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Toll-like receptors: From the discovery of NFκB to new insights into transcriptional regulations in innate immunity

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ABSTRACT

Toll-like receptors (TLRs) are key components of the innate immune system, functioning as pattern recognition receptors that recognise a wide range of microbial pathogens. TLRs represent a primary line of defence against invading pathogens in mammals, plants and insects. Recognition of microbial components by TLRs triggers a cascade of cellular signals that culminates in the activation of NFκB which leads to inflammatory gene expression and clearance of the infectious agent. The history of NFκB began with the TLR4 ligand lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, since this was the stimulus first used to activate NFκB in pre-B-cells. However, since those early days it has been a circuitous route, made possible by drawing on information provided by many different fields, that has led us not only to the discovery of TLRs but also to an understanding of the complex pathways that lead from TLR ligation to NFκB activation. In this review we will summarize the current knowledge of TLR-mediated NFκB activation, and also the recent discoveries that subtle differences in κB binding sequences and NFκB dimer formation result in specific gene expression profiles.

1. Introduction

Toll-like receptors (TLRs) are important initiators of innate immunity, recognising diverse microbial products which are collectively known as pathogen-associated molecular patterns (PAMPs). Signalling pathways are then activated which culminate in the induction of pro-inflammatory proteins that trigger both innate and adaptive immunity [1,2]. There are 10 human TLRs. The best characterised is TLR4, which recognises the Gram-negative product lipopolysaccharide (LPS). The other antibacterial TLRs are TLR2, which in combination with TLR1 recognises triacylated lipopeptides, or in combination with TLR6 recognises diacylated lipopeptides; and TLR5 which recognises bacterial flagellin. The anti-viral TLRs are TLR3 which recognises double stranded RNA, TLR7 and TLR8 which both recognise single stranded RNA, and TLR9 which recognises CpG motifs, that exist in both virus and bacteria. Finally various fungal and protozoal products are sensed by TLR2. Ligation of TLRs by their PAMPs results in different effector responses depending on the cell type involved. In terms of signalling one thing all TLRs have in common is that every TLR tested so far leads to NFκB activation. Once activated by a PAMP, a TLR triggers a cascade of cellular signals, culminating in the eventual activation of NFκB which binds to a discrete nucleotide sequence in the upstream regions of genes that produce proinflammatory cytokines, such as TNFα, IL-1, and IL-2, thereby regulating their expression [3]. The release of these cytokines and cytokines such as IFNγ is the hallmark of the cellular response to the activation of the innate immune system. NFκB has been studied in depth as a transcription factor

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target for TLR signalling and serves as a key "readout" for TLRs. More attention has been paid to the upstream regulation of the pathway to NFκB, with less work being done on subtleties in the NFκB system engaged by TLRs. More recently however a greater understanding of these subtleties has become evident with interesting new insights into specificities in the activation process of different NFκB subunits emerging, which may in turn explain specificities in the innate response to different pathogens.

2. NFκB: the early days

In 1986, in an effort to understand the mechanism of activation of the enhancer sequence of the immunoglobulin gene, Sen and Baltimore searched for the presence of trans-acting factors they assumed mediated enhancer function in nuclear extracts [4]. Three binding proteins were discovered, one of which bound the κ enhancer sequence only in cells within the lymphoid lineage, being expressed in mature B-cells and plasma cells but not in pre-B-cells or T-cells. This factor was termed Nuclear factor κB (NFκB). Its molecular identity was not known and it was defined by its presence in nuclear extracts which could bind the motif in the κB enhancer in a gel shift assay. Sen and Baltimore discovered that when a cell line representing pre-B-cells was treated with a B-cell mitogen and underwent apparent transition from a pre-B-cell to a mature B-cell NFκB was induced. Interestingly the B-cell mitogen they used was bacterial lipopolysaccharide (LPS). Therefore, the first ever stimulus shown to activate NFκB was the TLR4 ligand LPS. This finding laid the path for the discovery of the LPS receptor TLR4. Sen and Baltimore showed that LPS induced NFκB both in cell types where it was constitutively expressed and also in cell types where NFκB had to be induced. They also revealed that LPS induction of NFκB involves posttranslational modification of a pre-existing protein, because the induction took place in the presence of translational inhibitors [5].

Although Sen and Baltimore initially discovered that NFκB was an important regulator of κ gene expression in B-cells, they observed that the NFκB DNA motif was not restricted to the κ enhancer and furthermore that NFκB itself was not restricted to B-lymphoid cells, but could be induced transiently in a number of cell types by an appropriate stimulus. They therefore postulated that although the NFκB transcription factor and the κB enhancer site are used for a specific purpose during B-cell differentiation, (specifically, the activation of κ light chain transcription), this regulatory act was not dedicated to that one single event but was used more broadly in transcriptional regulatory circuits. Today we know NFκB to play crucial roles in regulating a wide variety of events including inflammation, immunity, cell proliferation and apoptosis.

In order to study the functions of various cytokines researchers needed a protocol to induce their production. It was found that the most effective means to induce immune cells, such as macrophages, to produce cytokines was to expose them to LPS. LPS evokes a powerful inflammatory response by stimulating cells to release the cytokines TNFα and IL-1. These cytokines in turn induce the expression of a range of other pro-inflammatory proteins. The signalling pathways activated by TNFα and IL-1 then became a focus for investigation. Osborn et al. demonstrated that out of eight cytokines tested, both TNFα and IL-1 specifically activated NFκB binding in a number of cell lines [6]. Not long after that Lenardo et al. [7] showed that virus infection of fibroblasts activated the binding and nuclear localization of NFκB and in pre-B-cells this resulted in the induction of interferon β (IFNβ).

IL-6 is a cytokine released by activated monocytes that plays a crucial role in the immune response, however IL-6 can also be induced in some T-cells, B-cells and fibroblasts [8]. IL-6 was also shown to be induced in response to LPS, viral infection, TNFα and IL-1. It did not take long for researchers to realize that NFκB becomes activated by a set of stimuli similar to those that induce IL-6 expression and to prove that these stimuli did in fact activate NFκB which subsequently led to IL-6 gene induction [9]. By this stage it was becoming clear that NFκB was involved in the control of a variety of genes activated upon inflammation, and that it might therefore play a central role in the inflammatory response to infection.

3. Toll and IL-1R

Concurrent with the work being performed on NFκB activation, other groups interested in the completely unrelated area of embryonic development were analyzing the generation of dorsoventral polarity in the early embryo of *Drosophila melanogaster*. A transmembrane protein termed Toll and a transcription factor termed Dorsal are both necessary for correct dorsoventral development [10]. Toll consists of an extracytoplasmic domain with many leucine-rich repeats (LRRs), and an intracellular domain, which by searching the database of all known gene sequences at the time, Gay and Keith [11] discovered was highly homologous to that of the human Type 1 IL-1 Receptor. Previous to this discovery in 1990, Dorsal was discovered to be related to NFκB [12]. As Toll signalled to Dorsal, and since IL-1 activated NFκB it was hypothesized that the biochemical nature of signal transduction by IL-1R and Toll would be similar.

A powerful way to chart the evolutionary design of regulatory pathways is by inferring their likely molecular components, and biological functions, through interspecies comparisons of protein sequences and structures. On studying the downstream signalling cascade of the IL-1R and the *Drosophila* Toll pathways it was discovered that many more components of the pathways displayed striking structural and functional similarities. IL-1R signalling involves a serine/threonine kinase called interleukin-receptor associated kinase (IRAK) [13]. Toll signalling induces the activation of the Pelle serine/threonine kinase a homologue of IRAK-1. As is now well known, NFκB is regulated by an inhibitory molecule called inhibitory κB (IκB) which retains NFκB in the cytoplasm under normal conditions. Upon cellular stimulation IκB releases NFκB allowing NFκB to translocate into the nucleus where it binds to its target promoters [14]. In *Drosophila*, Cactus, which is structurally related to IκB holds Dorsal in the cytoplasm. Degradation of Cactus causes activation of Dorsal and the related protein dorsal related immune factor (DIF) [15,16].

