Mammalian Toll-like receptors

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Toll-like receptors (TLRs) are essential in the host defense against microbial pathogens. Individual TLRs recognize distinct structural components of pathogens and evoke inflammatory responses. Recent evidence indicates that TLRs recognize not only bacteria and fungi but also viruses. The molecular mechanisms by which TLRs induce differential gene expression are now beginning to be clarified.

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Abbreviations

DC	dendritic cell
HSP	heat-shock protein
IFN	interferon
IL	interleukin
IRAK	IL-1 receptor-associated kinase
LPS	lipopolysaccharide
MAP	mitogen-activated protein
NF-κB	nuclear factor κB
NOD	nucleotide-binding oligomerization domain
PAMP	pathogen-associated molecular pattern
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNF	tumor necrosis factor

Introduction

The innate immune system recognizes conserved motifs in pathogens termed 'pathogen-associated molecular patterns' (PAMPs; [1]). Toll-like receptors (TLRs) have an essential role in the innate recognition of PAMPs and in triggering acquired immunity in higher organisms [2,3]. So far, ten mammalian Toll-like receptors (TLR1–TLR10) have been identified. The TLR family is characterized by the presence of an extracellular domain containing leucine-rich repeats and a cytoplasmic Toll/IL-1 receptor (TIR) domain similar to that of the interleukin 1 (IL-1) receptor family. These receptor families function through the same signaling molecules, including MyD88, IL-1 receptor-associated kinase (IRAK), TNF receptor associated factor (TRAF) 6, mitogen-activated protein (MAP) kinases and nuclear factor (NF)-κB.

Individual TLRs recognize distinct structural components of pathogens (Figure 1). In this review, I discuss the function and signaling pathways of the mammalian TLR family, focusing on recent progress in this area.

Recognition of PAMPs by mammalian Toll-like receptors

TLR4

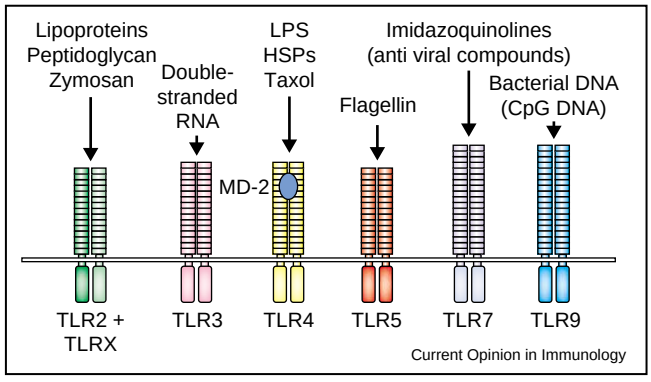
Lipopolysaccharide (LPS), a component of the outer membranes of Gram-negative bacteria, is a potent activator of macrophages and a causal agent of endotoxin shock. After being released into the bloodstream, LPS is captured immediately by LPS-binding protein, a specific lipid transfer protein that delivers LPS to CD14 present on the surfaces of mononuclear phagocytes. CD14 lacks a transmembrane domain and so is incapable of transducing signals, which suggests that other molecules must be responsible for LPS signaling.

Both the positional cloning of the locus responsible for LPS hyporesponsiveness in C3H/HeJ mice and the generation of TLR4 knockout mice have shown that TLR4 is essential for LPS signaling [4,5]. In addition, the interaction of LPS with TLR4 requires another molecule, MD-2, which associates with the extracellular domain of TLR4 [6]. Knockout mice have provided evidence that MD-2 is indispensable for and unique to TLR4 signaling; that is, MD-2 does not effect the response to peptidoglycan (a TLR2 ligand) or to DNA-containing CpG dinucleotides (a TLR9 ligand; [7•]).

Some mammalian species discriminate between different LPS structures. The lipid A analog lipid IVa is a potent antagonist in human cells but acts as an LPS mimetic in mouse cells. Another TLR4 ligand, taxol, is a plant-derived anticancer reagent that mimics the action of LPS in mice but not in humans. In addition, pentaacylated LPS stimulates murine but not human cells. These differences between humans and mice have been attributed to differences between the human and mouse TLR4 and/or MD-2 molecule [8–10].

