two distinct signal transduction pathways downstream of TLR activation based on the requirement for MyD88.

MyD88-dependent NF-κB activation

MyD88 is essential for downstream signaling from all TLRs with the exception of TLR3 (43). MyD88 interacts with TLRs 7, 8 and 9 *via* its C-terminal TIR domain and associates with all the other TLRs through the bridging adaptor TIRAP. MyD88 also binds to the kinase IRAK4 through death domains contained in both proteins. Receptor associated IRAK4 subsequently phosphorylates IRAK1, which in turn complexes with the E3 ubiquitin ligase TRAF6. Both IRAK4 and IRAK1 are essential for MyD88 dependent signaling (44, 45); however there are conflicting reports over whether IRAK4 kinase activity is essential for TLR signal transduction (46, 47). IRAK1 binding and activation of TRAF6 do not appear to require its kinase activity (48, 49). Of note, phosphorylated IRAK1 undergoes ubiquitination and proteasomal degradation which most likely serves as a negative feedback loop for TRAF6 activation (50). Activated TRAF6 undergoes K63-mediated polyubiquitination which differs from the K48 form of polyubiquitination in that it does not lead to proteasomal degradation (51). The polyubiquitination of TRAF6 appears to be mediated by an E2 complex containing UBC13 and UEV1A. This E2 complex is capable of activating NF-κB in cytoplasmic extracts and dominant negative forms of UBC13 inhibit NF-κB activation *in vitro* (52). However, UBC13 null mice do not show any defects in NF-κB activation suggesting possible compensation by alternative E2 proteins (53). The exact role of TRAF6 polyubiquitination is unclear but it appears to be necessary for the recruitment of TAB1, TAB2 and TAB3. TAB2 and TAB3 are related proteins and contain ubiquitin-binding Zinc-finger domains in their C-terminal regions which appear necessary for the activation of NF-κB (54). Analysis of TAB1 and TAB2 knockout mice, however, has failed to reveal a defect in NF-κB activation in cells lacking these proteins (55) but has instead suggested a role for TAB1 in TGF-β signaling (56). RNA interference experiments however, have demonstrated that suppression of both TAB2 and TAB3 expression is necessary in order to observe loss of NF-κB activity, suggesting functional redundancy between these structurally similar proteins (57). In addition to functional and structural similarities, both TAB2 and TAB3 proteins share the ability to interact with (51, 57, 58) and activate (54) the kinase TAK1. The mechanism of TAK1 activation by recruitment to the TRAF6/TAB2/TAB3 complex is unclear but it may involve a trans-autophosphorylation event induced by dimerisation or oligomerisation. Thus the K63-polyubiquitination of TRAF6 seems to recruit TAK1 through the TAB2 and TAB3 ubiquitin binding proteins. TRAF6 is itself an E3 ubiquitin ligase and is capable of K63-autoubiquitination (52); however the critical E3 RING-finger domain of TRAF6 is not necessary for TLR-induced NF-κB activation as demonstrated by expression of various TRAF6 mutants in TRAF6 knockout cells, indicating that another E3 ligase may be responsible for TLR mediated TRAF6 polyubiquitination (59). TAK1 was previously identified as a component of an NF-κB-inducing complex *in vitro* and is also activated upon stimulation of cells with TNF-α and IL-1 (60). Over-expression of TAK1 alone however, does not lead to NF-κB activation and the co-expression of other proteins such as TAB1, TAB2 and TAB3 is required (51, 58, 60, 61). TAK1 knockout mice are embryonic lethal, suggesting a critical role for this kinase not related to NF-κB activation (55). Support for a role for TAK1 in TLR signaling is provided by experiments using B cell-specific conditional TAK1 knockouts which display defective NF-κB activation in response to TLR4, TLR9 and TLR3 (62). Significantly, a TAK1 homolog has been identified in *Drosophila*, dTAK1, mutation of which results in increased susceptibility to bacterial infection (63). Furthermore, dTAK1 is required for activation of dIKK by

bacterial peptidoglycans (64), revealing a clear evolutionary conservation of TAK1 in TLR signaling pathways. TAK1 is an IKK kinase that phosphorylates critical serines in the activation loop of IKKβ leading to its activation, and subsequent phosphorylation, K48-polyubi-quitination and degradation of IκB proteins (51). In addition to NF-κB activation, TAK1 is also required for the activation of JNK and p38 and subsequent AP-1 activity following TLR stimulation (62).