However, the reason why a human protein involved in inflammation and a fly protein involved in dorsoventral

polarity would resemble each other so strikingly remained a mystery up until 1993, when a role for Toll in the immune respose of *Drosophila* was implicated. DIF was found to mediate the induction of bateriocidal cecropins in infected flies, and this factor translocated into the nucleus in a *Drosophila* mutant that had the Toll receptor constitutively activated [17]. Then in 1996 Lemaitre et al. discovered that Toll also functioned in the fly in defense against fungal infection [16]. These studies therefore revealed the first components in the signalling pathway activated by IL-1 leading to NFκB, and provided the first indications of how TLRs might function.

4. Conservation of an ancient host defence system and the history of TLRs

The homology between IL-1R and Toll was in their cytosolic regions, and the domain that was homolgous was termed the Toll/IL-1R resistence (TIR) domain. Using sequence and pattern searchs of the expressed-sequence tag (EST) database, a search with a sequence profile of the TIR domain identified a matching sequence in the EST database derived from human fetal liver/spleen library. The protein was called human Toll (hToll) [18]. Alignment of the sequences of the human and *Drosophila* Toll proteins showed homology over the entire length of the protein chains, and a similarity between the cytoplasmic domains that was higher than that between the hToll and IL-1R. The extracellular domain of hToll contained 21 tandemly repeated leucine rich motifs (LRRs) separated by a non LRR region, similar to that in *Drosophila* Toll (dToll). IL-1R on the otherhand had Ig domains coupled to the TIR domain. As the ligand for hToll was unknown, a dominant-positive mutant of hToll was generated, which was shown to induce activation of NFκB.

Rock et al. then identified five human Tolls, which they termed the Toll-like receptors (TLRs). One of these, TLR4, was the same hToll described by Medzhitov and Janeway [18,19]. At this point in time the ligands for TLRs were unknown and researchers only knew that by constitutively activating TLRs they could drive NFκB activation and cause the production of the proinflammatory cytokines TNFα, IL-1, IL-6 and IL-8. TLRs therefore contributed to human immunity, but it was not known how the TLRs were activated during an infection.

It had been known for over 30 years that the C3H/HeJ strain of mice had a defective response to LPS. Whereas normal mice die of septic shock brought on by LPS injection within an hour, these mutant mice survive and behave as though they have not been exposed to LPS at all. Speculations regarding the protein that is affected by the mutation in what was termed the *Lps* locus had for the most part centered on the hypothesis that it must be a part of the LPS signal transduction cascade. In fact the receptor for LPS itself was unknown. LPS was known to be concentrated from the plasma by LPS binding protein (LBP), and CD14 was identified as a receptor for LPS on the surface of the cells. However because CD14 lacks a cytoplasmic domain, it was assumed that a coreceptor for LPS must exist in order to permit transduction of the signal across the plasma membrane. Poltorak et al. performed a BLAST search of the *Lps* locus and of two authentic genes identified *Tlr4* turned out to be identical to *Lps*. Comparison of TLR4 cDNA cloned from C3H/HeJ with that from several LPS-responsive strains of mice showed that at position 712, within the cytoplasmic domain, a histidine is predicted to occur in the TLR4 protein of C3H/HeJ mice, whereas in LPS responsive mice, rats and humans a proline is in this position. This Pro-His point mutation was proven to exert a dominant negative effect on LPS signal transduction [20].

LPS was not the only microbial product that was known to activate NFκB and induce the production of inflammatory cytokines in the absence of a known receptor. There was mounting evidence for the activation of NFκB by many such microbial molecules. Soon after the initial discovery of NFκB, it was shown that NFκB played a central role in sendai virus and poly(I:C) induction of IFNβ [7]. In fact NFκB was known to be activated by numerous viral families including Human immunodeficiency virus (HIV), human T-cell leukaemia virus (HTLV), hepatitis B virus, hepititis C virus, Epstein Barr virus (EBV) and influenza virus (reviewed in [21]). It was shown that characteristic bacterial DNA sequences which include unmethylated CpG sequences activate a signalling cascade leading to the activation of NFκB and inflammatory gene induction [22,23]. Lipoteichoic acid (LTA) a component of the cell wall of gram-positive bacteria was also shown to cause the activation of NFκB [24]. In the past 6 years these pathogenic molecules have all been identified as ligands for various different TLRs.

5. TLR signalling

TLRs actually belong to a superfamily called the IL-1R/TLR superfamily all members of which possess cytoplasmic Toll/IL-1R resistence (TIR) domains. The TIR domain is approximately 160 amino acids long and is essential for signalling. The superfamily is divided into three subgroups. The members of the IL-1R sub-group have extracellular immunoglobulin domains, the TLR sub-group possess extracellular LRR domains, and there is also an adapter sub-group which are cytoplasmic proteins with no extracellular region [25].

In 1994, Dan Hultmark discovered that a protein known to be involved in myeloid differentiation MyD88, was related to the cytoplasmic domains of IL-1R and dToll and suggested that together with the IL-1R and dToll, MyD88 may define a family of signalling transducing molecules with an ancestral function in the activation of immunity [26]. Three years later his hypothesis was proven correct when Cao et al. showed that MyD88 binds both IRAK and the IL-1R cytoplasmic domain mediating the association of IRAK with the receptor. Therefore, MyD88 plays the same role in IL-1R signalling as Tube does in the dToll pathway coupling a serine/threonine kinase to the receptor complex [27]. MyD88 is the original member of the IL-1R/TLR adapter sub-group.

To date four more adapter proteins have been identified; Mal, also known as TIR domain containing adaptor protein (TIRAP) [28,29]; Toll/IL-1R domain containing adaptor inducing IFNβ (TRIF) also known as TIR containing adaptor molecule-1 (TICAM-1) [30–32]; TRIF-related adaptor molecule (TRAM) also known as TIR containing protein (TIRP) and TIR containing adaptor molecule-2 (TICAM-2) [30,33,34]; and SAM and ARM-containing protein (SARM) [35]. The differential recruitment of these adaptor proteins to the TLRs form the basis for specificity in the signalling processes activated by TLRs.

Due to the high degree of conservation between the IL-1R and the TLRs much of what is known about downstream signalling events mediated by TLRs was initially provided from studying the signalling mechanisms of IL-1R. Just as Poltorak et al. were pinpointing TLR4 as the receptor for LPS signalling, other groups were investigating the role and involvement of IL-1R and TNFα signal transducer molecules in LPS signalling. Indeed, Zhang et al. showed that MyD88, IRAK1, IRAK2 and TRAF6 were all involved in LPS-induced NFκB activity, and hypothesised from this that the LPS signalling cascade uses an analogous molecular framework for signalling as IL-1 [36]. Concurrently Medzhitov et al. published evidence that signalling through the hToll, which was identified as TLR4, employs MyD88 as an adaptor protein and induces activation of NFκB via IRAK1 and TRAF6 involvement [37] (Fig. 1).