Despite these data, there is no evidence for the direct binding of LPS to TLR4. Recently, a model has been proposed in which LPS is recognized by a cluster of receptors associated with lipid rafts [11]. The proteins CD14, heat-shock protein (HSP)70 and HSP90 are constitutively found in lipid rafts, whereas TLR4, the

Figure 1



Ligands recognized by TLRs. It is currently thought that TLR3, TLR4, TLR5, TLR7 and TLR9 deliver their signals by forming homodimers after interacting with their ligands, although there is no direct evidence for this. For TLR2, ligands are recognized by a heterodimer of TLR2 and another TLR (TLR1, TLR6 and probably TLR10). For TLR4, another secreted molecule, MD-2, is also required for ligand recognition.

chemokine receptor CXCR4 and growth differentiation factor 5 (GDF5) are recruited to lipid rafts after stimulation with LPS. This suggests that LPS stimulation generates the dynamic association of several receptors within lipid rafts. But whether the LPS-induced formation of a large receptor complex is linked to ligand-specific recognition of LPS remains unclear, as do the consequent cellular responses.

TLR2 (TLR1, TLR6)

TLR2 recognizes many different microbial components, including peptidoglycan from Gram-positive bacteria such as *Staphylococcus aureus*, lipoproteins and lipopeptides from several bacteria, glycophosphatidylinositol anchors from *Trypanosoma cruzi*, lipoarabinomannan from *Mycobacterium tuberculosis*, porins from *Neisseria meningitides*, and the yeast cell-wall component zymosan.

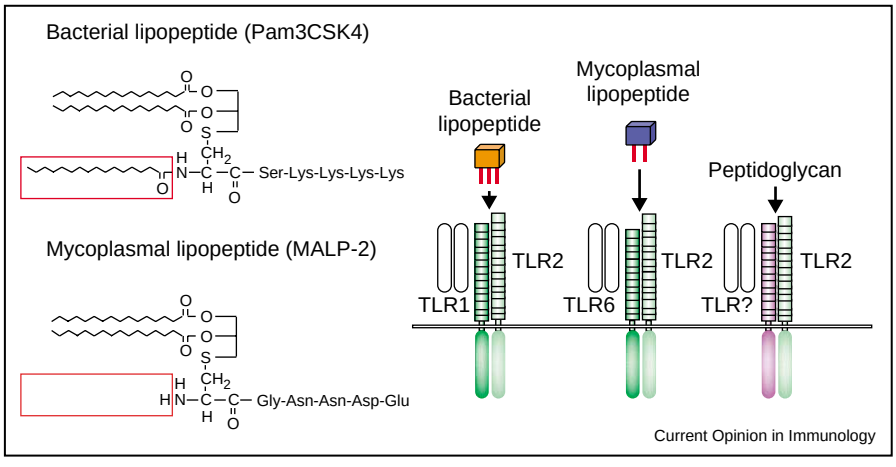
Aderem and co-workers [12] first suggested the possibility that TLR2 ligands are recognized by heterodimers formed between TLR2 and other TLRs. This idea has been confirmed by analyses of knockout mice [13,14•]: neither TLR2-deficient nor TLR6-deficient macrophages respond to the synthetic mycoplasmal lipopeptide MALP-2, whereas TLR6-deficient but not TLR2-deficient macrophages respond normally to another synthetic lipopeptide PAM3CSK4.

All lipoproteins contain a lipolyated amino-terminal residue, and it is this lipid moiety that is responsible for their immunostimulatory activities. PAM3CSK4 contains a triacylated cysteine residue at its amino terminus, whereas the cysteine residue in MALP-2 is only diacylated. Replacement of the lipid portion of MALP-2 with that of PAM3CSK4 results in the activation of TLR6-deficient macrophages, showing that TLR6 can discriminate between the subtle differences in the lipid portions of these lipopeptides (O Takeuchi, S Akira, unpublished data, [54]). Recent studies have shown that triacylated lipoproteins or lipopeptides, such as the 19 kDa lipoproteins of *M. tuberculosis*, the outer-surface lipoprotein of spirochete *Borrelia burgdorferi*, PAM3CSK4 and N-PAM-S-Lau$_2$CSK, are preferentially recognized by a heterodimer formed between TLR2 and TLR1 (Figure 2; [14•,15•]).

TLR5

Flagellin is a 55 kDa monomer obtained from bacterial flagella, polymeric rod-like appendages extending from the outer membrane of Gram-negative bacteria that propel the organisms through their aqueous environment.

