The history of Toll-like receptors - redefining innate immunity.

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Abstract

The discovery of Toll-like receptors (TLRs) was an important event for immunology research and was recognized as such with the awarding of the 2011 Nobel Prize in Physiology or Medicine to Jules Hoffmann and Bruce Beutler, who, together with Ralph Steinman, the third winner of the 2011 Nobel Prize and the person who discovered the dendritic cell, were pioneers in the field of innate immunity. TLRs have a central role in immunity — in this Timeline article, we describe the landmark findings that gave rise to this important field of research.

Introduction

Before the discovery of Toll-like receptors (TLRs), innate immunity was seen as a crude and unsophisticated part of the immune system; its main purpose was considered by immunologists to be the initiation of the more sophisticated adaptive immune response, which was thought to confer protection on the infected organism. In addition, innate immunity was implicated in systemic responses such as fever. The molecular basis for innate immunity was not known, particularly with respect to how innate immune agents such as the cytokines interleukin-1 (IL-1), tumour necrosis factor (TNF) and IL-6 were induced. In addition, the signalling events that promote the expression of the antiviral interferons (IFNs) were unclear. The characterization of TLRs has provided molecular insights into these processes and has also allowed the discovery of other families of innate immune receptors, revealing new perspectives to researchers in immunology.

In this Timeline article, we describe the beginnings of this field of research, following the identification of the IL-1 receptor (IL-1R) in mammals and the cell surface protein Toll inDrosophila melanogaster. We discuss the identification of the mammalian Toll-like receptor 4 (TLR4) as the lipopolysaccharide (LPS) receptor, the subsequent discovery of further ligands for different TLRs and the elucidation of TLR signalling pathways (Timeline). Finally, we briefly review the evolution of our understanding about the role of TLRs in disease and the therapeutic applications of TLR agonists and antagonists.

TIR domains: flies, plants and mammals

TLRs are prototype pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) from microorganisms or danger-associated molecular patterns (DAMPs) from damaged tissue. The idea that such innate immune receptors exist dates back to 1989, when Charles Janeway1 predicted, in an important monograph, that PRRs recognizing

microbial products link innate and adaptive immunity1. The question of how research into TLRs began can be answered in several ways, but the first molecule of relevance to be identified was IL-1R type 1 (IL-1R1). IL-1 is a pleiotropic pro-inflammatory cytokine, and it was reported by several laboratories in the 1980s to be involved in T cell activation, pyrogenicity, the promotion of cartilage breakdown and the activation of the acute phase response2. In 1988, the gene encoding IL-1R1 was cloned, but the predicted sequence gave no clues as to the mechanism by which it might signal, as there were no recognizable motifs in the cytosolic domain3. In 1991, this domain was shown to be homologous to the cytosolic domain of a D. melanogaster protein termed Toll4. This was unexpected, as at that time the only known function of Toll was to promote dorsoventral polarity in the developing D. melanogaster embryo5.

Notably, the establishment of dorsoventral polarity in flies had been shown to involve a protein termed Dorsal, which shares a REL homology domain with the other members of the nuclear factor-κB (NF-κB) family of transcription factors6. At that time, NF-κB, which was first identified in B cells as a factor that was activated by the Gram-negative bacterial cell wall constituent LPS7, was emerging as a major component of inflammation and infection. Indeed, remarkable numbers of genes with roles in immunity and inflammation were shown at that time to be regulated by NF-κB. Moreover, IL-1 had been found to activate NF-κB signalling8. This suggested that the same highly efficient signalling mechanism might be used both in development in D. melanogaster and in pro-inflammatory signalling in mammals. So, it was established that the highly similar proteins Toll and IL-1R1 have important NF-κB-dependent roles in two different contexts; this was particularly evident after Toll and IL-1R1 were shown to share common amino acids that are essential for NF-κB signalling9.