5.1. *The MyD88 dependent pathway*

MyD88 is involved in NFκB activation by every TLR identified so far with the exception of TLR3, since poly(I:C)/TLR3-induced NFκB activation was normal in the absence of MyD88 [38]. The MyD88-dependent pathway is analogous to signalling pathways through the IL-1R. MyD88, which has a C-terminal TIR domain, and an N-terminal death domain associates with the TIR domain of the TLRs and upon binding of ligand to the receptor MyD88 recruits IRAK4 to the TLR receptor complex

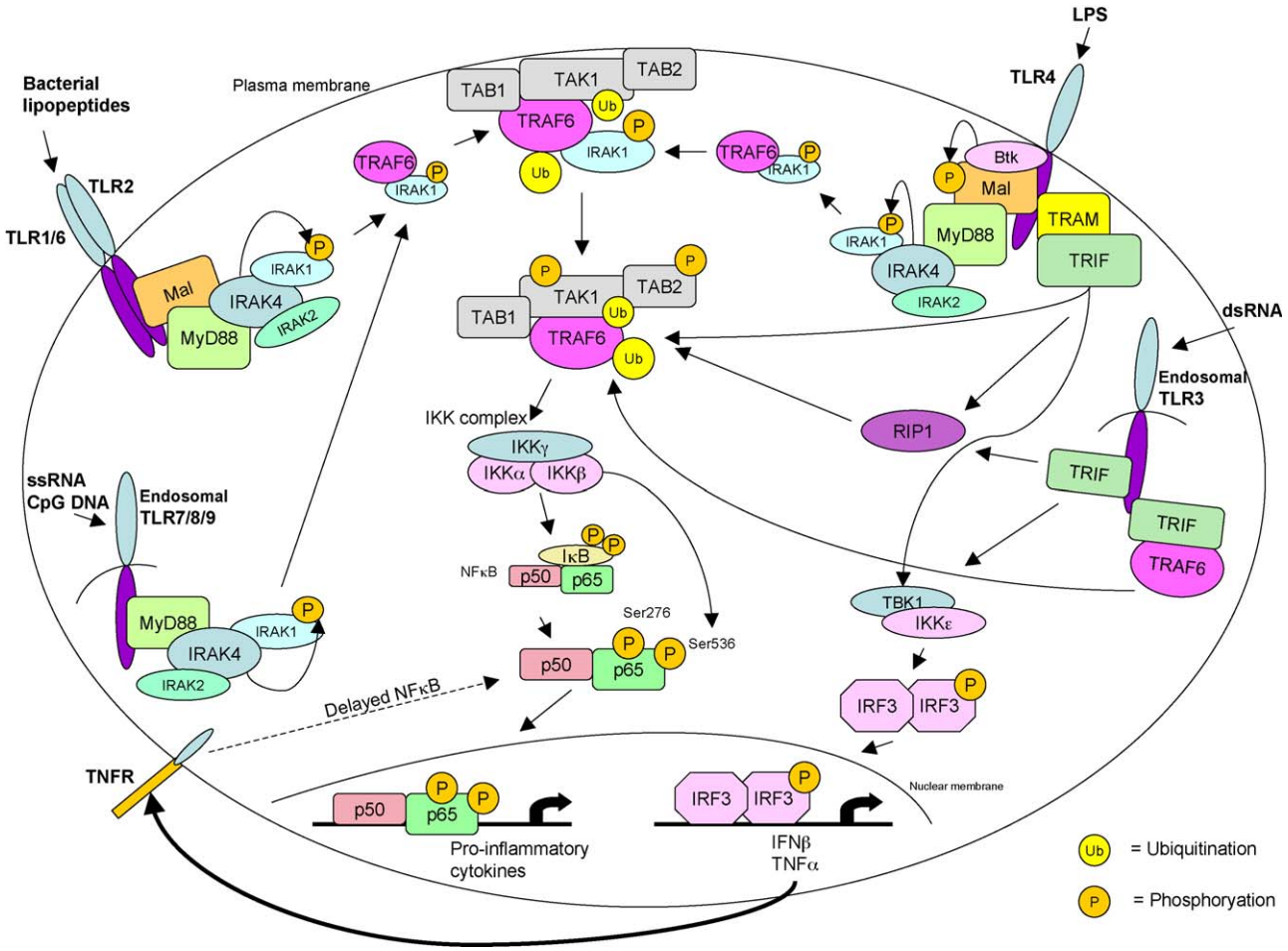

Fig. 1 – Overview of the TLR signalling pathway to NFκB and IRF3. On TLR ligation, TLR4 dimers and TLR2 heterodimerised with TLR1 or TLR6 recruit Mal and MyD88 to their TIR domains, TLR7/8/9 recruit MyD88 alone. MyD88 binds IRAK4 which in turn phosphorylates IRAK1 [43]. IRAK1 binds TRAF6 which in turn binds a pre-formed membrane bound complex of TAB1/TAK1/TAB2. Ubiquitination of TRAF6 and TAK1 activate the complex, and phosphorylation of TAK1 and TAB2 initiates the release of the complex from the membrane [46–48]. Active TAK1 phosphorylates the IKK complex, which in turn phosphorylates IκB targeting IκB for degradation [50]. IKKβ phosphorylates S536 on p65 [95]. Btk interacts with the TIR domain of TLR4, Btk phosphorylates Mal and leads to the phosphorylation of p65 on S536 [77–79]. NFκB is then free to translocate into the nucleus, bind its target DNA and promote the expression of pro-inflammatory cytokines. Once activated by a ligand, TLR3 recruits TRIF directly to its TIR domain while TLR4 recruits TRAM to its TIR domain which in turn recruits TRIF [33]. TRIF binds TRAF6 directly leading to TAK1 activation in an IRAK1/4 independent manner [62,63]. TRIF also binds directly to RIP1 which binds TRAF6 and leads to the activation of the IKK complex [64]. TRIF can also interact with TBK1/IKKε which leads to the activation of IRF3 [61]. IRF3 then translocates into the nucleus and binds its target genes. TNFα expression is induced in an NFκB-independent IRF3-dependent manner, binds to the TNF receptor and induces NFκB activation providing delayed NFκB induction [68,69].

through the interaction of the death domains of both molecules and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Phosphorylated IRAK-1 subsequently dissociates from the receptor complex and associates with TRAF6 [39–41] (Fig. 1).

The presence of IRAK-4 is required for the recruitment of IRAK-1 to the receptor complex and for the activation and subsequent degradation of IRAK-1 protein. However the molecular mechanism of IRAK-4 signalling and the role of its kinase activity remain elusive. Qin et al. have shown that the IRAK-4 kinase-inactive mutant had the same ability as wildtype IRAK-4 in restoring IL-1-mediated signalling in human IRAK-4 deficient cells, whereas Lye et al. showed by reconstitution of IRAK-4 deficient murine cells with wildtype or kinase inactive IRAK-4, that the kinase activity of IRAK-4 is required for the optimal transduction of IL-1 induced signals [42,43]. However with the exception of TLR3 all TLRs activate NFκB via a pathway involving IRAK-4 and IRAK-1.

While IRAK-1 is essential for IL-1 signalling, when IRAK-1 deficient macrophage cells are stimulated with LPS they still exhibit LPS-induced TNFα production albeit at depleted levels, implying that another IRAK can compensate for the lack of IRAK-1 [44,45]. This could possibly be IRAK-2, a homologue of IRAK-1, which similar to IRAK-1 lies upstream of TRAF6. Using the technique of making deletion mutants it has now been demonstrated that LPS signalling requires both IRAK-1 and IRAK-2 to function downstream of MyD88 [36].

TRAF6 is the next signalling molecule after IRAK. It was found to have role in the IL-1R signalling pathway when a dominant negative form of the protein inhibited activation of NFκB by IL-1 [40]. Phosphorylated IRAK1 is active and somehow activates TRAF6. TRAF6 and IRAK1 then dissociate from the receptor complex and interact with a membrane bound pre-associated complex of TGF-β activated kinase (TAK1) and two TAK1-binding proteins (TAB), termed TAB1 and TAB2. A series of ubiquitination reactions then occur on TRAF6 itself and on its substrate TAK1. The activation of TAK1/TAB1/TAB2 complex by TRAF6 depends on the nonclassical polyubiquitination of TRAF6 [46]. Phosphorylation of TAK1 and TAB2 occurs initiating the dissociation of TRAF6/TAK1/TAB1/TAB2 from the membrane to the cytosol, and IRAK-1 degradation [47,48] (Fig. 1). TAK1 is subsequently active and can then phosphorylate downstream targets such as the IκB kinases (IKKs). Once activated the IKK complex induces phosphorylation and subsequent degradation of IκB, which leads to the translocation of NFκB culminating in NFκB activation and the expression of pro-inflammatory cytokines [49–53].

