

## Cooperation of Toll-like receptor signals in innate immune defence

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**Abstract** | The mechanisms by which the recognition of Toll-like receptor (TLR) ligands leads to host immunity remain poorly defined. It is now thought that to induce an effective immune response, microorganisms must stimulate complex sets of pattern-recognition receptors, both within and outside of the TLR family. The combined activation of these different receptors can result in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity. Therefore, a complete understanding of the role of TLRs in host resistance to infection requires 'decoding' of these multiple receptor interactions. This Review highlights recent advances in the newly emerging field of TLR cooperation and discusses their implications for the development of adjuvants and immunotherapies.

### Pattern-recognition receptor

A receptor that recognizes unique structures that are shared by different microorganisms. Signalling through these receptors typically leads to the production of pro-inflammatory cytokines and chemokines and to the expression of co-stimulatory molecules by antigen-presenting cells. The expression of co-stimulatory molecules, together with the presentation of antigenic peptides, by antigen-presenting cells couples innate immune recognition of pathogens with the activation of adaptive immune responses.

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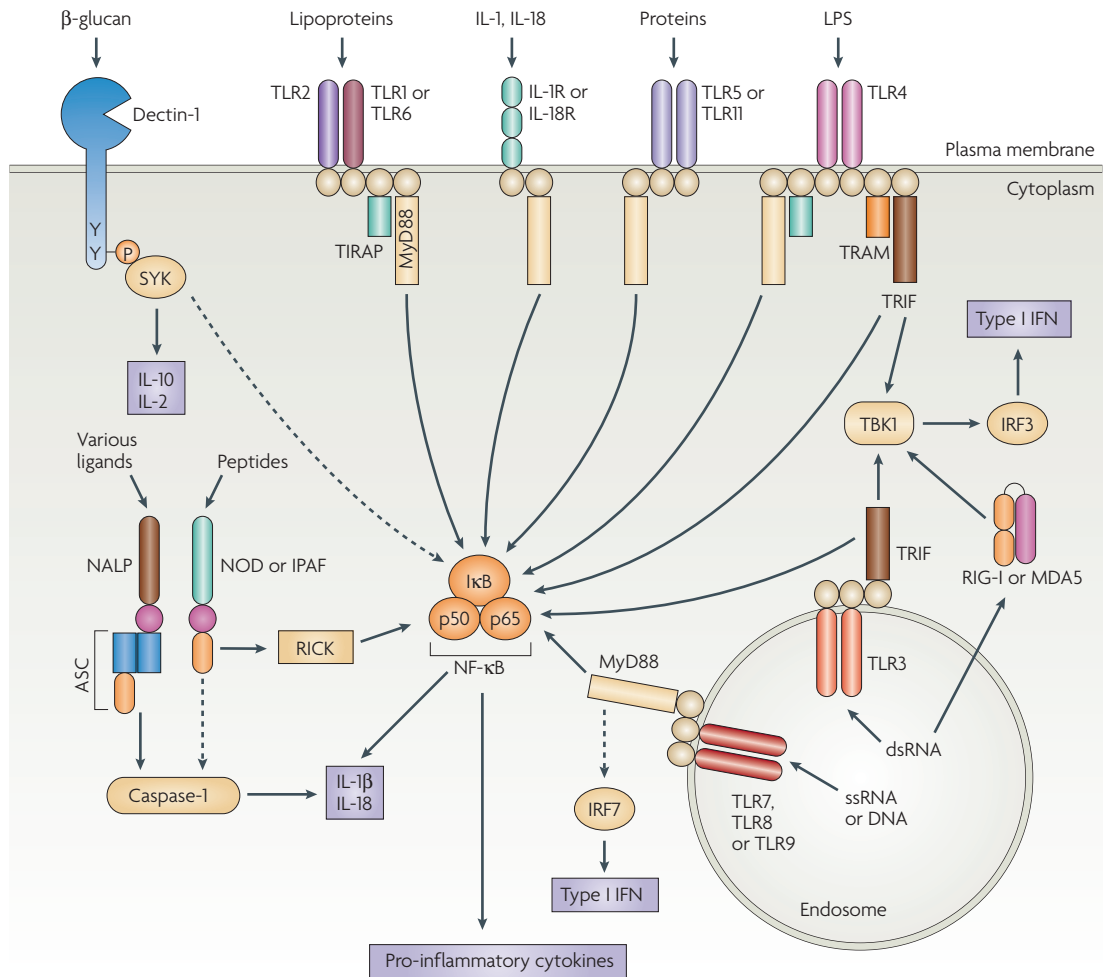
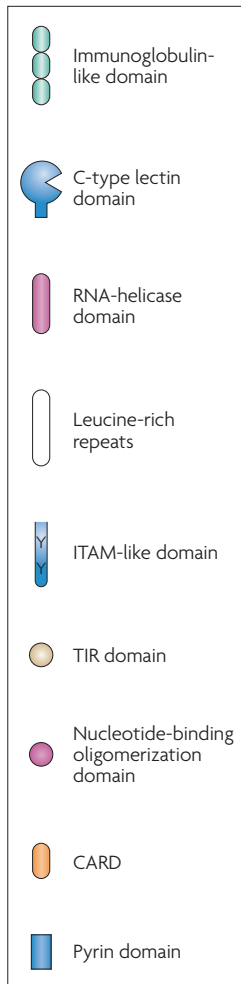
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Mammalian Toll-like receptors (TLRs) are a family of at least 12 membrane proteins that trigger innate immune responses through nuclear factor- $\kappa$ B (NF- $\kappa$ B)-dependent and interferon (IFN)-regulatory factor (IRF)-dependent signalling pathways<sup>1</sup> (FIG. 1). TLRs are evolutionarily conserved molecules and were originally identified in vertebrates on the basis of their homology with *Toll*, a molecule that stimulates the production of antimicrobial proteins in *Drosophila melanogaster*<sup>2,3</sup>. It is of note that *Toll* was functionally defined on the basis of its crucial role in determining the resistance of *D. melanogaster* to infection with fungi and Gram-positive bacteria<sup>2,4</sup>. By contrast, mammalian TLRs have been functionally characterized and distinguished mainly on the basis of their stimulation by different ligands *in vitro*<sup>1</sup>, and mice that are deficient in a single TLR rarely show the extreme susceptibility to pathogenic infection that was originally observed in *D. melanogaster* with a functional deletion of *Toll*<sup>5</sup>. These considerations have led to questions concerning whether the role of *Toll* in host resistance to infection in *D. melanogaster* is comparable to the role of TLRs in mammalian immune defence, and the functional significance of TLR diversification in vertebrate hosts.