MyD88-independent NF-κB activation
As mentioned above, the TIR domain containing adaptor MyD88 is involved in downstream signaling of all TLRs with the exception of TLR3 (43). TLR3 instead exclusively uses the adaptor protein TRIF which binds directly to the receptor upon activation and is critical in the activation of NF-κB and the induction of interferon-β expression through activation of IRF3 (65). However, TRIF is also employed by TLR4 which associates with the receptor indirectly through another adaptor protein called TRAM (66, 67). Thus, the TLR4 pathway contains both MyD88-dependent and MyD88-independent signaling elements. Activation of NF-κB by TRIF occurs independently of IRAK1 and IRAK4 (68). NF-κB activation by TRIF appears to require the kinase RIP1 (69). In this case, RIP1 binds to a RIP homotypic interaction motif (RHIM) in TRIF to trigger downstream NF-κB activating events. Interestingly, analogous to IRAK1, the kinase activity of RIP1 is not essential for its downstream activation of NF-κB (70). This is supported by the ability of a RIP1 dominant negative mutant to inhibit TRIF-mediated NF-κB activation as well as a failure of RIP1 knockout mice to adequately activate NF-κB in response to TLR3 activation (69). The exact mechanism of RIP1-mediated NF-κB activation in the TLR3 pathway is unknown but studies on the role of RIP1 in the activation of NF-κB by the TNFR have shed some light on this issue. RIP1 has been reported to be K63-polyubiquitinated upon recruitment to the TNFR which facilitates its interaction with IKKγ *via* a C-terminal ubiquitin binding domain in IKKγ (71-73). Activation of IKKβ is then postulated to occur following TAK1/TAB2 recruitment to the K63-polyubiquitinated RIP1 and TAK1 phosphorylation of IKKβ (74). It should be noted, however, that thus far experimental evidence is lacking for the interaction of TAK1/TAB2 and RIP1. The significance of these findings from TNFR-mediated NF-κB activation in relation to TRIF-mediated NF-κB activation lies in the absence of TRAF6 involvement. This is of relevance as recent studies have demonstrated normal TLR3 activation of NF-κB in TRAF6 knockout cells (75, 76) in contrast to earlier dominant negative overexpression studies which suggested a TRAF6 requirement in TRIF-mediated NF-κB activation (77, 78). This is further supported by the demonstration of LPS-inducible NF-κB activity in MyD88/TRAF6 double knockout mice, presumably through a RIP1 dependent pathway (79). These findings, however, do not preclude a role for TRAF6 in TRIF-mediated NF-κB activation, which may contain a certain degree of redundancy.

Mediating TLR specific responses

The collation of a large number of DNA microarray datasets of pathogen-host interactions has revealed a common host response to pathogens as well as cell and stimulus specific responses (80). The common response is found in a range of cell types, such as DCs, macrophages, endothelial and epithelial fibroblasts, challenged with various bacteria, viruses or other pathogens. The overwhelming pre-ponderance of NF-κB activated genes present in the common response emphasizes the critical role played by NF-κB as the central mediator of TLR signals in the host response to pathogens. The responses to various pathogens contain both a common and a pathogen specific elements (81-83). While some of these pathogen specific genes, such as influenza-induced interferons and subsequent interferon-induced genes (83, 84), are downstream of TRIF mediated IRF3 activation and limited to TLR4 and TLR3 signaling (85), many pathogen- specific genes are regulated by NF-κB (83). Thus the question arises as to how TLR signaling pathways that converge on common signaling intermediates and transcription factors, such as NF-κB, induce distinct transcriptional outputs. There is no simple answer to this question, but emerging evidence supports a role for signal-specific post-translational modification of NF-κB as well as the interaction of NF-κB with co-operating transcription factors.