TRAF6 has been shown to have a role in all TLR pathways to NFκB tested to date, functioning as a central signalling molecule that can dock with multiple effectors and thus lead to NFκB and MAP kinase activation by different routes.

It was a database search for molecules structurally similar to MyD88 that led to the identification of Mal [28,29]. Dominant negative forms of Mal inhibit LPS/TLR4-mediated NFκB activation, but not NFκB activated by IL-1R or IL-18R [54]. However, Mal knockout mice responded normally to the TLR3, TLR5, TLR7 and TLR9 ligands, as well as to IL-1 and IL-18, but were severely impaired in their responses to ligands for TLR2 and TLR4. These data showed that Mal has a crucial role in the MyD88-dependent pathway shared by TLR2 and TLR4 [55].

In MyD88 deficient mice there was no production of inflammatory cytokines and no NFκB activation in response to TLR2, TLR7 and TLR9 ligands. LPS induced production of inflammatory cytokines is not observed either, however LPS induced activation of NFκB is observed albeit with delayed kinetics when compared with wildtype mice [56]. These findings indicate that while TLR4-mediated production of inflammatory cytokines depends completely on MyD88 and Mal, a MyD88-independent component exists downstream of TLR4. When Mal knockout mice were generated they were also found to have delayed kinetics in the activation of NFκB in response to LPS, similar to that observed in MyD88 deficient mice. Another transcription factor IRF3 was activated in a Mal and MyD88 independent manner in response to LPS [56–58]. IFN inducible genes are induced via the activation of IRF3 and other interferon inducible factors. LPS induction of IFN-inducible genes encoding IP-10, GARG-16 and IRG-1 was comparable in wild type, Mal-deficient and MyD88-deficient macrophages [58]. However results from the Mal knockout and MyD88 knockout mice did not preclude the possibility that MyD88 might compensate for the Mal-deficiency and vice versa during the LPS response. Mal–MyD88 double knockout mice were therefore generated. Genes encoding IP-10, IRG-1 and GARG-16 were induced normally in response to LPS in double knockout macrophages, and LPS-induced expression of co-stimulatory molecules was observed in double knockout dendritic cells. These studies suggested that there must be another adapter, which would function in TLR4 signalling on the MyD88–Mal-independent pathway. In addition to TLR4 signalling, TLR3 signalling also activates IRF3 and induces IFNα/β in a MyD88 and Mal independent manner. This suggested that another adaptor needed to exist for TLR3 signalling also [58].

5.2. *The MyD88 independent/TRIF dependent pathway*

So far it appears that all the human TLRs with the exception of TLR3 and TLR4 require MyD88 to signal. Moreover, TLR3 seems to be unique in that it signals exclusively through the third adapter to be discovered, TRIF [58]. In contrast to TLR3, TLR4 signalling independent of MyD88 has been shown to require both TRIF and the fourth adapter to be found, TRAM. Overexpression of TRIF activated the NFκB dependent promoter in a similar fashion to MyD88 and Mal. However, TRIF, but neither Mal nor MyD88, activated the IFN-β promoter [58]. It has also been shown that the noncanonical IKKs, IKKε and TANK-binding kinase-1 (TBK1), mediate activation of IRF3 through interaction with TRIF and TRAM, thereby inducing the IFNβ promoter [59,60]. TRIF also associated with TLR3, and a dominant negative version of TRIF comprising its TIR domain alone inhibited TLR3-dependent activation of both the NFκB and IFN-β promoters [58]. These data indicated that TRIF is involved in MyD88-independent activation of TLR3 signalling. TRIF binds to TLR3 and recruits TRAF6 directly through a TRAF6-binding motif in its N-terminal domain. This leads to TAK1 activation and subsequent NFκB activation in an IRAK1 and IRAK4 independent manner (Fig. 1) [61–63].

TRIF can also activate NFκB through an alternative pathway. The C-terminal of TRIF possesses a RIP homotypic interaction motif (RHIM), and it associates with receptor interacting protein 1 (RIP1) through homophilic interaction of

RHIM domains [64] (Fig. 1). A dominant negative form of RIP1 inhibited TRIF-mediated NFκB activation. Furthermore, MEFs from RIP1 knockout mice showed impaired TLR3-mediated NFκB activation, indicating that RIP1 is responsible in part for TRIF-mediated NFκB activation. Thus, TRAF6 and RIP1 are involved in TRIF-dependent activation of NFκB. However, although TRAF6 is involved in the TLR3 induced TRIF-mediated NFκB activation, TLR4-mediated NFκB activation was still inducible in MyD88/TRAF6 double knockout mice [65] indicating the presence of TRAF6-independent NFκB activation in the TRIF-dependent pathway. There is also one study where it was shown that TLR3-mediated activation of NFκB was not affected in TRAF6 deficient macrophages [66].

In TRIF- and TRAM-deficient mice, normal inflammatory cytokine production induced by TLR2, TLR7 and TLR9 ligands was observed, and TLR4-mediated phosphorylation of IRAK was induced normally, indicating that activation of the MyD88-dependent pathway was unaffected. However, TLR4-mediated inflammatory cytokine production, which is believed to be mainly induced by the MyD88-dependent pathway, was defective in TRIF- and TRAM-knockout mice [67]. Therefore, TRIF must be involved in TLR4-mediated induction of inflammatory cytokines although the mechanisms remain to be elucidated.

Since the discovery of the MyD88-independent pathway it has been known that NFκB activation mediated through the MyD88-dependent pathway occurs earlier than activation mediated by the MyD88-independent pathway. Recently Covert et al. [68] tackled this discrepancy using a systems-based computational approach. A lag in NFκB activation could occur in two ways. The kinetics of the MyD88-independent pathway could be much slower than the kinetics of the MyD88-dependent pathway, or both pathways could display similar kinetics but the initiation of the MyD88-independent pathway could be delayed. The model put forward by Covert et al. indicated that both pathways are likely to have similar kinetics, but that the MyD88-independent pathway requires approximately a half an hour time delay before it is activated. In fact Covert et al. explain that this delay occurs because NFκB activation by the MyD88-independent pathway requires protein synthesis. In MyD88-deficient cells pretreated with the protein synthesis inhibitor cyclohexamide, LPS stimulation failed to activate NFκB. By comparing early gene expression levels in LPS stimulated MyD88-deficient cells Covert et al. were able to show that transcripts of TNFα were increased. Pretreatment of MyD88-deficient cells with soluble TNF receptor blocked LPS-stimulated activation of NFκB. Therefore they have shown that the TRIF-dependent pathway activates TNFα production and secretion, in an NFκB-independent manner, and that the secretion and subsequent binding of TNFα to its receptors leads to NFκB activation. Furthermore, they have shown that the MyD88-independent pathway specific transcription factor IRF3 mediates this activation of TNFα, as depletion of IRF3 impairs the activation of NFκB. Covert et al. therefore suggest that the activation of NFκB by the MyD88-independent/TRIF-dependent pathway results by means of a secondary response through TNFα and IRF3, establishing an autocrine pathway for delayed NFκB activation. The combination of the two pathways leading to NFκB activation allow for the stable and consistent early NFκB response to LPS. Werner et al. have similarly demonstrated that TNFα mediates a feedforward mechanism in response to LPS that produces positives feedback on IKK activity [69] (Fig. 1). This secondary response through TNFα might also explain why LPS-stimulated TRAF6 deficient macrophages are still capable of delayed NFκB activation, as mentioned earlier.