The TLR-family members are pattern-recognition receptors (PRRs) that collectively recognize lipid, carbohydrate, peptide and nucleic-acid structures that are broadly expressed by different groups of microorganisms. Some TLRs are expressed at the cell surface, whereas others are expressed on the membrane of endocytic vesicles or other intracellular organelles. TLRs are composed of an ectodomain of leucine-rich repeats

(LRRs), which are involved directly or through accessory molecules in ligand binding, and a cytoplasmic Toll/interleukin-1 (IL-1) receptor (TIR) domain that interacts with TIR-domain-containing adaptor molecules<sup>6</sup>. The structural basis of ligand binding by TLRs is poorly understood and several unexpected cross-specificities (for example, the recognition by **TLR9** of both CpG-containing oligodeoxynucleotides (ODNs) and malaria haemozoin) have now been described<sup>1</sup>. The early hypothesis that individual TLRs might have evolved to recognize distinct phylogenetic groups of pathogens has been mainly abandoned because of the well-documented, broad recognition of microbial products (both pathogenic and non-pathogenic) belonging to diverse species and phyla by many of the TLRs. In addition, the recognition of endogenous ligands by TLRs is now thought to have an important role in the regulation of inflammation, both in infectious and non-infectious diseases. However, the precise identification of these endogenous ligands has remained controversial because of their possible contamination with microbial products<sup>7</sup>. The function of TLRs is further diversified by the different signalling pathways that can be induced by ligand interaction<sup>1</sup> (BOX 1).

Although TLRs are an important system for microbial sensing, they are not the only PRRs with this function (BOX 2). At the cell-surface, C-type lectin-like molecules, such as the mannose receptor and the  $\beta$ -glucan receptors (for example, **dectin-1**), also participate in the binding and uptake of microbial components<sup>8,9</sup>. Components of bacteria and viruses that gain entry into the cytoplasm are recognized by cytosolic receptors, through which



**Figure 1 | Schematic representation of the structure and main signalling pathways of the PRR families.** Only the adaptor molecules and the main signalling pathways that differentiate the different classes of pattern-recognition receptor (PRR) are shown. In reality, the pathways that are activated by the different receptors are multiple and complex (BOXES 1, 2). For example, Toll-like receptor (TLR) signalling involves not only nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation, but also mitogen-activated protein kinases, phosphatidylinositol 3-kinase and several other pathways that markedly affect the overall biological response to the activation of TLRs. Dectin-1 (a  $\beta$ -glucan receptor) is shown as an example of various cell-surface PRRs, some belonging to the lectin-like family and some linked with the immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor Fc receptor  $\gamma$ -chain (FcR $\gamma$ ), the activation of which, as described in the text, can markedly affect TLR signalling. ASC, apoptosis-associated speck-like protein containing a CARD (caspase-recruitment domain); ds, double-stranded; IFN, interferon; I $\kappa$ B, inhibitor of NF- $\kappa$ B; IL, interleukin; IPAF, ICE-protease-activating factor; IRF, IFN-regulatory factor; LPS, lipopolysaccharide; MDA5, melanoma-differentiation-associated gene 5; MyD88, myeloid differentiation primary-response gene 88; NALP, NACHT-, LRR- and pyrin-domain-containing protein; NOD, nucleotide-binding oligomerization domain; RICK, receptor-interacting serine/threonine kinase; RIG-I, retinoic-acid-inducible gene I; ss, single-stranded; TBK1, TANK-binding kinase 1; TIRAP, Toll/IL-1R (TIR)-domain-containing adaptor protein; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor protein inducing IFN $\beta$ ; SYK, spleen tyrosine kinase.

**Haemozoin**

The crystalline product resulting from digestion of haemoglobin by the intraerythrocytic replicative stage of malaria parasites (*Plasmodium* spp.).