Figure 2



Recognition of TLR2 ligands by heterodimers. In cooperation with TLR2, TLR1 and TLR6 recognize the structural difference between bacterial lipopeptide and mycoplasmal lipopeptide. Peptidoglycan is likely to be recognized by a TLR2 homodimer or a heterodimer of TLR2 and an unknown TLR.

Flagellin is also a potent pro-inflammatory factor, whose signaling has been shown to be mediated through TLR5 [16].

TLR5 is expressed on the basolateral, but not apical, surface of intestinal epithelia [17]. Therefore, flagellin activates pro-inflammatory gene expression only if it crosses intestinal epithelia and contacts the basolateral membrane, which may explain in part why commensal microbes can secrete flagellin into the intestinal lumen without inducing inflammation.

TLR9

TLR9 is essential for responses to bacterial DNA (and viral DNA) and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG DNA). These oligonucleotides have been shown to stimulate the proliferation of B cells and to activate macrophages and dendritic cells (DCs; [18]). The optimal immunostimulatory CpG DNA motifs differ between mouse and human; this difference is due to amino acid sequence differences between the extracellular regions of the human and mouse TLR9s [19].

TLR3

Viral replication within infected cells often results in the generation of double-stranded RNA that can stimulate immune cells. Recently, TLR3-deficient mice have been shown to have reduced responses to double-stranded RNA, as well as to the viral RNA mimic poly(I–C), suggesting that TLR3 is involved in the recognition of double-stranded RNA [20••].

TLR7

TLR7 recognizes several types of imidazoquinoline [21••]. Imiquimod (also known as Aldara, R-837, S-26308) and R-848 (also known as resiquimod, S-28463) are low-molecular mass compounds of the imidazoquinoline family that possess potent antiviral and antitumor properties. The activity of imiquimod is mainly dependent on its ability to induce cytokines, including IFN-α and IL-12.

Topical imiquimod therapy is now approved for the treatment of external genital and perianal warts caused by papilloma virus infection. R-848 is a more potent analog of imiquimod currently under development. In addition, it has been shown that TLR7 recognizes other synthetic chemicals, including loxoribine and broprimine (H Hemmi, S Akira, unpublished data; Figure 3).

Heat-shock proteins and innate immune responses

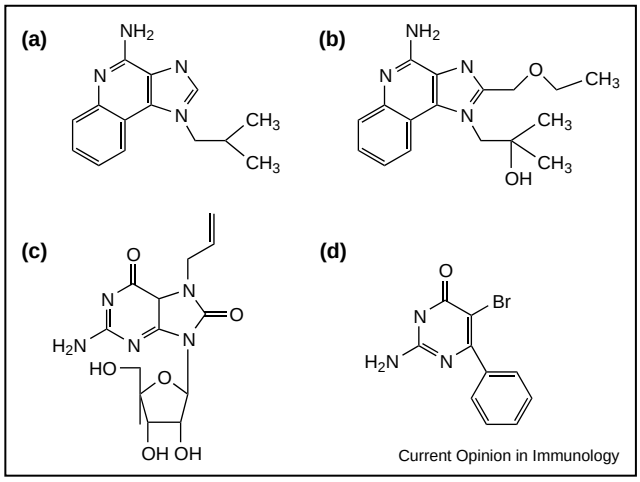
Heat-shock proteins (HSPs) associate with several different peptides in cells and function as chaperones. In addition, HSPs themselves stimulate immune cells to secrete pro-inflammatory cytokines. Recent studies have indicated that HSPs, in particular HSP60, HSP70, HSP90 and GP96, activate macrophages and DCs through TLR2 and TLR4 [22], although there is some concern about the possibility of endotoxin contamination in these studies. Although many publications have ruled out this possibility, other reports have provided evidence that suggests that HSPs do not function as direct activators of the innate immune system [22].

Several reports have shown that TLR4 recognizes fragments of the proteoglycan heparan sulfate, the extra domain A of cellular fibronectin, hyaluronan oligosaccharides and fibrinogen, all of which are generated during the course of inflammation and tissue damage [23–26]. Although this finding is very attractive for supporting the 'danger theory of immune activation' proposed by Matzinger [27], there remains the possibility that these endogenous ligands might also be contaminated with a true TLR4 ligand such as LPS.

Signal initiation sites and trafficking of TLRs

There are fundamental differences in the signals elicited by the various TLRs [28]. For TLR4, the TLR4–MD2 complex is localized at the cell surface and LPS signaling is initiated at the cell membrane. Similarly, TLR2 is also expressed on the cell surface.