Post-translational modification of NF-κB
While IKKβ activation, IκB phosphorylation and degradation are necessary for NF-κB activation in response to TLR stimulation, further post-translational modifications of NF-κB are required for a maximal transcriptional response. The p65 subunit of NF-κB appears to be the primary target of signal induced phosphorylation by a number of kinases. Phosphorylation of S276 in the Rel homology domain of p65 by protein kinase A is an important regulatory event which occurs during TLR4 signaling (86, 87). Phosphorylation of S276 modulates the oligomerisation and DNA binding properties of p65 to enhance the NF-κB transcriptional response (88, 89). In addition, S276 phosphorylation enhances the binding of the transcriptional enhancers CBP and p300 to p65, leading to effective removal of repressive histone deacetylase complexes from target genes (88, 89). Two other sites of phosphorylation located in the transcriptional activation domain of p65 have been identified as S529 and S536 (90-94). IKKβ has been identified as the key kinase in the TLR4-induced phosphorylation of p65 on S536 (95). Phosphorylation at these sites also enhances transcriptional activity. Although the mechanism is unclear at present, it may involve a reduced interaction of p65 with IκBα (96). In addition to the TLR activated NF-κB kinases and their phosphorylation sites outlined above, a number of kinases have been described which appear to modulate NF-κB activities in response to a range of stimuli (97, 98).

However, a role for these additional kinases in TLR signaling has yet to be determined. Furthermore, there is no evidence yet that different TLR signaling pathways lead to distinct patterns of NF-κB phosphorylation. Despite this, however, it remains an attractive mechanism for the shaping of specific transcriptional responses.

Acetylation of NF-κB has also been demonstrated to be important for the nuclear function of NF-κB (99, 100). Three primary acetylation sites have been identified in p65, i.e., K218, K221 and K310, each of which has a distinct modulatory effect on the transcriptional activities of NF-κB (101). Acetylation of p65 on K218 and K221 significantly reduces its ability to interact with IκBα, thereby extending the duration of the NF-κB response in the nucleus by preventing the nuclear export of NF-κB/IκBα complexes (99). In contrast, the acetylation of p65 at K310 does not affect its interaction with IκBα nor its ability to bind DNA but is required for optimal transcriptional activity (99). Acetylation at K122 and K123 of p65 has also been described and interestingly appears to have an inhibitory effect on NF-κB mediated transcription by reducing DNA binding affinity (100). The p50 subunit of NF-κB is also acetylated, at K431 and K440, which appears to enhance the transcriptional activity and DNA binding activity of modified heterodimers (102, 103). Furthermore, recent studies have provided a link between the phosphorylation and acetylation of p65 with the finding that increased acetylation at K310 results from phosphorylation at S276 and S536 (104). Taken together, studies on phosphorylation and acetylation suggest a key role for post-translational modification in the regulation of TLR induced NF-κB activation. The further elucidation of how such modifications are regulated in a signal specific manner will clarify their contribution to shaping the transcriptional response.

NF-κB and co-operating factors

Although NF-κB is an essential factor for the expression of many genes, it does not act in isolation and it is just one component in a complex series of molecular events required for gene transcription. NF-κB has been reported to interact with a number of transcription factors, including C/EBP (105, 106), AP-1 (107), Sp-1 (108, 109), SRF (110), STAT6 (111) and SEF (112). More recently, NF-κB has been shown to directly interact with TRIF-activated IRF3 on target promoters (113). This interaction appears to be limited to TLR4-induced IRF3 complexes and is negatively regulated by the binding of glucocorticoids at target promoters (113, 114). This latter example of signal specific transcription factor crosstalk clearly illustrates the potential for fine tuning a specific transcriptional response at the promoter level through the interplay of distinct downstream targets.