**C-type lectins**

Animal receptor proteins that bind carbohydrates in a calcium-dependent manner. The binding activity of C-type lectins is based on the structure of the carbohydrate-recognition domain (CRD), which is highly conserved between members of this family.

they induce cytokine production and cell activation<sup>1,5</sup>. These cytosolic receptors are grouped in two main families: the NLR family (nucleotide-binding oligomerization domain (NOD)-like receptor family), which includes at least 23 members that are either NOD receptors or NALPs (NACHT-, LRR- and pyrin-domain-containing proteins); and a family of receptors that have an RNA-helicase domain joined to two caspase-recruitment domains (CARDs), such as retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiated

gene 5 (MDA5). The name RIG-I-like receptor (RLR) family has been proposed by Creagh and O'Neill<sup>10</sup> for the latter family of receptors, for consistency in nomenclature with the TLR and NLR families.

There is growing evidence that these additional PRRs can cooperate with TLRs in the innate immune response to pathogens. The main sentinel cells of innate immunity (epithelial cells, phagocytic cells and dendritic-cell (DC) subsets) simultaneously express overlapping but not identical combinations of TLRs

**Box 1 | Toll-like receptor signalling pathways**

Toll-like receptors (TLRs) (with the exception of TLR3), interleukin-1 receptor (IL-1R) and IL-18R induce nuclear factor- $\kappa$ B (NF- $\kappa$ B)-dependent cytokine production through a pathway involving the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88)<sup>6</sup>. However, TLR3 (and TLR4) uses a MyD88-independent signalling pathway that involves the adaptor molecule Toll/IL-1R (TIR)-domain-containing adaptor protein inducing interferon- $\beta$  (TRIF; also known as TICAM1)<sup>6</sup>. Further complexity in the TLR signalling pathways results from the obligatory use of the adaptor molecule TIR-domain-containing adaptor protein (TIRAP; also known as MAL) in association with MyD88 by TLR2 and TLR4, and of the adaptor TRIF-related adaptor molecule (TRAM; also known as TICAM2) in association with TRIF by TLR4 (REF. 6) (FIG. 1).

Activation of TLR3 or TLR4 induces type I interferon (IFN) production through the adaptor TRIF, which associates with the kinase TANK-binding kinase 1 (TBK1) and induces the phosphorylation and nuclear translocation of IFN-regulatory factor 3 (IRF3)<sup>89</sup>. The production of type I IFNs is further regulated by a positive-feedback loop. IFN $\beta$  (in mice and humans) and IFN $\alpha$ 4 (in mice), which are produced in response to activated IRF3, can induce the transcription of genes encoding other signalling proteins, such as IRF7 and IRF8 (REFS 90,91). Once phosphorylated, these transcription factors drive the expression of all type I IFN genes, thereby amplifying type I IFN production.

The outcome of TLR signalling can also be influenced by the spatio-temporal regulation of ligand-TLR interactions. For example, in plasmacytoid dendritic cells, ligation of TLR9 by D-type CpG-containing oligodeoxynucleotides (ODNs) activates a signalling complex present in early endosomes that is composed of MyD88, IL-1R-associated kinase 1 (IRAK1) and tumour-necrosis factor receptor-associated factor 6 (TRAF6). This complex physically associates with IRF7 and results in the production of type I IFNs<sup>92,93</sup>. By contrast, recognition of D-type CpG ODNs by all other cell types that express TLR9 results in the activation of IRF5 (which forms a complex with MyD88 and TRAF6 downstream of TLR9 in late endosomes) and the transcription of pro-inflammatory genes<sup>34,94,95</sup>.

and other PRRs<sup>11</sup>, and this might result in tissue-specific responses to microbial stimulation. For example, CD11c<sup>+</sup> DCs in the lamina propria of the intestines, in contrast to DCs at other anatomical locations, express TLR5 but not TLR4, a property that might help the intestinal innate immune system to distinguish between pathogenic and commensal flora<sup>12</sup>. Invading microorganisms are likely to interact with many TLRs

and non-TLR PRRs, and both the magnitude and quality of the subsequent immune response are likely to depend on the distribution of receptors on the innate immune cells encountered by the microorganisms and the coordinated sum of the signals induced by these different receptors. Here, we review the emerging information about how individual TLRs interact with each other and with members of the non-TLR PRR families, and how such interactions influence the generation of host resistance to infection.

**TLR cooperation in cellular responses**

Initial studies of the cooperation between different TLRs expressed by haematopoietic cells showed that the simultaneous activation of TLR2 and TLR4 resulted in a synergistic induction of tumour-necrosis factor (TNF) production<sup>13,14</sup>. However, the induction of cross-tolerance between the two receptors and the use of muramyl dipeptide as a putative TLR2 ligand, which has recently been formally identified as a ligand for NOD2 (REF. 15), in one of these studies complicates the interpretation of these early reports<sup>13,14</sup>. A subsequent study showed that stimulation of mouse macrophages with both polyI:C (polyinosinic-polycytidylic acid; a TLR3 ligand) and CpG DNA (a TLR9 ligand) induced more-than-additive levels of TNF, IL-6 and IL-12 p40 (REF. 16), which confirmed that cooperation between certain TLRs does exist.