Figure 3



Structures of imidazoquinolines, loxoribine and bropirimine. **(a)** Imiquimod and **(b)** R-848 are members of a novel group of low molecular mass compounds, the imidazoquinolinamines. They have antiviral and antitumor activity as inducers of IFN-α and other cytokines *in vivo*. Imiquimod is administered as a 5% cream (Aldara) and is currently used to treat anogenital warts. **(c)** Loxoribine (7-allyl-8-oxoguanosine) enhances the activity of natural killer cells and the proliferation of B lymphocytes, and induces the production of IFNs and cytokines. **(d)** Bropirimine (2-amin-5-bromo-6-phenyl-4(3)-pyrimidinone) is an orally active immunomodulator that increases endogenous amounts of IFN-α and other cytokines, and is used clinically against *in situ* carcinomas in the bladder.

By contrast, TLR9 is present in the cytoplasm, and both the internalization of CpG DNA and endosomal maturation are prerequisites for immune activation triggered by CpG DNA [29]. Consistent with this latter finding, the response to CpG DNA can be abolished by inhibitors of endosomal maturation, such as chloroquine or bafilomycin, as well as by wortmannin, an inhibitor of phosphatidylinositol-3-OH (PI3) kinases that facilitate phagocytosis, endocytosis and endosomal maturation. By contrast, inhibition of endocytosis or endosomal maturation does not affect LPS-induced signaling. Thus, the expression of TLR must be routed specifically to those cellular sites that are best suited to sense their physiological ligands.

GP96 is a paralogue of HSP90 that is localized in the endoplasmic reticulum and is required for chaperoning proteins to the cell surface. In a murine cell line deficient in GP96, TLRs are retained intracellularly and the cells are unresponsive to the TLR ligands LPS and peptidoglycan. The reintroduction of GP96 into the mutant cell results in restored expression of TLR1, TLR2 and TLR4 at the cell surface, as well as their responsiveness to LPS, showing that GP96 is required for the cell-surface export of TLRs [30]. MD-2 is also involved in the intracellular transport of TLR4. In MD-2-deficient embryonic fibroblasts, TLR4 is not expressed on the plasma membrane but is retained predominantly in the Golgi apparatus. It is highly likely that GP96 is responsible for the association of TLR4 with MD-2 in the endoplasmic reticulum.

LPS is also internalized by several types of cell, and this process is generally thought to be involved in the detoxification and clearance of endotoxin. Recent studies have shown, however, that in several types of cell LPS internalization may be an obligatory event that is linked directly to ligand recognition and cell activation. In one LPS-hyperesponsive intestinal cell line, TLR4 is not expressed on the cell surface but is located in the Golgi apparatus [31]. After the cells are exposed to LPS, TLR4 becomes co-localized with internalized LPS. Similarly, a link between LPS internalization and the activation of signal transduction in cardiomyocytes has been reported [32].

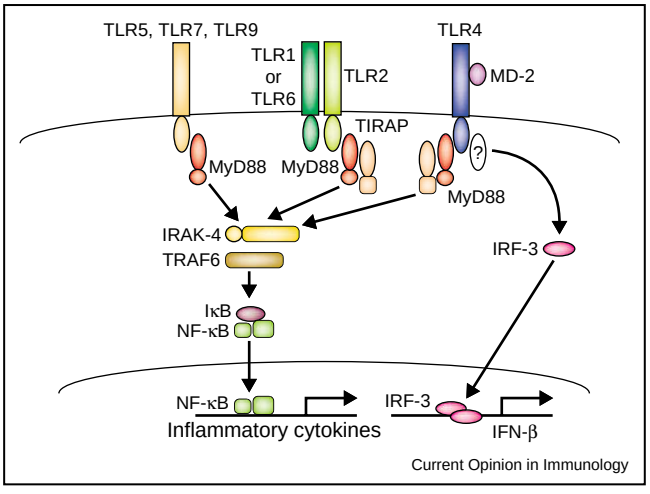
Recently, an intracellular system of LPS recognition has been proposed that involves the nucleotide-binding oligomerization domain (NOD) family of proteins, NOD1 and NOD2 [33]. These are homologs of the Apaf1/Ced4 caspase activators and each contains a nucleotide-binding site and a leucine-rich repeat. They bind to LPS and mediate the activation of nuclear factor (NF)-κB in response to LPS in a TLR4-independent manner, suggesting that the NOD family may act as intracellular receptors for invading bacteria and LPS. Susceptibility to Crohn's disease has been reported to be associated with frameshift and missense mutations in NOD2 [33,34]. The mutant NOD2 proteins cannot activate NF-κB in response to LPS, but the mechanism by which mutations in NOD2 lead to the development of Crohn's disease remains unclear.