In 1994, the next important step in the development of the TLR field involved the characterization of a plant protein that confers resistance to tobacco mosaic virus — the N protein10. The amino-terminal domain of the N protein was found to be similar to the cytoplasmic domains of Toll and IL-1R1, which indicated that this conserved domain is involved in host defence in two disparate kingdoms — the plant and the animal kingdoms. This conserved domain was named the Toll–IL-1-resistence (TIR) domain.

Importantly, at around the same time (in 1993) Michael Levine and colleagues11 reported that Dif, another member of the NF-κB family in D. melanogaster, translocated from the cytoplasm to the nucleus following bacterial infection or injury in the larval fat body. This study also

demonstrated that Dif binds to a κB-like sequence in the promoter of the gene that encodes the antimicrobial peptide cecropin. Furthermore, it was shown that Dif was activated by the constitutively active mutant of Toll, termed Toll10b (Ref. 11). These studies built on earlier work by Hans Boman12, who was the first to describe the antimicrobial peptides cecropin and attacin in the moth Hyalophora cecropia12.

The apparent association of a Toll mutant with the Dif-dependent induction of antimicrobial peptides, together with the earlier described link between Toll and Dorsal, led Jules Hoffmann's laboratory to postulate that Toll might regulate not only developmental processes but also immune gene expression. Definitive proof for this hypothesis was provided in 1996 by Bruno Lemaitre13, a member of the Hoffman laboratory, in the context of the Toll-mediated induction of the antifungal peptide Drosomycin13. Indeed, Lemaitre showed that, after microbial infection, Drosomycin expression was upregulated following activation of the Toll pathway14 — work for which the Nobel Prize was awarded. It was subsequently shown that Dif, and not Dorsal, was the main regulator of antimicrobial peptide expression in adult flies15.

Mammalian proteins that were more similar to Toll than to IL-1R1 had been spotted in the PubMed database as early as 1994 (Ref. 16). These proteins were predicted to have TIR domains, as well as leucine-rich repeats that are similar to those of Toll, and to differ from IL-1R1 in terms of their lack of immunoglobulin domains. In 1997, one of these mammalian Toll homologues, which was termed hToll at the time, was cloned and studied by Ruslan Medzhitov and Janeway17. They showed that transfection of human monocytes with a CD4–hToll chimeric protein (predicted to be constitutively active in the absence of ligand) led to the activation of NF-κB and to the expression of NF-κB-dependent genes, including the gene encoding CD80 (Ref. 17). CD80 is a protein that provides co-stimulation via CD28 to T cells, and this highly important finding provided one of the first observed links between innate and adaptive immunity, as innate hToll signalling in antigen-presenting cells is associated with CD80 expression and T cell activation. This landmark discovery of the function of hToll fulfilled the criterion that had been postulated by Janeway for the identification of PRRs1 — that they would provide an important link between innate and adaptive immunity.

In 1998, five mammalian Toll homologues were described and named Toll-like receptors (TLRs) — these included hToll which was renamed as TLR4 (Ref. 18). At the time, no function was ascribed to mammalian TLRs but, as Toll was involved in innate immunity in D. melanogaster,

there was a strong suspicion that TLRs would be involved in innate immunity — TLRs were the ideal PRR candidates1. Moreover, LPS was an obvious candidate PAMP that might be sensed by a TLR, given its ability to activate NF-κB. So, almost 10 years after the publication of his monograph, Janeway's contribution to the field of TLRs was shown to be crucial, as it had inspired several researchers (including Medzhitov, who was working with Janeway) to engage in the search for PRRs.

The LPS receptor uncovered

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LPS had been studied in great detail as an important component of endotoxin — the substance that had been identified as the ill-defined causative agent of Gram-negative bacteria-induced sepsis. However, it was a challenging reagent to work with, as there is diversity in the polysaccharides present on any given individual strain of bacteria. Few receptors in the history of immunology had received more attention than the LPS receptor, but attempts to determine the identity of this receptor were hampered by the difficulties in working with a ligand that was so structurally heterogeneous and impossible to purify to homogeneity. The importance of lipid A — the lipid moiety of LPS — for the inflammatory activity of LPS was first described in 1954 (Ref. 19). However, the correct structure of lipid A was not properly recognized until 1983 (Ref. 20). Synthetic forms of lipid A, which were devoid of impurities and much easier to work with than natural LPS, became available in 1985 and provided an important way to determine whether previously reported effects of endotoxin were mediated by LPS or simply by biological contaminants.