In addition, the binding of nuclear IκB proteins to NF-κB dimers may also shape the transcriptional response. IκBζ is itself a TLR inducible gene which associates with p50 and is critical for the induction of the pro-inflammatory cytokines IL-6 and IL-12 following TLR stimulation in macrophages (115). IκBNS is also a TLR-inducible gene, which in contrast appears to limit the expression of a subset of inflammatory genes in response to TLR stimulation (116), similar to the related Bcl-3 protein which also has an inhibitory effect on certain TLR inducible genes (117, 118). The mechanism of action of these nuclear IκB interactions is unclear at present but may involve additional protein-protein interactions.

Negative regulation of TLR induced NF-κB activity

While rapid induction of NF-κB is essential to the host response to pathogens, the ability to switch off the expression of potentially damaging pro-inflammatory genes is equally important. The down-regulation of TLR-induced NF-κB activity occurs through two key mechanisms. The primary mechanism has been described above and entails the NF-κB mediated expression of its cognate inhibitors, the IκB proteins, which are proteasomaly degraded during the activation of NF-κB. The *de novo* synthesis of the IκB proteins in an NF-κB-dependent manner constitutes a negative feedback loop for NF-κB activity. Newly synthesized IκB proteins enter the nucleus *via* a nuclear localization signal where they bind free NF-κB dimers and translocate to the cytoplasm as an IκB/NF-κB complex using a nuclear export sequence, thereby terminating NF-κB transcriptional activity (7). The second mechanism of limiting NF-κB activity was initially discovered in studies of IκBα knockout cells and involves the ubiquitin targeted proteasomal degradation of promoter bound NF-κB (119). Subsequent analysis has revealed a key role for IKKα in this mode of NF-κB activity termination. IKKα is not required for the activation of NF-κB by TLRs (12) but is critical in limiting the duration and strength of the TLR-induced NF-κB transcriptional response (120, 121). IKKα phosphorylation of p65 and c-Rel targets them for proteasomal degradation in the nucleus and is a key event in the termination of the NF-κB response to TLR stimulation.

Other mechanisms of terminating the TLR mediated activation of NF-κB target sites upstream of the IKK complex primarily involve competitive inhibition by homologs or splice variants of TLR signaling components. These include the LPS inducible genes IRAKM, a homolog of IRAK1 (122), and IRAK2c/d, a splice variant of IRAK2 (123), which interfere with the function of IRAK1 and IRAK4. MyD88s is a splice variant of MyD88 with a limited expression pattern that is unable to interact with IRAK4 (124). Other negative regulatory factors of TLR-induced NF-κB activation are TOLLIP and A20. TOLLIP interacts with TLR2 and TLR4 as well as IRAK1 and interferes with the activation of NF-κB through MyD88-dependent pathways (125). A20 is an LPS-inducible gene previously identified as a potent inhibitor of TNF-α-induced NF-κB activity (124), which has recently been attributed to play a critical role in the termination of TLR-induced NF-κB activity (126). The NF-κB regulatory functions of A20 are mediated through two ubiquitin-editing domains (127). The

N-terminal domain of A20 contains a de-ubiquitinating activity which removes K63-polyubiquitin chains from both RIP1 and TRAF6, thereby inhibiting the recruitment and activation of the TAK1/TAB2/TAB3 complex. In addition, a C-terminal ubiquitin ligase domain of A20 mediates the K48-polyubiquitination of RIP1 leading to its proteasomal degradation. Other negative regulators of TLR signaling such as decoy receptors have been identified and are discussed in depth elsewhere (128, 129).

Future perspectives

The intense research of TLR signaling pathways in recent years reflects the enormous significance of these receptors as gateways to innate and adaptive immunity. Given the importance of TLRs in the immune response it is perhaps not surprising that NF-κB plays such a central role in converting these receptor signals into the appropriate transcriptional responses. The emerging role of TLRs in a range of human diseases, including sterile inflammatory disorders, provides new therapeutic targets and opportunities for the treatment of these disorders. Moreover, the potential for targeted intervention in specific pathways, without a broad inhibition or suppression of the immune system, is a very exciting prospect indeed. The continued study of regulation of TLR-induced NF-κB activity and the mechanisms of signal specific gene expression will prove critical in the translation of our knowledge of TLR regulated immunity into therapeutic tools.

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