Diversity and complexity in the signaling pathways triggered by TLRs

Much evidence now indicates that the TLR signaling pathways differ from one another and elicit different biological responses (Figure 4). For example, stimulation of DCs by *Escherichia coli* LPS specifically induces the production of the IL-12 p70 variant and IP-10 through TLR4, whereas stimulation through TLR2 results instead in the release of the IL-12 p40 homodimer [35].

The analysis of cytokine gene expression in macrophages has identified differences between the genes induced by TLR4 ligands and those induced by TLR2 ligands [36,37]. When compared with TLR2 ligands, TLR4 ligands preferentially induce the production of IL-1β, IFN-γ, IL-12 p40 and monocyte chemoattractant protein (MCP)-5, as well as the release of nitric oxide. Similar patterns have been observed in mast cells [38]. Mast cells stimulated by TLR2 ligands release more IL-4 and IL-5, less TNF-α and no IL-1β, as compared to mast cells stimulated by LPS. In addition, the stimulation of mast cells with TLR2 ligands but not TLR4 ligands results in the degranulation of mast cells and the mobilization of calcium.

Figure 4



Signaling pathways triggered by TLRs. TLR4 ligands such as LPS induce inflammatory cytokines as well as IFN-β. The induction of inflammatory cytokines is dependent on the adaptor molecules MyD88 and TIRAP, whereas the induction of IFN-β is independent of these molecules and is regulated through the phosphorylation and nuclear translocation of IFN regulatory factor 3 (IRF-3). TLR2 ligands such as mycoplasma lipoprotein and peptidoglycan induce inflammatory cytokines through the MyD88/TIRAP-dependent pathway, but do not induce IFN-β as they do not activate the MyD88-independent pathway. Cytokine induction through TLR5, TLR7 or TLR9 depends on MyD88 but not on TIRAP.

MyD88 is an adaptor molecule that recruits the kinase IRAK to the IL-1 receptor or the TLR4 receptor complexes after stimulation by IL-1 or LPS, respectively. Indeed, MyD88-deficient mice are unresponsive to IL-1, LPS and other microbial cell-wall components such as peptidoglycan and lipopeptides [39,40]. But there is a difference in the signaling pathways triggered by LPS and those triggered by these latter types of stimuli. Mycoplasmal lipopeptide activation of NF-κB and MAP kinases, which is mediated by TLR2, is completely abolished in TLR2-deficient or MyD88-deficient macrophages. By contrast, LPS activation of MAP kinases and NF-κB remains intact in MyD88-deficient macrophages, although activation is delayed in comparison to wild-type mice [39]. This indicates that the LPS response is mediated by both MyD88-dependent and MyD88-independent pathways, each of which leads to the activation of MAP kinases and NF-κB. The MyD88-dependent pathway is essential, however, for the inflammatory response mediated by LPS.

Recent studies have shown that the MyD88-independent pathway is responsible for the activation of IFN regulatory factor 3 (IRF-3) and the subsequent induction of IFN-β and IFN-inducible genes [41•,42•]. The MyD88-independent pathway also leads to the induction of costimulatory molecules such as CD40, CD80 and CD86 [43•]. Recently, another adaptor molecule, known as TIR domain-containing adapter protein (TIRAP) or Mal, has been cloned and shown to associate specifically with TLR4. It was suggested that this molecule might be responsible for the MyD88-independent response [42•,44••,45••]. Analyses with TIRAP-deficient mice have shown, however, that TIRAP is not specific to TLR4 signaling nor does it seem to participate in the MyD88-independent pathway. Instead, TIRAP has a crucial role in the MyD88-dependent signaling pathway shared by TLR2 and TLR4 (M Yamamoto, K Takeda, S Akira, unpublished data; see Now in press). The distinct gene expression induced by individual TLRs may be due to the generation of 'signalsomes' that comprise different combinations of adaptors after the stimulation of individual TLRs.