Between 1960 and 1965, a spontaneous mutation occurred in the C3H/HeJ mouse colony at The Jackson Laboratory that rendered the colony resistant to LPS toxicity. The genetic basis of that resistance was determined to be under the control of a single autosomal gene named Lpsd. In 1978, Lpsd was mapped to mouse chromosome four by backcross-linkage analysis between the C3H/HeJ and the C57BL/6J mouse strains.

In the early 1980s, a binding protein for LPS was discovered in serum and was termed lipopolysaccharide-binding protein (LBP)21. Later, in 1986, LPS was shown to activate NF-κB7; subsequently, Jiahuai Han et al.22 reported that LPS also activated p38 mitogen-activated protein kinase (MAPK)22. It was known that p38 MAPK could also be activated by IL-1, as had been shown to be the case for NF-κB, which suggested that the receptor for LPS resembled IL-1R1 in terms of their downstream-activated signalling pathways.

Many individuals attempted to identify a signalling receptor for LPS. Scavenger receptors, such as scavenger receptor A (SRA), were shown to bind to the lipid A moiety of LPS, but were subsequently shown to be involved in the degradation of lipid A rather than in LPS-induced signalling23. In 1990, an 18–25 kDa LPS-binding protein was identified that was almost certainly the LPS co-receptor myeloid differentiation factor 2 (MD2), but at that time there was little further investigation into this observation24. Shortly after this, seminal papers showed that LBP works together with CD14 to initiate LPS signalling, and that CD14 increases the sensitization of cells to LPS by a factor of 1,000–10,000 (Refs 25, 26). A popular concept at the time was that CD14 — which is a glycosylphosphatidylinositol (GPI)-anchored protein — initiates signalling via its GPI anchor, perhaps by directly activating a protein kinase. Later reports provided strong evidence that, contrary to what had been initially thought, CD14 was not a signalling receptor, as both a soluble and a transmembrane form of CD14 were able to mediate LPS responses27, 28. These observations initiated a thorough search for a CD14-associated signal transducer.

Despite considerable effort, the immunological community only began to make real progress in the hunt for the LPS receptor following the identification in 1997 of TLR4 (Ref. 17) (discussed above) — and the subsequent finding about a year later by Ruey-Bing Yang and colleagues29 that expression of a TLR conferred cellular responsiveness to preparations of endotoxin29. The study by Yang and colleagues was extremely important for the immunological community because it demonstrated that TLRs were in fact true PRRs. However, this work proved to be partly flawed, as the LPS receptor was misidentified as TLR2. Expression of TLR2 did in fact confer LPS responsiveness to transfected HEK293 cells, but only because the LPS preparations that had been used were contaminated with small but active amounts of bacterial lipoprotein30, which is a potent TLR2 agonist. Indeed, despite the strength of this early paper by Yang and colleagues, Craig Gerard31 warned in an accompanying news piece that, on the basis of the chromosomal location of the known TLRs18, the Lpsd gene was likely to encode TLR4 (Ref. 31).

The breakthrough came later, in 1998, when Bruce Beutler's32 group positionally cloned the Lpsd gene — work for which the Nobel Prize was awarded. This study definitively identified Lpsd as Tlr4 (Ref. 32). Shortly after that, Danielle Malo's33 group also reported that Lpsd was in fact Tlr4 (Ref. 33). Furthermore, a study published later, in 1999, by ShizuoAkira and colleagues34 showed that TLR4-knockout mice failed to respond to LPS, which confirmed that TLR4 is the signalling receptor for LPS34.