6. The role of protein tyrosine kinases in TLR-mediated signalling to NFκB

An intensive period of research had therefore revealed the major protein components linking TLRs to the IKK complex. Additional proteins were then shown to participate, particularly tyrosine kinases. It was first suggested that protein tyrosine kinases (PTKs) were involved in LPS signalling when it was reported that expression of Src kinases was enhanced following stimulation of myeloid cells with LPS [70,71]. Soon afterwards it was reported that PTKs were required for the induction of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα in response to LPS in murine macrophages [72]. Orlicek et al., directly implicated Src kinases in LPS signalling to TNFα and iNOS (inducible nitric oxide synthase) induction with the use of the Src-selective inhibitor PP1, in murine macrophages [73]. However, the relative importance of Src kinases in LPS signalling in macrophages has been directly questioned by the generation of $hck^{-/-}fgr^{-/-}lyn^{-/-}$ mice, which showed no major impairment in LPS-induced activation, indicating that the Src kinases Hck, Fgr and Lyn are not obligatory for LPS-initiated signal transduction [74].

The possibility that Bruton's tyrosine kinase (Btk) may be involved in LPS signalling was suggested by a study in xid mice, which lack a functional Btk. These studies implicated Btk in macrophage effector functions in response to LPS [75]. Macrophages from xid mice showed poor nitric oxide (NO) induction and reduced production of the pro-inflammatory cytokines IL-1β and TNFα in response to LPS challenge, when compared with wild-type controls. As NFκB is a key transcription factor regulating the expression of IL-1β and TNFα they examined the ability of LPS to induce expression of Rel-family proteins. Levels of Rel-family members were reduced in xid mice stimulated with LPS compared with control mice. More recently it has been found that Btk interacts with the TIR domains of many of the TLRs including that of TLR4 and also with Mal and MyD88, the adapters used by TLR4. LPS induces tyrosine phosphorylation of Btk and activates its kinase activity. It has since been shown that Btk directly phosphorylates Mal and functions on the pathway from TLR4 to the phosphorylation of the p65 subunit of NFκB, promoting transactivation [76–79].

7. TLRs and the promotion of transactivation of NFκB

The p65 subunit of NFκB is constitutively phosphorylated in unstimulated cells [80,81], and this basal phosphorylation is further increased by a broad range of stimuli including hydrogen peroxide, TNFα, phorbol myristate acetate (PMA), IL-1, LPS and other TLR ligands such as CpG DNA, R848 a synthetic ligand for

TLR7/8 and LTA [81–88]. The p65 subunit has a unique C-terminus which contains two transactivation (TA) domains, TA1 and TA2 [89]. The majority of p65 phosphorylation occurs at serine residues within these TA domains. This phosphorylation is known to be required for transactivation of gene expression because mutating these serine residues greatly impairs NFκB-dependent gene transcription (reviewed in [90]).

To date, five distinct serine residues have been identified on the p65 NFκB subunit that are inducibly phosphorylated in response to LPS, TNFα, and IL-1. Serine 276 in the RHD on p65 is phosphorylated by protein kinase A (PKA) in response to LPS [91,92]. Zhong et al. [93] found that LPS stimulated PKA-dependent phosphorylation of p65 on serine 276 promotes its interaction with the coactivator CREB-binding protein (CBP), potentiating NFκB transcription [91]. Phosphorylation of serine 536 was originally reported by Sakurai et al. [86]. They found that overexpression of IKKα or IKKβ induced the phosphorylation of serine 536 in vivo and in vitro. Since LPS is a potent activator of IKK, Yang et al. [94] investigated whether LPS could induce phosphorylation of serine 536 on p65. They found that IKKβ plays an essential role in LPS-induced serine 536 phosphorylation in MEFs, while IKKα is only partially required for serine 536 phosphorylation (Fig. 1). In contrast they also found that although impaired, IKKβ was not essential for TNF-induced phosphorylation of serine 536 in MEFs. More recently Buss et al. [95] reported that phosphorylation of p65 in response to IL-1 at serine 536 may be mediated by IKKα, IKKβ and IKK-related kinases IKKε and TBK1, and an as yet unknown kinase identified by chromatographic fractionation of cell extract. Furthermore they suggest that phosphorylation of serine 536 favours IL-8 transcription mediated by TATA-binding protein associated factor II31, a component of TFIID. As stated above Btk has also been shown to play a crucial role in LPS induced p65 phosphorylation. Xid mice which lack a functioning Btk do not exhibit phosphorylation of p65 on serine 536 in response to LPS when compared with wild-type mice [78].

The importance of regulatory phosphorylation on p65 is evident from the analysis of cells lacking the protein kinases PKCζ [96], NIK [97], IKKε [98], TBK1/NAK [99,100], or GSK3β [101]. In all of these cells the IκB degradation pathway is normal but there is impaired activation of NFκB-dependent gene expression. In fact a further function for GSK3β has recently been identified as a critical modulator of TLR-mediated production of pro-inflammatory versus anti-inflammatory cytokines in vivo [102]. Stimulation of peripheral blood mononuclear cells (PBMCs) with TLR2, TLR4, TLR5 or TLR9 agonists induced substantial increases in IL-10 production while suppressing the release of pro-inflammatory cytokines including IL-1β, IL-6, TNFα, IL-12 and IFNγ, after GSK3β inhibition. GSK3β regulated the inflammatory response by differentially affecting the nuclear amounts of the NFκB subunit p65 and CREB interacting with the co-activator CBP.

8. Specificity at the NFκB subunit level: a variation on a theme

Recent work on TLRs and the NFκB system has revealed specificity in the regulation of different NFκB subunits. A new nuclear IκB protein, IκBζ, arrived on the scene relatively recently, which has been shown to interact with NFκB via a C-terminal ankyrin repeat domain in the nucleus [103]. IκBζ is barely detectable in resting cells but it is dramatically induced in response to TLR ligands such as LPS, lipopeptides and CpG DNA, in a MyD88 dependent manner. NFκB activation is essential for this induction, however NFκB activation alone is not sufficient for IκBζ induction, as TNFα also activates NFκB yet IκBζ induction by TNFα was marginal. As the stability of the IκBζ mRNA was specifically upregulated by stimulation with LPS or IL-1β but not with TNFα, Yamazaki et al. have suggested that the other TIR domain dependent signal required for IκBζ induction, apart from NFκB activation, leads to mRNA stabilisation. The induced IκBζ localises in the nucleus and preferentially interacts with the NFκB p50 subunit rather than the p65 subunit (Fig. 2). The most recent studies on IκBζ have made use of IκBζ-deficient mice and have demonstrated that IκBζ is essential for the induction of a subset of inflammatory genes including IL-6 and the IL-12p40 subunit. The transcriptional activity of IκBζ depends on the p50 subunit as it binds the target DNA without transactivation potential [103,104]. Interestingly overexpression of IκBζ results in an augmentation of IL-6 production in response to LPS, but inhibits TNFα production [105].

Another study that highlights the subtleties in TLR and NFκB signalling is a very recent investigation into the mechanism of glucocorticoid immunosuppression. It is well known that glucocorticoids are potent immunosuppressive agents, however a very recent study discovered that glucocorticoids do not globally inactivate NFκB, but target only a subset of the TLR-inducible NFκB dependent genes in macrophages, indicating that the mechanisms of transrepression in the macrophage must operate in a promoter specific manner [106]. The difference between the signalling pathway activated by TLR3 and that by TLR4 leading to IRF3-dependent gene expression can explain the inhibition of TLR dependent gene induction by glucocorticoids. TLR3 activates IRF3 homodimers which then bind the Interferon sensitive response element (ISRE) on target genes. However when TLR4 is activated, a complex of IRF3 and the NFκB subunit p65 is formed and so NFκB plays a key role in the functioning of IRF3 during TLR4 signalling [107]. Glucocorticoids have therefore been found to repress the induction of ISRE-containing genes, but only when activated by TLR4 as opposed to TLR3, because they specifically target and disrupt the p65/IRF complex (Fig. 2). This mechanism enables glucocorticoids to differentially regulate pathogen-specific programs of gene expression.