Further studies indicated that gene expression and protein secretion of TNF, IL-1 $\beta$ , IL-6, IL-10, IL-12, IL-23 and cyclooxygenase-2 were several-fold higher in DCs stimulated with combinations of TLR ligands than in cells stimulated with a single agonist<sup>17,18</sup>. Microarray analysis showed that of the genes induced by a single TLR ligand, the expression of only 1% of these was increased in a clearly synergistic manner when a combination of TLR ligands was used<sup>18</sup>. However, the expression of a few genes was also downregulated in a synergistic manner

**Box 2 | Signalling pathways of pattern-recognition receptors other than TLRs**

The members of the NLR (NOD-like receptor) family of intracellular pattern-recognition receptors (PRRs) include NALPs (NACHT-, LRR- and pyrin-domain-containing proteins), IPAF (ICE-protease-activating factor; also known as CARD12 and CLAN) and NOD proteins. NLRs have a tripartite domain structure composed of an amino-terminal effector domain, which is usually a pyrin domain (PYD) or a caspase-recruitment domain (CARD), followed by a nucleotide-binding oligomerization domain (NOD), which might be involved in self-oligomerization, and finally a carboxy-terminal series of leucine-rich repeats (LRRs) that are involved in ligand binding<sup>10</sup>. Signalling through members of the NLR family is complex, but in general, activation of several NALPs or IPAF results in the activation of caspase-1. By contrast, activation of NOD proteins induces nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation through receptor-interacting serine/threonine kinase (RICK; also known as RIP2 or CARDIAK)<sup>10</sup>.

The RLR (RIG-I-like receptor) family is formed by at least two members — retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA5) — which are composed of effector CARDS and an RNA-helicase domain that is involved in the recognition of double-stranded RNA. Their activation results in recruitment of the kinase TANK-binding kinase 1 (TBK1), activation of NF- $\kappa$ B and interferon (IFN)-regulatory factor 7 (IRF7) and/or IRF3, and the induction of type I IFN production<sup>10</sup>.

Other surface receptors on innate immune cells that are involved directly or indirectly in the recognition of microbial structures include the mannose and  $\beta$ -glucan receptors<sup>96</sup>, and other C-type lectins<sup>97</sup>. The  $\beta$ -glucan receptor (also known as dectin-1 in mice) has an immunoreceptor tyrosine-based activation motif (ITAM)-like motif in its intracellular portion that activates the kinase SYK (spleen tyrosine kinase)<sup>96</sup>. The modulation of innate immune responses is also mediated by other cell-surface receptors, some with as-yet-undefined ligands, that are associated with signalling molecules that contain ITAMs, such as Fc receptors (FcRs)<sup>98</sup>, TREMs (triggering receptors expressed on myeloid cells)<sup>63,99</sup> and OSCAR (human osteoclast-associated receptor)<sup>96</sup>.

**Cross-tolerance**

Cross-tolerance is observed when the addition of a Toll-like receptor (TLR) ligand induces tolerance to subsequent challenge with the same stimulus used for priming and also to subsequent challenges with other stimuli that signal through one or more different TLRs.

**Microarray analysis**

A technique for measuring the transcription of genes. It involves hybridization of fluorescently labelled cDNA prepared from a cell or tissue of interest with glass slides or other surfaces dotted with thousands of oligonucleotides or cDNA, ideally representing all expressed genes in the species.

by the combined TLR stimulation<sup>18</sup>. Cytokine production can also be negatively regulated by simultaneous signalling through certain TLRs<sup>19</sup>. In particular, the production of IL-10 after TLR2 stimulation was shown to block the expression of IL-12 p35 and CXC-chemokine ligand 10 (CXCL10; also known as IP10) by human DCs in response to either TLR3 or TLR4 ligands. Therefore, the positive or negative regulation of genes by the combination of TLR ligands is quite selective and it seems to affect mainly gene products that are involved in the regulation of the innate and adaptive immune responses to pathogens, which often express multiple TLR ligands.

**TLR regulation of IL-12 p70 production.** The synergistic effect of certain TLR ligands is particularly marked for the production of the immune-regulating cytokine IL-12 p70, which is the biologically active, heterodimeric form of IL-12 composed of the **IL-12 p35** and **IL-12 p40** chains<sup>17</sup>. Stimulation of DCs with single TLR, NLR or RLR ligands normally induces low levels of the IL-12 p40 homodimer and negligible levels of biologically active IL-12 p70. By contrast, stimulation of mouse or human DCs with the **TLR7** and **TLR8** ligand R848 (also known as resiquimod) and either polyI:C or the TLR4 ligand lipopolysaccharide (LPS) results in amounts of IL-12 p70 that are one or two orders of magnitude higher than the amounts induced by the individual TLR ligands<sup>17,18,20</sup>. A more-than-additive effect on the production of IL-6 and IL-12 p40 was also observed in these studies, although the effect was less marked than that shown for IL-12 p70 (REFS 17,18,20).

It is known that for high levels of IL-12 p70 to be produced by DCs in response to a single TLR ligand, co-stimulation by either secreted (for example, IFN $\gamma$ ) or membrane-bound (for example, CD40 ligand, CD40L) signals derived from T cells or natural killer (NK) cells is required<sup>21,22</sup>. Therefore, it could be hypothesized that IL-12 will be present only at later times during an immune response when NK cells and/or T cells are already activated<sup>23</sup>. This then raises the question of whether IL-12 p70 can be the initial start-up signal for the T helper 1 (T<sub>H</sub>1)-cell response to pathogens. However, levels of IL-12 as high as or higher than those produced in response to a single TLR ligand and co-stimulation with IFN $\gamma$  and/or CD40L are rapidly produced by simultaneous stimulation with two TLR ligands, even in the absence of NK cells and T cells<sup>17,24</sup> (F. Yarovinsky, A.S. and G.T., unpublished observations).