However, TLR4 was soon found to lack LPS-binding activity and, therefore, it was unclear how it would function as an LPS receptor until MD2 was shown in 1999 to confer LPS responsiveness to TLR4 (Ref. 35). Initially, MD2 was thought to be a chaperone for TLR4, but in 2001 it was demonstrated that a soluble (extracellular) form of MD2 functions as a TLR4 co-receptor, which suggested that the MD2–LPS complex was the essential ligand for TLR4 (Ref. 36). Indeed, co-crystallization structures of the TLR4–MD2 complex with a rough mutant form of LPS bound in the lipid A binding pocket were reported in 2009 (Ref. 37) and showed TLR4–MD2 dimers to be the basic signalling units for the LPS receptor. Umeharu Ohto et al.38 more recently published findings using co-crystallization structures of the mouse and human forms of the TLR4–MD2 complex bound to a synthetic lipid A precursor known as lipid IVa38. This study demonstrated that an inducible conformational change in MD2, which is brought about by the binding of the ligand to MD2, results in the formation of an 'm'-shaped complex; the authors showed that this is the principal event that is required for LPS activity38. A clear molecular understanding of the mechanism of recognition of LPS, the causative agent of Gram-negative bacteria-induced septic shock, had finally been achieved and the role of TLR4 as a PRR had been established.

Mammalian TLR ligands

In 2001, TLR5 was shown to sense bacterial flagellin — a protein component of flagella — and further work showed that TLR5 could regulate both innate and adaptive responses to bacteria in the intestine45, 46, 47. A role for TLR10 has not yet been shown, although the sequence of this receptor is known to be most similar to TLR1, and, therefore, TLR10 might heterodimerize with TLR2. Mouse TLR11 has been shown to detect a component of uropathogenic bacteria48 and, in cooperation with mouse TLR12, to bind to the Toxoplasma gondii profilin protein49, 50, 51. Moreover, mouse TLR13 has been very recently shown to recognize bacterial ribosomal RNA52, 53, 54.

Before the discovery of TLR3 in 2001, it was thought that TLRs were not involved in antiviral responses; however, the previous discovery, in 2000, that viral proteins antagonized TLR4

signalling (Ref. 55), together with the observation that the fusion protein from respiratory syncytial virus mediated responses via TLR4 (Ref. 56), had already prompted the idea that TLR ligands might be derived from viruses as well as from bacteria. Indeed, in 2001, TLR3 was shown to recognize double-stranded RNA, which is a major component of many viruses, and to mediate activation of the NF-κB and type I IFN signalling pathways57 (Fig. 1). Structural studies indicated that a common mechanism might underly the activation of all TLRs, as the receptor–ligand structures for TLR2 and TLR3 (Ref. 58) complexes both adopted an 'm'-shaped TLR dimer, as was reported for TLR4 (Ref. 59). In 2012, the crystal structure of zebrafish Tlr5 in complex with a fragment of flagellin was resolved, and, although the receptor–ligand interaction modes were distinct from those of other non-protein TLR ligands, flagellin binding also caused the formation of an 'm'-shaped receptor dimer60.

TLR9 was the next TLR to be implicated in antiviral responses. In 2000, TLR9 had been identified as the receptor for CpG-rich hypomethylated DNA motifs61, which are frequent in bacteria but rare in vertebrates. It was later found that TLR9 also responds to herpesvirus DNA62, 63, 64. In 2002, TLR7 was characterized as another antiviral TLR, as it was shown to sense the synthetic chemical imiquimod, which was known to stimulate antiviral responses65. Subsequently, in 2004, both TLR7 and the related receptor TLR8 were shown to sense single-stranded viral RNA66, 67, 68.

In recent years, TLRs have also been shown to recognize endogenous ligands. From 2002 to 2005, host nucleic acids were found to function as ligands for TLR9, TLR7 and TLR8 in certain contexts69, 70, 71, 72. All of these findings established that TLRs are a family of receptors that can initiate innate immunity and inflammation in response to danger signals in the form of infection or tissue damage.