Several signaling molecules downstream of MyD88 have been analyzed recently through gene targeting. IRAK-4, a novel IRAK molecule that is closely related to the *Drosophila* protein Pelle, has been found to be indispensable for responses to IL-1 and to various TLR ligands [46••]. IRAK-M has been shown to be a negative regulator of TLR signaling [47••]. But the mechanism by which these signaling molecules mediate TLR signaling is not understood completely.

Antiviral immunity and TLRs

Mammalian TLRs have been established as essential receptors in the induction of immunity to several microbes, including Gram-positive and Gram-negative bacteria, mycobacteria and fungi. But the role of TLRs in antiviral immunity is not so well established. Recent evidence indicates that TLRs may be involved in the detection and elimination of viruses. First, TLR4 has been shown to recognize a surface glycoprotein of respiratory syncytial virus. TLR4-deficient mice show a reduced inflammatory response against, and impaired clearance of, this virus [48]. Second, mouse mammary tumor virus has been shown to activate B cells by an interaction between the mammary tumor viral envelope protein and TLR4 [49]. Last, vaccinia virus encodes two proteins, A46R and A52, that inhibit signal transduction mediated by the IL-1 receptor, IL-18 receptor and TLR4, showing that vaccinia virus is likely to evade the host immune response by suppressing TIR-domain-dependent intracellular signaling [50].

Recent findings that TLR3 and TLR7 recognize synthetic double-stranded RNA (which mimics viral RNA) and antiviral chemical compounds, respectively, further support the involvement of TLRs in viral recognition. Plamacytoid DCs are a unique subset of immature antigen-presenting cells that secrete type I IFN. Plamacytoid DCs express TLR7 and TLR9, and produce a large amount of IFN-α in response to imidazoquinolines or CpG DNA [51,52]. Although the natural ligand for TLR7 has not been identified, it is likely that TLR7 recognizes viral products or endogenous molecules that are produced during the course of viral infection. Taken together, the above findings strongly suggest that the mammalian immune cells may recognize viral invasion through TLR3, TLR4, TLR7 and TLR9.

Conclusion

Since the discovery of TLRs a few years ago, much progress has been made in our understanding of their role in microbial recognition. But many questions are still unanswered. How and where do TLRs recognize microbial components? How does the activation of individual TLRs elicit differential gene expression and biological responses? Does the inappropriate activation of TLRs result in autoimmune disease?

The last question arises from a recent study that has shown that immune complexes containing self-DNA activate self-IgG-specific B cells as a result of two distinct signals acting through the B cell receptor and through TLR9. This indicates the possible role of TLR9 in the pathogenesis of systemic lupus erythematosus (SLE; [53••]) and may also explain why chloroquine (an endosomal inhibitor and consequently an inhibitor of TLR9 function) has some efficacy in the treatment of SLE. Further understanding of innate immunity will definitely provide the basis for more rational means to treat many infectious diseases, immune disorders and cancers.

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This paper shows that a MyD88-independent pathway activates IRF-3, with the subsequent induction of several IFN-inducible genes such as IP-10.

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The authors show that TLR4 signaling, but not TLR2 signaling, induces IFN-β, thereby giving rise to secondary induction of IFN-inducible genes via activation of STAT1. This study also indicates that the adaptor molecule TIRAP (also known as Mal) is involved in the MyD88-independent pathway; however, this latter finding has been shown subsequently to be incorrect through the analysis of TIRAP knockout mice (M Yamamoto, K Takeda, S Akira, unpublished data; see Now in press).

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See annotation [45••].

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These two papers [44••,45••] report the cloning of a novel adaptor molecule that specifically associates with the cytoplasmic portion of TLR4, and that may be a candidate adaptor involved in MyD88-independent pathway. But more recent studies of TIRAP knockout mice rule out this speculation and demonstrate the essential role of the protein in the MyD88-dependent pathway shared by TLR4 and TLR2 (M Yamamoto, K Takeda, S Akira, unpublished data; see Now in press).

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The IgG/chromatin immune complex activates autoreactive B cells that express an antigen receptor specific for self-IgG through synergistic activation of the antigen-receptor-dependent and the MyD88-dependent signaling pathways. These findings suggest that TLR activation is involved in the pathogenesis of autoimmune diseases.

Now in press

The work referred to in the text as (M Yamamoto, K Takeda, S Akira, unpublished data) is now in press:

54. Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, Hoshino K, Takeuchi O, Kobayashi M, Fujita T *et al.*: **Essential role of TIRAP/Mal for activation of the signaling cascade shared by TLR2 and TLR4**. *Nature* 2002, in press.