In addition to IFN $\gamma$ , type I IFNs have also been shown to have a complex role in regulating cytokine production; type I IFNs upregulate IL-12 p70 production while partially inhibiting the production of IL-12 p40. Indeed, DCs derived from mice that are genetically deficient for the type I IFN receptor (IFNAR) and human DCs or monocytes treated with a neutralizing antibody specific for IFNAR have decreased IL-12 p70 production. Specifically, in response to either a single TLR ligand or a combination of TLR ligands, the production of IL-12 p70 and CXCL10, as well as IL-12 p35 mRNA accumulation, but not the production of IL-12

p40 and IL-6, or IL-12 p40 mRNA accumulation, were dependent on type I IFN signalling<sup>17,24</sup>.

The promoter of the gene encoding IL-12 p35 is partially responsive to IFN $\gamma$  signalling, which indicates that type I IFN signalling (which partially overlaps with IFN $\gamma$  signalling) might also participate in the transcriptional regulation of IL-12 p70 — for example by inducing the expression of IRF-family members such as IRF1 and IRF8 that are involved in transcription of the gene encoding IL-12 p35 (REF. 25). However, it is also possible that type I IFNs remove negative-feedback mechanisms for IL-12 production. For example, endogenous type I IFNs might inhibit the activation by TLR3 or TLR4 ligands of the phosphatidylinositol 3-kinase (PI3K)–AKT pathway, which is known to limit IL-12 production<sup>26</sup>. Although low levels of type I IFN production are observed in response to the single TLR ligands or combinations of TLR ligands that induce IL-12 p70 production, the combination of TLR ligands did not result in synergistic induction of signal transducer and activator of transcription 1 (STAT1) phosphorylation or of CXCL10 production, two responses that are strictly dependent on endogenous type I IFN production in purified DCs<sup>17</sup>. Furthermore, in the absence of endogenous type I IFN signalling, the overall level of IL-12 p70 production by purified DCs is markedly decreased, but a more-than-additive induction of IL-12 p70 production by a combination of TLR ligands is still observed<sup>17</sup>. Therefore, type I IFN is required for optimal IL-12 p70 production but it is clearly not the response element that accounts for TLR synergism. Nevertheless, the role of endogenous type I IFN in IL-12 p70 production requires further investigation.

During innate and adaptive immune responses to a pathogen, the IFN $\gamma$  and other cytokines produced by NK cells, natural killer T (NKT) cells and antigen-specific T cells are important for amplifying IL-12 production by DCs, which is required for optimal T<sub>H</sub>1-cell responses. In addition, activated T cells also increase IL-12 p70 production by interacting with DCs through CD40L–CD40 interactions<sup>27</sup>. Therefore, signals from accessory cells are important to amplify and maintain IL-12 p70 production by DCs. Although signalling through IFNAR is also required for optimal IL-12 p70 production, type I IFN, unlike IFN $\gamma$ , is produced endogenously by DCs in response to TLR signalling, and combined TLR ligands can induce high levels of IL-12 p70 in the absence of accessory cells and their products. Overall, these results indicate that IL-12 production in response to combined TLR signalling can be an autonomous DC function.

Because many pathogens express ligands for several TLRs (TABLE 1), it is likely that these ligands can cooperate to activate multiple TLRs during infection, resulting in the production of sufficient levels of IL-12 p70 and other pro-inflammatory cytokines early in infection. These pro-inflammatory cytokines then initiate and amplify the innate immune response and adaptive T<sub>H</sub>1-cell immunity. The possible successive involvement of different cytokines and cell-surface receptors in regulating IL-12 p70 production during the response to infection is shown in FIG. 2.



Table 1 | Examples of pathogens expressing ligands for multiple TLRs

Pathogen	Toll-like receptor (TLR)	TLR ligand
<i>Mycobacterium tuberculosis</i>	TLR2	Lipoarabinomannan
	TLR4	Phosphatidylinositol mannosides
	TLR9	DNA
<i>Salmonella typhimurium</i>	TLR2	Bacterial lipoprotein
	TLR4	Lipopolysaccharide
	TLR5	Flagellin
<i>Neisseria meningitidis</i>	TLR2	Porin
	TLR4	Lipopolysaccharide
	TLR9	DNA
<i>Haemophilus influenzae</i>	TLR2	Lipoprotein
	TLR4	Lipopolysaccharide
<i>Candida albicans</i>	TLR2	Phospholipomannan
	TLR4	Mannan
	TLR9	DNA
Murine cytomegalovirus	TLR2	Viral protein
	TLR3	Double-stranded RNA
	TLR9	DNA
Herpes simplex virus	TLR2	Viral protein
	TLR3	Double-stranded RNA
	TLR9	DNA
Influenza virus	TLR7, TLR8	Single-stranded RNA
	TLR3	Double-stranded RNA
	TLR4	Not determined
Respiratory syncytial virus	TLR3	Double-stranded RNA
	TLR4	Envelope F protein
<i>Trypanosoma cruzi</i>	TLR2	Glycosylphosphatidylinositol anchor
	TLR4	Glycoinositolphospholipid-ceramides
	TLR9	DNA
<i>Toxoplasma gondii</i>	TLR2	Glycosylphosphatidylinositol anchor?
	TLR11	Profilin