Elucidation of TLR signalling

The intricate signalling pathways that are downstream of mammalian TLRs have been resolved over the past 15 years (Fig. 1). In 1997, myeloid differentiation primary-response protein 88

(MYD88) — a protein that was initially recognized to be involved in myeloid cell differentiation — was shown to bind to IL-1R1 to drive signalling through the NF-κB pathway73, 74. MYD88 was found to have a TIR domain, which suggested that TIR-homotypic interactions with TIR domain-containing receptors were involved in MYD88 signalling. MYD88 was also shown to have a death domain that interacts with the death domain of a protein kinase — termed IL-1R-associated kinase 1 (IRAK1). Following on from this finding, other IRAKs were discovered and IRAK4 was shown to be the most important receptor-proximal kinase (reviewed in Ref. 75). IRAK1 was shown to interact with TNF receptor-associated factor 6 (TRAF6), which is a member of the TRAF family of proteins that are known to activate the NF-κB pathway. Signalling to NF-κB, p38 MAPK and JUN N-terminal kinase (JNK) was shown to occur downstream of TRAF6, and the kinase cascades, adaptor proteins and ubiquitylation reactions that are involved in these signalling pathways have now been well characterized. The same signals were shown to be induced downstream of TLR2, TLR5, TLR7 and TLR9 activation (Fig. 1), which is consistent with the idea that these TLRs are all MYD88-dependent TLRs76.

At the same time, it was realized that certain TLRs signal from endosomes. Initially, it was observed that CpG-DNA localizes to lysosomal compartments, where it recruits MYD88 (Ref. 77), and, subsequently, biochemical evidence confirmed that TLR3, TLR7 and TLR9 all localize to endosomes78. CpG-DNA was shown to induce the trafficking of TLR9 from the endoplasmic reticulum to the endolysosome for signalling79. Following this, interferon-regulatory factor 7 (IRF7) was identified as an important MYD88-dependent transcriptional regulator that was downstream of endosomal TLR7 and TLR9 and that has a role in the induction of type I IFN gene expression80, 81 (Fig. 1).

The next TIR adaptor protein to be discovered was MYD88-adaptor-like protein (MAL; also known as TIRAP)82, 83. In 2001, important studies using MYD88-deficient mice showed that the induction of the type I IFN response via IRF3, as well as the delayed NF-κB activation and dendritic cell (DC) activation that are downstream of TLR4, were MYD88-independent responses84, 85, 86, suggesting that MYD88-independent signalling can be activated downstream of TLRs. However, MAL was not shown to fulfil this MYD88-independent role, but was instead shown to be a bridging adaptor that links MYD88 to TLR4 and, to a lesser extent, to TLR2 (Refs 87, 88, 89, 90). By contrast, TIR domain-containing adaptor protein inducing IFNβ (TRIF; also known as TICAM1), a third TIR adaptor that was characterized between 2002 and 2003, was shown to be involved in the MYD88-independent TLR4 pathway, as well as in the MYD88-independent TLR3 signalling pathway91, 92, 93, 94 (Fig. 1). Following this, a fourth TIR

adaptor, TRIF-related adaptor molecule (TRAM; also known as TICAM2), was shown to link TRIF to TLR4 (Refs 95, 96, 97). Therefore, TLR4 was shown to have the most complex signalling arrangement of all the TLRs, activating either the MAL–MYD88 pathway to induce NF-κB signalling or the TRAM–TRIF pathway to induce IRF3 signalling (Fig. 1). The TRAM–TRIF pathway is activated downstream of endosomal TLR4, TLR7 and TLR9, which illustrates the importance of subcellular localization for differential signalling by TLRs (Fig. 1). In 2006, a fifth TIR adaptor, sterile-α- and armadillo-motif-containing protein 1 (SARM1), was shown to inhibit TRIF98. In addition, as SARM1 is the most evolutionarily conserved of the TIR adaptor molecules, it is thought that there are additional functions for SARM1 that remain to be discovered. Interestingly, SARM1 was recently shown to control axonal degeneration in both mice and flies99. Finally, a protein termed B cell adaptor for PI3K (BCAP; also known as PIK3AP1), which is produced by B cells, has a domain that is related to the TIR domain and has been proposed as a sixth TIR adaptor molecule. BCAP has been shown to modulate B cell activation by TLRs100, 101. In addition to BCAP, a large number of negative regulators of TLRs have been identified over the past decade; this, as well as the fact that overactivation of TLR-dependent innate immune responses can kill the host, shows the importance of TLR modulation.