In a related study recent work on the κB consensus site carried out by Leung et al. [108] has shown that although the consensus DNA sequence for NFκB binding is very broad, the sites active in any one gene exhibit remarkable evolutionary stability. In this study the κB site sequences of eleven genes were studied. The κB site sequences were shown to be 100% conserved between mouse and human in all 11 genes. In contrast to this, the regions surrounding the κB sites often displayed less than 85% conservation. Leung et al. showed that swapping sites between κB-dependent genes that have different properties altered NFκB-dimer specificity of the promoters and revealed that two κB sites can function together as a module to regulate gene activation. Furthermore they discovered that the consensus sequence itself

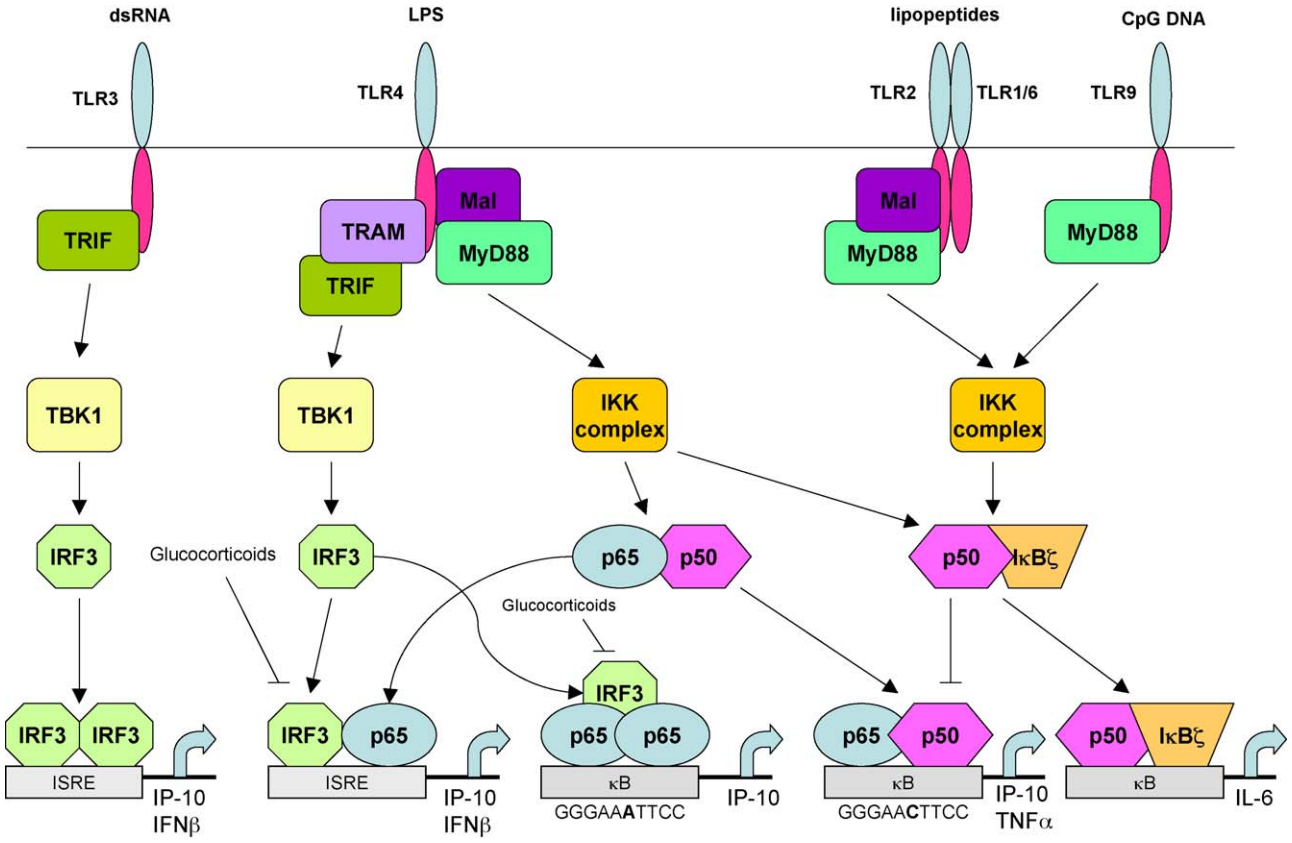

Fig. 2 – Specificity at an NFκB subunit level. TLR3 recruits TRIF to its TIR domain which interacts with TBK1 activating IRF3 which binds as a dimer to the ISRE site of the IFNβ and IP-10 genes [33]. TLR4 recruits TRAM to its TIR domain which in turn recruits TRIF and activates TBK1, TLR4 also recruits Mal and MyD88 which signal to activate the IKK complex. TBK1 activates IRF3, and IKK activates the p65/p50 NFκB dimer [53,61]. IRF3 and p65 then form a complex that binds the ISRE element [107]. This IRF3/p65 complex is susceptible to glucocorticoid inhibition [106]. The heterodimer p65/p50 binds to the κB site – 5′-GGGAACTTCC-3′ – in response to LPS stimulation and induces the expression of IP-10 and proinflammatory genes. However a homodimer of p65/p65 can also bind a κB site with one nucleotide difference 5′-GGGAAATTCC-3′ and induce IP-10 expression, but only when in a complex with IRF3 acting as a coactivator [108]. TLR2/4/9-driven IKK activation induces IκBζ to interact with p50 [104]. This IκBζ/p50 dimer binds the κB site and is essential for the induction of a subset of inflammatory genes including IL-6. Formation of this dimer inhibits TNFα production [105].

contains information that is interpreted by the bound NFκB-dimer, changes dimer configuration and determines which coactivators will form functional interactions with the NFκB-dimer.

When IP-10 and MCP-1 genes respond to TNFα they have different NFκB family member requirements. Both hetero- and homodimers of NFκB are able to function on the MCP-1 promoter while only heterodimers of NFκB appear able to activate IP-10 [109]. Leung et al. were able to show that the heterodimer requirement of the IP-10 promoter was due to a single nucleotide difference at the sixth nucleotide of the κB sites. Therefore, this single nucleotide is responsible for the difference in κB family member requirements. Surprisingly, although inactive, p65 homodimers were shown to bind the IP-10 promoter in response to TNFα, which in turn even bound the coactivator CBP/p300. It was then discovered that the IP-10 requirement for a NFκB heterodimer for activation by TNFα was not necessary after LPS stimulation, since LPS induced IP-10 mRNA in both wt and p50/p52 deficient cells. This result suggested that the unresponsiveness of the IP-10 gene to homodimers in the TNFα-treated cells is a consequence of the lack of a cofactor that LPS can induce. On TNFα stimulation this κB site sequence might not support transcription because the conformation of the p65 homodimers might not recruit the appropriate coactivators to the promoter. In fact, chromatin immunoprecipitation assays demonstrated that IRF3 is recruited to the IP-10 promoter but not the MCP-1 promoter in an LPS-dependent manner. The requirement of IP-10 for IRF3 in p50/p52 deficient cells can explain the stimulus specific requirements for κB family members. Under LPS stimulation p65 homodimers appear to interact with IRF3 to activate the IP-10 gene (Fig. 2). Under TNFα stimulation IRF3 is not activated and p65 homodimers, although bound are unable to function and the IP-10 gene is not activated. All these studies have furthered our understanding of NFκB induced specificity. Not only does the κB site sequence determine κB dimer specificity, it also determines coactivator requirements.

9. Future perspectives

NFκB has its origin in the effect of LPS on pre-B-cells. Remarkable progress has been made since then in our understanding of the signalling pathways and molecular components in the NFκB system. The regulation of the NFκB system by TLRs continues to reveal new information on the intricacies of the regulation of innate immunity, and we can anticipate yet more insights into a transcription factor system that still has more to tell us about the complexities of gene expression in health and disease.