**Molecular mechanisms.** The synergy between different TLRs could in part be due to the preferential induction of IL-12 p40 transcription by myeloid differentiation primary-response gene 88 (MyD88)-associated TLRs and of IL-12 p35 transcription by TIR-domain-containing adaptor protein inducing IFN $\beta$  (TRIF; also known as TICAM1)-associated TLRs. The different signalling pathways activated by receptors that use these two adaptor molecules (MyD88 and TRIF) have been largely characterized but the interaction of the signalling pathways is not well understood. In both human and mouse DCs, the production of IL-12 p70 was synergistically induced when certain MyD88-associated TLR ligands were combined with TRIF-associated TLR ligands<sup>17,18,20,24</sup> (FIG. 3), which indicates that the two signal-transduction pathways downstream of MyD88 and TRIF might cooperate. Indeed, it has recently been shown that MyD88-associated TLRs synergize with TRIF-associated TLRs

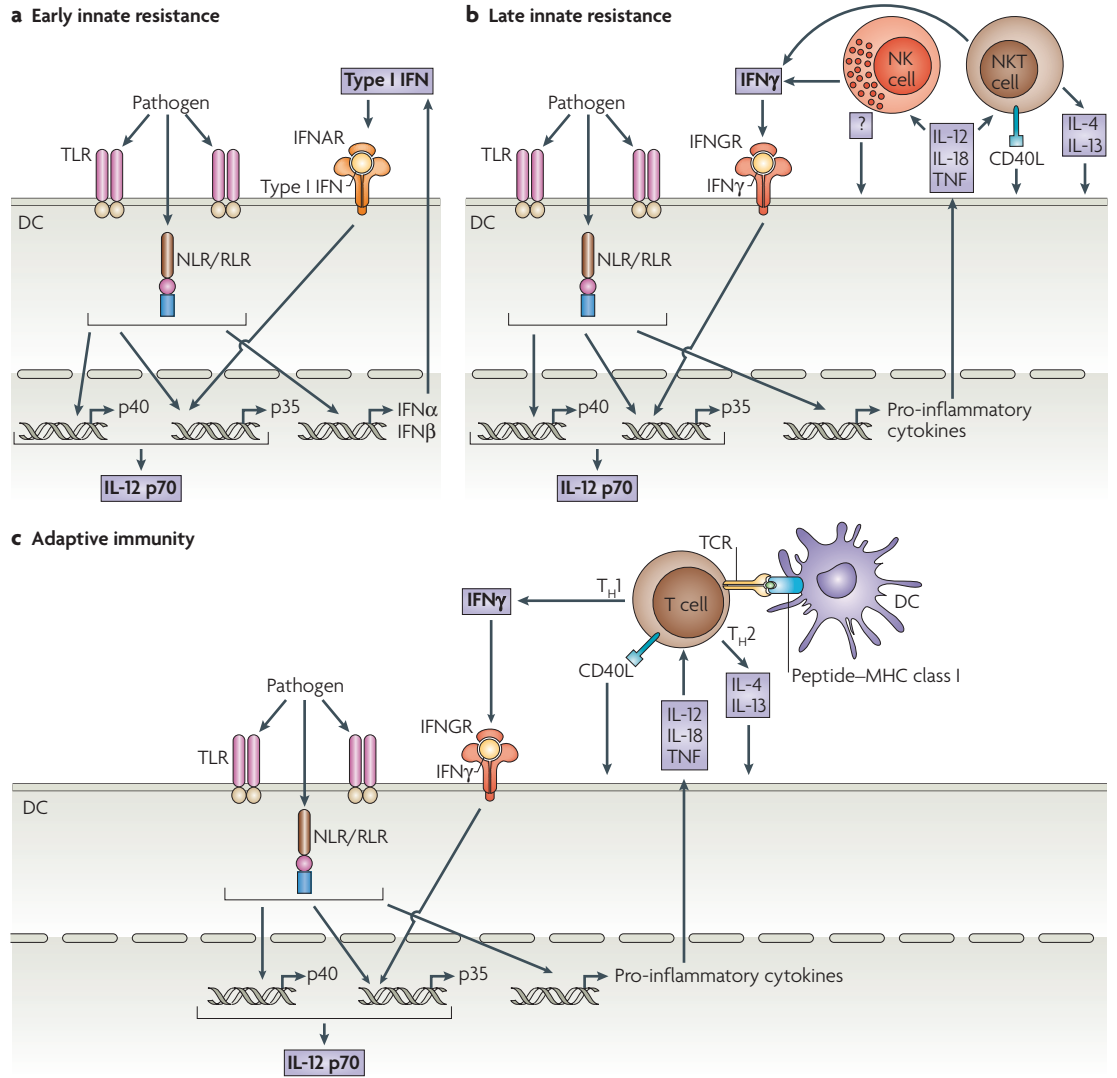
for the induction of several pro-inflammatory cytokines, including IFN $\beta$ , in part by increasing NF- $\kappa$ B activation and nuclear translocation. By contrast, sequential stimulation with ligands for either MyD88- or TRIF-associated TLRs results in cross-tolerance for the responsiveness of TLRs using the same adaptor<sup>28</sup>.