TLRs in disease

As soon as it was realized that TLR4 was involved in LPS sensing and, therefore, that it could have a role in sepsis, it was predicted that targeting of TLRs might be important for the treatment of several diseases. In addition to interfering with TLR responses to treat pathogen infections, an obvious clinical application of the knowledge gained from TLR studies was to use TLR ligands as vaccine adjuvants102. Indeed, in 2005, it was demonstrated that TLR activation is an important aspect of adjuvancy in vaccines, as antigens alone fail to induce an antibody response (unless they are haptenated or aggregated)103. This understanding followed earlier studies that provided the first indications that TLR signalling is an important link between the innate and adaptive immune responses. Another earlier demonstration of the link between TLRs and adaptive immunity came from the finding that the vaccine adjuvant monophosphoryl lipid A, which is a less toxic version of LPS, promotes antibody responses via TLR4 activation104. Moreover, in 2000, TLRs were shown to be expressed on the major antigen-presenting cells, DCs105; and the BCG (bacillus Calmette–Guérin) vaccine against tuberculosis was shown to cause DC maturation via TLR2 and TLR4 signalling106. Bacterial lipopeptides were also shown to stimulate DC maturation via TLR2 1 year later (Ref. 107). Furthermore, Medzhitov and colleagues17 had demonstrated that, in human monocytes, overexpression of what became known as TLR4 could induce expression of the co-stimulatory molecule CD80 (Ref. 17) and mechanistically this was shown to involve TRIF108. Finally, imiquimod, which was already being used to treat genital warts caused by the papilloma virus, was identified as a TLR7 ligand in 2002

(Ref. 65). These and other findings showed the importance of the activation of TLRs in promoting adaptive immunity against pathogens and, consequently, in sustaining host defence.

TLR inhibition has also been attempted in the clinic, the goal of which is to limit excessive inflammation that is presumably driven by the overactivation of a particular TLR. Therapies involving the synthetic small-molecule inhibitor of TLR4 eritoran (also known as E5564) were trialed in patients with sepsis as early as in 2007. However, it ultimately had only marginal effects109, 110, possibly because it was administered at a late time point in the disease course. In 2010, oligonucleotide-based inhibitors of TLR7 and/or TLR9 were shown to have therapeutic potential in animal models of systemic lupus erythematosus, which is known to involve aberrant immune responses to host nucleic acids111. Finally, in 2012, an inhibitory antibody to TLR2 was shown to efficiently limit ischemia–reperfusion injury in the hearts of pigs112 and in the kidneys of mice113. This indicates that endogenous danger signals that are generated by ischemia-induced tissue damage are sensed by TLR2, which, in turn, promotes inflammation and tissue necrosis.

An important insight into the role of TLRs in human disease has come from the analysis of human genetic variation (reviewed in Ref. 114). Although single nucleotide polymorphisms in the genes encoding TLRs have been shown to confer a greater risk of developing infectious diseases, the overall effects are modest. By contrast, loss-of-function mutations in TLR-related genes have been strongly associated with susceptibility to infection; in 2007 TLR3 mutations were linked with increased risk of herpesvirus infection115, whereas, at approximately the same time, mutations in the genes encoding MYD88 (Ref. 116) and IRAK4 (Ref. 117) were found to correlate with increased susceptibility to pyogenic infections. Moreover, MYD88 mutations are frequently found in certain lymphomas, as was first reported in 2011 (Ref. 118). Notably, MYD88 deficiency can be lethal in childhood but it seems that if patients survive until adulthood, adaptive immunity is sufficient to protect them in later life116. The fact that individuals with deletions in the important signalling molecule MYD88 are only susceptible to a restricted range of pathogens challenges our view of the importance of TLRs and IL-1 in human host defence; indeed, it might indicate that there is redundancy among TLRs and other PRRs, such as the NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). It is also possible that TLRs have a more important role in repairing damaged tissue than in antimicrobial defence; this TLR function might be involved in inflammatory conditions, as in the case of ischemia–reperfusion injury (discussed above). Therefore, the true therapeutic potential of TLRs has not yet been realized.