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REFERENCES

[1] Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17(1):1–14.
[2] O'Neill LA. How Toll-like receptors signal: what we know and what we don't know. Curr Opin Immunol 2006;18(1):3–9.
[3] Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994;12:141–79.
[4] Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 1986;46(5):705–16.
[5] Sen R, Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell 1986;47(6):921–8.
[6] Osborn L, Kunkel S, Nabel GJ. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci USA 1989;86(7):2336–40.
[7] Lenardo MJ, Fan CM, Maniatis T, Baltimore D. The involvement of NF-kappa B in beta-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. Cell 1989;57(2):287–94.
[8] Wong GG, Clark SC. Multiple actions of interleukin 6 within a cytokine network. Immunol Today 1988;9(5):137–9.
[9] Libermann TA, Baltimore D. Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Mol Cell Biol 1990;10(5):2327–34.
[10] Anderson KV, Nusslein-Volhard C. Information for the dorsal–ventral pattern of the Drosophila embryo is stored as maternal mRNA. Nature 1984;311(5983):223–7.
[11] Gay NJ, Keith FJ. Drosophila Toll and IL-1 receptor. Nature 1991;351(6325):355–6.
[12] Ghosh S, Gifford AM, Riviere LR, Tempst P, Nolan GP, Baltimore D. Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal. Cell 1990;62(5):1019–29.
[13] Cao Z, Henzel WJ, Gao X. IRAK: a kinase associated with the interleukin-1 receptor. Science 1996;271(5252):1128–31.
[14] Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science 1988;242(4878):540–6.
[15] Grosshans J, Bergmann A, Haffter P, Nusslein-Volhard C. Activation of the kinase Pelle by Tube in the dorsoventral signal transduction pathway of Drosophila embryo. Nature 1994;372(6506):563–6.
[16] Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 1996;86(6):973–83.
[17] Ip YT, Reach M, Engstrom Y, Kadalayil L, Cai H, Gonzalez-Crespo S, et al. Dif, a dorsal-related gene that mediates an immune response in Drosophila. Cell 1993;75(4):753–63.
[18] Medzhitov R, Preston-Hurlburt P, Janeway Jr CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 1997;388(6640):394–7.
[19] Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci USA 1998;95(2):588–93.
[20] Poltorak A, He X, Smirnova I, Liu MY, Huffel CV, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282(5396):2085–8.
[21] Bowie AG, Haga IR. The role of Toll-like receptors in the host response to viruses. Mol Immunol 2005;42(8):859–67.
[22] Stacey KJ, Sweet MJ, Hume DA. Macrophages ingest and are activated by bacterial DNA. J Immunol 1996;157(5):2116–22.
[23] Sparwasser T, Miethke T, Lipford G, Erdmann A, Hacker H, Heeg K, et al. Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-alpha-mediated shock. Eur J Immunol 1997;27(7):1671–9.
[24] Kengatharan M, De Kimpe SJ, Thiemermann C. Analysis of the signal transduction in the induction of nitric oxide synthase by lipoteichoic acid in macrophages. Br J Pharmacol 1996;117(6):1163–70.
[25] O'Neill LA, Dinarello CA. The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. Immunol Today 2000;21(5):206–9.
[26] Hultmark D. Macrophage differentiation marker MyD88 is a member of the Toll/IL-1 receptor family. Biochem Biophys Res Commun 1994;199(1):144–6.
[27] Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity 1997;7(6):837–47.
[28] Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, et al. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 2001;413(6851):78–83.
[29] Horng T, Barton GM, Medzhitov R. TIRAP: an adapter molecule in the Toll signaling pathway. Nat Immunol 2001;2(9):835–41.
[30] Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TICAM-2: a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 2003.
[31] Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3- mediated interferon-beta induction. Nat Immunol 2003;4(2):161–7.
[32] Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol 2002;169(12):6668–72.
[33] Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 Signaling to IRF-3/7 and NF-KB Involves the Toll Adapters TRAM and TRIF. J Exp Med 2003.
[34] Bin LH, Xu LG, Shu HB. TIRP: a novel TIR domain-containing adapter protein involved in Toll/interleukin-1 receptor signalling. J Biol Chem 2003;28:28.
[35] O'Neill LA, Fitzgerald KA, Bowie AG. The Toll-IL-1 receptor adaptor family grows to five members. Trends Immunol 2003;24(6):286–90.

[36] Zhang FX, Kirschning CJ, Mancinelli R, Xu XP, Jin Y, Faure E, et al. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J Biol Chem 1999;274(12):7611–4.
[37] Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, et al. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell 1998;2(2):253–8.
[38] Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413(6857):732–8.
[39] Li S, Strelow A, Fontana EJ, Wesche H. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. Proc Natl Acad Sci USA 2002;99(8):5567–72.
[40] Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV. TRAF6 is a signal transducer for interleukin-1. Nature 1996;383(6599):443–6.
[41] Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, et al. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature 2002;416(6882):750–6.
[42] Qin J, Jiang Z, Qian Y, Casanova JL, Li X. IRAK4 kinase activity is redundant for interleukin-1 (IL-1) receptor-associated kinase phosphorylation and IL-1 responsiveness. J Biol Chem 2004;279(25):26748–53.
[43] Lye E, Mirtsos C, Suzuki N, Suzuki S, Yeh WC. The role of interleukin 1 receptor-associated kinase-4 (IRAK-4) kinase activity in IRAK-4-mediated signaling. J Biol Chem 2004;279(39):40653–8.
[44] Li X, Commane M, Jiang Z, Stark GR. IL-1-induced NFkappa B and c-Jun N-terminal kinase (JNK) activation diverge at IL-1 receptor-associated kinase (IRAK). Proc Natl Acad Sci USA 2001;98(8):4461–5.
[45] Swantek JL, Tsen MF, Cobb MH, Thomas JA. IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. J Immunol 2000;164(8):4301–6.
[46] Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 2001;412(6844):346–51.
[47] Qian Y, Commane M, Ninomiya-Tsuji J, Matsumoto K, Li X. IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFkappa B. J Biol Chem 2001;276(45):41661–7.
[48] Jiang Z, Ninomiya-Tsuji J, Qian Y, Matsumoto K, Li X. Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. Mol Cell Biol 2002;22(20):7158–67.
[49] Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 2000;18:621–63.
[50] Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, et al. IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science 1997;278(5339):860–6.
[51] Regnier CH, Song HY, Gao X, Goeddel DV, Cao Z, Rothe M. Identification and characterization of an IkappaB kinase. Cell 1997;90(2):373–83.
[52] Woronicz JD, Gao X, Cao Z, Rothe M, Goeddel DV. IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. Science 1997;278(5339):866–9.
[53] Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell 1997;91(2):243–52.
[54] Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, et al. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 2001;413(6851):78–83.
[55] Horng T, Barton GM, Flavell RA, Medzhitov R. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature 2002;420(6913):329–33.
[56] Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999;11(1):115–22.
[57] Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 2002;420(6913):324–9.
[58] Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol 2002;169(12):6668–72.
[59] Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med 2003;198(7):1043–55.
[60] Sharma RP, He Q, Johnson VJ. Deletion of IFN-gamma reduces fumonisin-induced hepatotoxicity in mice via alterations in inflammatory cytokines and apoptotic factors. J Interferon Cytokine Res 2003;23(1):13–23.
[61] Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, et al. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol 2003;171(8):4304–10.
[62] Jiang Z, Zamanian-Daryoush M, Nie H, Silva AM, Williams BR, Li X. Poly(I-C)-induced Toll-like receptor 3 (TLR3)-mediated activation of NFkappa B and MAP kinase is through an interleukin-1 receptor-associated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. J Biol Chem 2003;278(19):16713–9.
[63] Jiang Z, Mak TW, Sen G, Li X. Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. Proc Natl Acad Sci USA 2004;101(10):3533–8.
[64] Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, et al. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 2004;5(5):503–7.
[65] Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol 2001;167(10):5887–94.
[66] Gohda J, Matsumura T, Inoue J. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not toll/IL-1 receptor domain-containing adaptor-inducing IFN-beta (TRIF)-dependent pathway in TLR signaling. J Immunol 2004;173(5):2913–7.
[67] Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;301(5633):640–3.
[68] Covert MW, Leung TH, Gaston JE, Baltimore D. Achieving stability of lipopolysaccharide-induced NF-kappaB activation. Science 1854-;309(5742):7.