However, when a TLR2 ligand (which uses MyD88 as a signalling adaptor) was combined with any other TLR ligand (including TRIF-associated TLR ligands), the synergy for IL-12 p70 production was moderate or absent. Only a low-level synergy for IL-12 p40, TNF and IL-6 production was observed when a TLR2 ligand was combined with a TLR3, TLR4 (REFS 17,24) or TLR9 (REFS 29,30) ligand. In addition, no significant synergy for IL-12 p70 production was observed when TLR3 and TLR4 ligands were combined<sup>17,20</sup>. The use of the co-adaptor TIR-domain-containing adaptor protein (TIRAP; also known as MAL) with MyD88 in TLR2 and TLR4 signalling, and of the co-adaptor TRIF-related adaptor molecule (TRAM; also known as TICAM2) with TRIF in TLR4 signalling, might distinguish the TLR2 and TLR4 signalling pathways from the pathways induced by other TLRs that do not use these co-adaptors.

Although TIRAP has been shown to be required for the recruitment of MyD88 to TLR4 and probably TLR2, but not to other MyD88-associated TLRs, no evidence of a direct role for TIRAP in TLR4 or TLR2 signalling has been reported<sup>31</sup>. However, tyrosine phosphorylation of STAT1, which in purified DCs indicates at least low levels of type I IFN secretion<sup>17</sup>, is observed in DCs stimulated with ligands for all TLRs except TLR2 (REFS 17,32). This indicates that differences might exist between receptors that signal through MyD88 alone and those that signal through MyD88 in association with TIRAP.

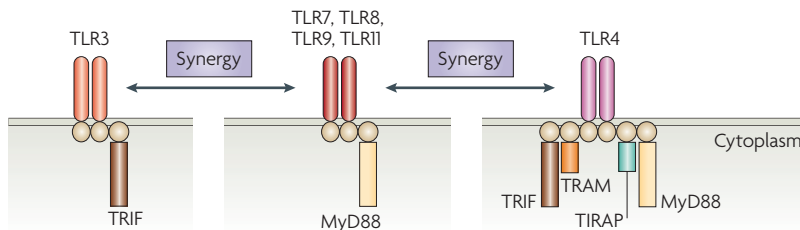
The synergy between different TLRs might also be the result of interactions between the different signalling pathways that affect the production of IL-12 p70 at both the transcriptional and post-transcriptional stages. Interestingly, it has been shown that IRF3, which is activated by TRIF-associated signalling, suppresses the transcription of IL-12 p35 mRNA, which indicates the existence of another possible mechanism of IL-12 p70 regulation that might be affected by the interaction of different TLR signalling pathways<sup>33</sup>.

Another important difference between the various TLRs is their cellular localization and/or the association of TLRs with other receptors or accessory molecules that modulate TLR signalling properties and might affect the ability of TLRs to interact synergistically. An example is provided by the ability of TLR7 and TLR9 to associate with different molecular complexes and to activate different signalling pathways when ligands interact with the receptors in early or late endosomes<sup>34</sup>. It will also be important to evaluate how simultaneous triggering of different receptors can affect the negative-regulatory mechanisms that modulate TLR responsiveness<sup>35</sup>. The molecular basis of some of these negative-feedback mechanisms is well characterized and includes decoy receptors, ST2 (an IL-1R-like molecule), the short form of MyD88, IL-1R-associated kinase M (IRAK-M),



**Figure 2 | Regulation of IL-12 p70 production by dendritic cells during innate and adaptive immune responses.**

**a | Early innate resistance.** Optimal production of interleukin-12 (IL-12) p70 by dendritic cells (DCs) occurs when ligands for different pattern-recognition receptors (PRRs) are combined<sup>17,18</sup>. Many pathogens express ligands for several PRRs and therefore efficiently induce IL-12 p70 production. In addition, high-level production of IL-12 p70 by purified DCs requires endocrine production of type I interferon (IFN)<sup>17</sup>. The level of endogenous type I IFN that is required for optimal IL-12 p70 production is low and sufficient type I IFN can be induced by most single PRR ligands. Therefore, the induction of type I IFN is not responsible for the synergy of different PRRs that is observed for the activation of IL-12 p70 production<sup>17</sup>. The production of IL-12 p70 by DCs can be cell autonomous and the cooperation of other cell types that eventually mediate a marked increase in IL-12 p70 production is not an absolute requirement for the early production of this cytokine. In an *in vivo* model of endotoxaemia, this early production of IL-12 p70 is observed within 2–3 hours after the injection of lipopolysaccharide (LPS)<sup>100</sup>. In response to Toll-like receptor 7 (TLR7) and TLR9 ligands, plasmacytoid DCs produce high levels of type I IFN within a few hours<sup>101</sup> that might replace the need for the autocrine synthesis of type I IFN by conventional DCs (not shown). However, high levels of type I IFN inhibit IL-12 production<sup>102</sup>, which indicates that type I IFN has a complex role in the regulation of IL-12 production. **b | Late innate resistance.** The early production of IL-12 and other pro-inflammatory cytokines by DCs rapidly induces natural killer (NK) cells, natural killer T (NKT) cells and activated/effector T cells (not shown) to produce IFN $\gamma$  and, in the case of NKT cells, IL-4 and IL-13. IFN $\gamma$  effectively replaces the need for type I IFN for IL-12 p70 production, probably by using similar molecular mechanisms<sup>21</sup>. In addition, activated NK cells and NKT cells increase IL-12 p70 production by DCs through membrane receptor–ligand interactions<sup>27,103,104</sup>. In an experimental model of endotoxaemia, IL-12-dependent production of IFN $\gamma$  is observed 6–7 hours after the injection of LPS<sup>100</sup>. **c | Adaptive immunity.** When an adaptive immune response to a pathogen is established at 5 to 7 days after infection, the production of IFN $\gamma$  by antigen-specific T helper 1 (T<sub>H</sub>1) cells is important for amplifying the production of IL-12 p70 by DCs, which is required for optimal T<sub>H</sub>1-cell responses. In addition, activated T cells also increase IL-12 p70 production by interacting with DCs through CD40 ligand (CD40L)–CD40 interactions<sup>27</sup>. IL-4 and IL-13 produced by T<sub>H</sub>2 cells are also important co-stimulators of IL-12 p70 production by DCs<sup>105</sup> although neither the mechanism nor the biological significance of this interaction is clear. IFNAR, type I IFN receptor; IFNGR, IFN $\gamma$  receptor; NLR, nucleotide-binding oligomerization domain (NOD)-like receptor; RLR, retinoic-acid-inducible gene I (RIG-I)-like receptor; TCR, T-cell receptor; TNF, tumour-necrosis factor.