Concluding comments

It was an exciting event for many immunologists when the 2011 Nobel Prize for Medicine or Physiology was partly awarded for the discovery of TLRs. However, the choice of researchers to whom the prize was awarded proved to be somewhat controversial — perhaps no surprise given the number of investigators who were involved in the many important discoveries in the field. It is worth noting that other prestigious prizes have also been awarded for the discovery of TLRs: Medzhitov and Hoffmann shared the 2009 Rosenstiel prize; Medzhitov, Hoffmann and Beutler shared the 2011 Shaw Prize; and Hoffmann and Akira shared both the 2011 Canadian Gairdner and the 2010 Keio prizes (Box 1). Overall, the field has benefited from many successful collaborations between laboratories all over the world, including the United States, Europe and Japan. We can all be grateful for the discoveries that have been made in the TLR field and that have led to a renaissance of interest in innate immunity, and we anticipate many more discoveries to come in a field that is in many ways still in its infancy. These discoveries will hopefully have major clinical implications for many diseases.

Figure 1

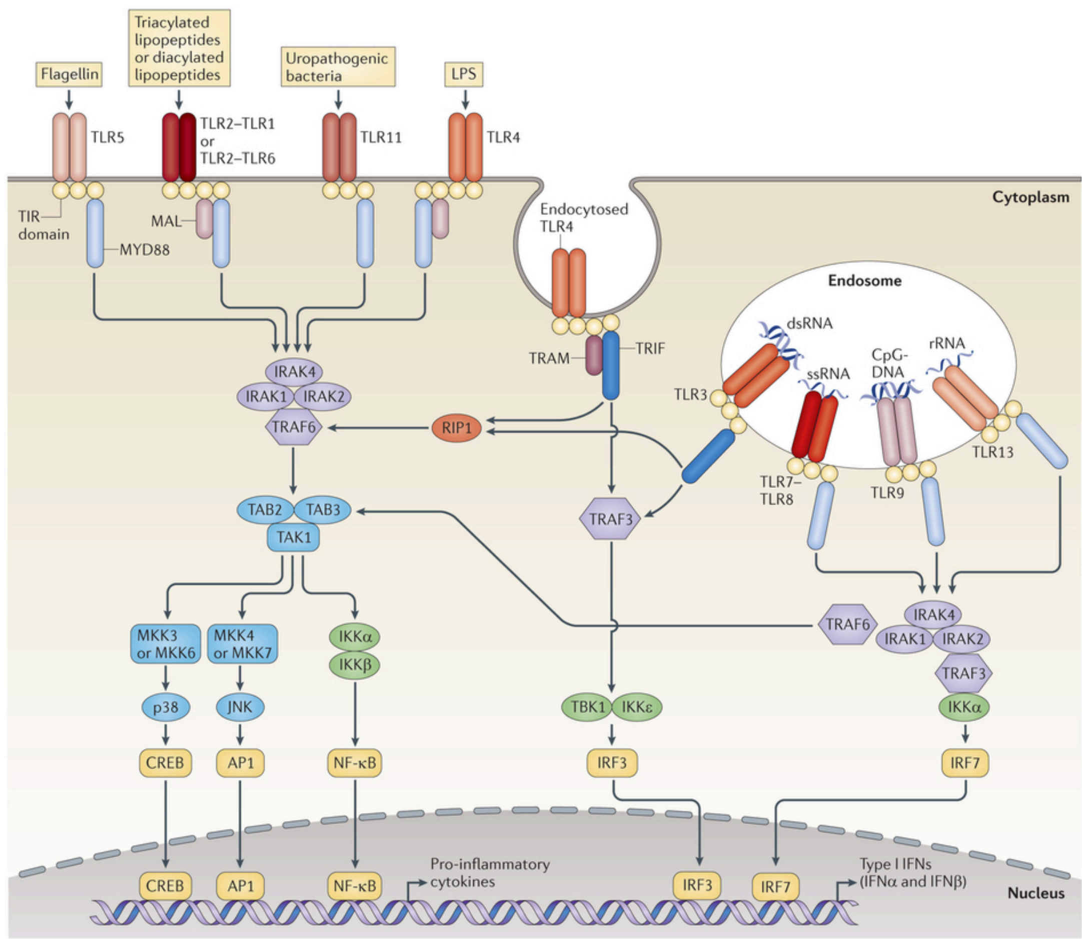
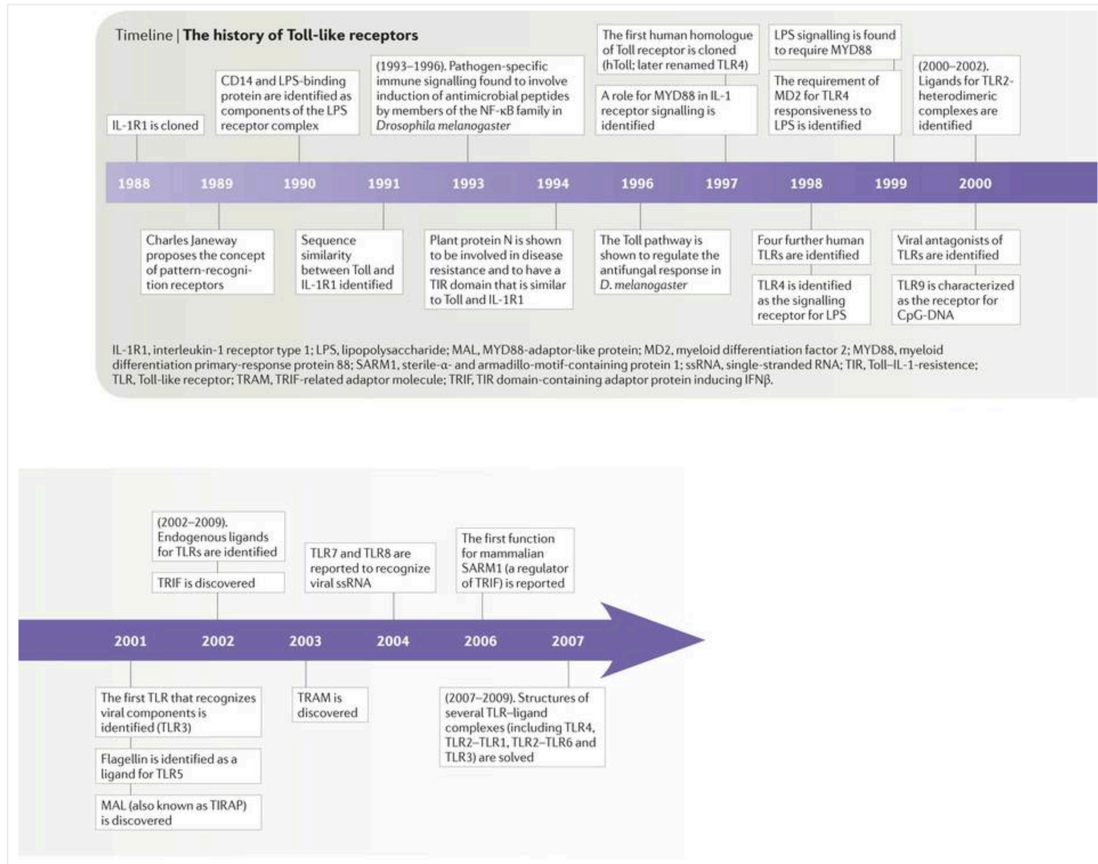


Figure 2



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