[69] Werner SL, Barken D, Hoffmann A. Stimulus specificity of gene expression programs determined by temporal control of IKK activity. Science 2005;309(5742):1857–61.
[70] Ziegler SF, Wilson CB, Perlmutter RM. Augmented expression of a myeloid-specific protein tyrosine kinase gene (hck) after macrophage activation. J Exp Med 1988;168(5):1801–10.
[71] Boulet I, Ralph S, Stanley E, Lock P, Dunn AR, Green SP, et al. Lipopolysaccharide- and interferon-gamma-induced expression of hck and lyn tyrosine kinases in murine bone marrow-derived macrophages. Oncogene 1992;7(4):703–10.
[72] Geng Y, Zhang B, Lotz M. Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. J Immunol 1993;151(12):6692–700.
[73] Orlicek SL, Hanke JH, English BK. The src family-selective tyrosine kinase inhibitor PP1 blocks LPS and IFN-gamma-mediated TNF and iNOS production in murine macrophages. Shock 1999;12(5):350–4.
[74] Meng F, Lowell CA. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. J Exp Med 1997;185(9):1661–70.
[75] Mukhopadhyay S, Mohanty M, Mangla A, George A, Bal V, Rath S, et al. Macrophage effector functions controlled by Bruton's tyrosine kinase are more crucial than the cytokine balance of T cell responses for microfilarial clearance. J Immunol 2002;168(6):2914–21.
[76] Jefferies CA, O'Neill LA. Bruton's tyrosine kinase (Btk)-the critical tyrosine kinase in LPS signalling? Immunol Lett 2004;92(1–2):15–22.
[77] Jefferies CA, Doyle S, Brunner C, Dunne A, Brint E, Wietek C, et al. Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor kappaB activation by Toll-like receptor 4. J Biol Chem 2003;278(28):26258–64.
[78] Doyle SL, Jefferies CA, O'Neill LA. Bruton's tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of p65 on serine 536 during NFkappaB activation by lipopolysaccharide. J Biol Chem 2005;280(25):23496–501.
[79] Gray P, Dunne A, Brikos C, Jefferies CA, Doyle SL, O'Neill LA. MyD88 adapter-like (Mal) is phosphorylated by Bruton's tyrosine kinase during TLR2 and TLR4 signal transduction. J Biol Chem 2006;281(15):10489–95.
[80] Li CC, Korner M, Ferris DK, Chen E, Dai RM, Longo DL. NF-kappa B/Rel family members are physically associated phosphoproteins. Biochem J 1994;303(Pt 2):499–506.
[81] Naumann M, Scheidereit C. Activation of NF-kappa B in vivo is regulated by multiple phosphorylations. EMBO J 1994;13(19):4597–607.
[82] Heissmeyer V, Krappmann D, Wulczyn FG, Scheidereit C. NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes. EMBO J 1999;18(17):4766–78.
[83] Schmitz ML, dos Santos Silva MA, Baeuerle PA. Transactivation domain 2 (TA2) of p65 NF-kappa B. Similarity to TA1 and phorbol ester-stimulated activity and phosphorylation in intact cells. J Biol Chem 1995;270(26):15576–84.
[84] Bird TA, Schooley K, Dower SK, Hagen H, Virca GD. Activation of nuclear transcription factor NF-kappaB by interleukin-1 is accompanied by casein kinase II-mediated phosphorylation of the p65 subunit. J Biol Chem 1997;272(51):32606–12.
[85] Anrather J, Csizmadia V, Soares MP, Winkler H. Regulation of NF-kappaB RelA phosphorylation and transcriptional activity by p21(ras) and protein kinase Czeta in primary endothelial cells. J Biol Chem 1999;274(19):13594–603.
[86] Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. J Biol Chem 1999;274(43):30353–6.
[87] Wang D, Baldwin Jr AS. Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. J Biol Chem 1998;273(45):29411–6.
[88] Wang D, Westerheide SD, Hanson JL, Baldwin Jr AS. Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. J Biol Chem 2000;275(42):32592–7.
[89] Schmitz ML, Baeuerle PA. The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. EMBO J 1991;10(12):3805–17.
[90] Schmitz ML, Bacher S, Kracht M. I kappa B-independent control of NF-kappa B activity by modulatory phosphorylations. Trends Biochem Sci 2001;26(3): 186–90.
[91] Zhong H, Voll RE, Ghosh S. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Mol Cell 1998;1(5):661–71.
[92] Zhong H, SuYang H, Erdjument-Bromage H, Tempst P, Ghosh S. The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. Cell 1997;89(3):413–24.
[93] Zhong H, May MJ, Jimi E, Ghosh S. The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. Mol Cell 2002;9(3):625–36.
[94] Yang F, Tang E, Guan K, Wang CY. IKK beta plays an essential role in the phosphorylation of RelA/p65 on serine 536 induced by lipopolysaccharide. J Immunol 2003;170(11):5630–5.
[95] Buss H, Dorrie A, Schmitz ML, Hoffmann E, Resch K, Kracht M. Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. J Biol Chem 2004;279(53):55633–4.
[96] Leitges M, Sanz L, Martin P, Duran A, Braun U, Garcia JF, et al. Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. Mol Cell 2001;8(4):771–80.
[97] Yin L, Wu L, Wesche H, Arthur CD, White JM, Goeddel DV, et al. Defective lymphotoxin-beta receptor-induced NF-kappaB transcriptional activity in NIK-deficient mice. Science 2001;291(5511):2162–5.
[98] Kravchenko VV, Mathison JC, Schwamborn K, Mercurio F, Ulevitch RJ. IKKi/IKKepsilon plays a key role in integrating signals induced by pro-inflammatory stimuli. J Biol Chem 2003;278(29):26612–9.
[99] Tojima Y, Fujimoto A, Delhase M, Chen Y, Hatakeyama S, Nakayama K, et al. NAK is an IkappaB kinase-activating kinase. Nature 2000;404(6779):778–82.
[100] Bonnard M, Mirtsos C, Suzuki S, Graham K, Huang J, Ng M, et al. Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. EMBO J 2000;19(18):4976–85.
[101] Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature 2000;406(6791):86–90.
[102] Martin M, Rehani K, Jope RS, Michalek SM. Toll-like receptor-mediated cytokine production is differentially

regulated by glycogen synthase kinase 3. Nat Immunol 2005;6(8):777–84.
[103] Yamazaki S, Muta T, Takeshige K. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. J Biol Chem 2001;276(29):27657–62.
[104] Yamazaki S, Muta T, Matsuo S, Takeshige K. Stimulus-specific induction of a novel nuclear factor-kappaB regulator, IkappaB-zeta, via Toll/Interleukin-1 receptor is mediated by mRNA stabilization. J Biol Chem 2005;280(2):1678–87.
[105] Motoyama M, Yamazaki S, Eto-Kimura A, Takeshige K, Muta T. Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein. J Biol Chem 2005;280(9):7444–51.
[106] Ogawa S, Lozach J, Benner C, Pascual G, Tangirala RK, Westin S, et al. Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. Cell 2005;122(5):707–21.
[107] Wietek C, Miggin SM, Jefferies CA, O'Neill LA. Interferon regulatory factor-3-mediated activation of the interferon-sensitive response element by Toll-like receptor (TLR) 4 but not TLR3 requires the p65 subunit of NF-kappa. J Biol Chem 2003;278(51):50923–31.
[108] Leung TH, Hoffmann A, Baltimore D. One nucleotide in a kappaB site can determine cofactor specificity for NF-kappaB dimers. Cell 2004;118(4):453–64.
[109] Hoffmann A, Leung TH, Baltimore D. Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. EMBO J 2003;22(20):5530–9.