**Figure 3 | TLR ligand combinations synergistically induce production of IL-12 p70.** Ligands for Toll-like receptor 7 (TLR7), TLR8, TLR9 and TLR11 can synergize with ligands for TLR3 or TLR4 for interleukin-12 (IL-12) p70 production. Although no or very little IL-12 p70 is synergistically induced by the combination of ligands for either TLR2 and TLR3 or TLR4, or TLR3 and TLR4, other pro-inflammatory cytokines are induced more than additively by these receptor combinations. MyD88, myeloid differentiation primary-response gene 88; TIRAP, Toll/IL-1 receptor (TIR)-domain-containing adaptor protein; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adaptor protein inducing interferon- $\beta$ .

members of the suppressor of cytokine signalling (SOCS) family, Toll-interacting protein (TOLLIP) and IRF4 (a negative regulator of IRF5-mediated TLR signalling)<sup>35</sup>. Therefore, these molecules are obvious candidates for analysis to determine a possible effect of TLR cooperation on the negative regulation of TLR signalling. In addition to intracellular regulatory molecules, secreted cytokines such as type I and type II IFNs and IL-10 have regulatory effects on TLR-mediated cellular responses and they might participate in the cooperative effects of triggering of different PRRs.

#### TLR cooperation with other PRRs

In addition to the synergy between ligands of different TLRs, synergistic induction of cytokine production has also been observed for DCs or macrophages activated by a TLR ligand combined with ligands for other PRRs. A large amount of evidence supports the observation that ligands of **NOD1** and **NOD2** can synergize with many TLR ligands, including TLR2 ligands, for the induction of TNF and IL-12 p40 production<sup>36–39</sup>. Because bacterial peptidoglycans (TLR2 ligands) are degraded to compounds that can activate NOD proteins, the synergy between TLR- and NLR-family receptors can amplify the response not only to a single pathogen but also to a single component of a pathogen<sup>15</sup>.

However, when the production of IL-12 by human DCs was analysed, combined stimulation of NOD2 and TLR2 resulted in only low-level production of IL-12 p70, whereas NOD2 activation effectively increased IL-12 p70 production in response to the activation of other TLRs, such as TLR7 and TLR8 (F. Gerosa and G.T., unpublished observations). Interestingly, the production of cytokines induced by TLR2 ligands, but not by other TLR ligands, was shown to be higher in mice deficient for NOD2 compared with wild-type mice<sup>40</sup>. But, discordant results were reported in a later study<sup>41</sup>, possibly as a result of the different experimental conditions in the two studies. However, the activation of NOD2 might involve differential signal-transduction pathways that result in either amplification or attenuation

of the responses mediated by TLRs (TLR7 and TLR8, or TLR2, respectively)<sup>42</sup>. This has important implications because of the increased frequency of functional mutations in one or both *NOD2* alleles in patients with Crohn's disease<sup>43</sup>. Therefore, careful dissection of the molecular mechanisms involved in the interaction between TLRs and NOD2 and of their role in mucosal immunity should improve our understanding of the pathogenesis of inflammatory colitis<sup>44</sup>.

A different molecular mechanism of cooperation between PRRs occurs for the simultaneous activation of TLRs and NALPs. The stimulation of the latter in a multiprotein complex containing the adaptor **ASC** (apoptosis-associated speck-like protein containing a CARD; also known as CARD5), which is known as the 'inflammasome'<sup>45</sup>, results in the activation of caspase-1. Active **caspase-1** cleaves pro-IL-1 $\beta$  and pro-IL-18 to produce the active forms of the two cytokines<sup>46,47</sup>. However, TLR stimulation is required for the production of these pro-cytokines, indicating an essential cooperation between the TLR and NALP families of receptors for the production of IL-1 and IL-18 (REFS 46,47).

**NALP3** is essential for the ATP-driven activation of caspase-1 in LPS-stimulated macrophages. In addition, ASC- and NALP3-deficient mice have an impaired contact hypersensitivity response to the hapten trinitrophenylchloride and have decreased secretion of IL-1 $\beta$  and IL-18 when cultured with the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes*<sup>48–50</sup>. NALP3 is also essential for IL-1 $\beta$  and IL-18 production in response to small-molecule agonists of TLR7 and TLR8, to double-stranded RNA, and to viruses such as Sendai virus and influenza virus<sup>48</sup>. Interestingly, endogenous ligands, such as gout-associated uric-acid crystals, also activate the NALP3-containing inflammasome and caspase-1, resulting in the production of IL-1 $\beta$  and IL-18 in inflammatory and autoimmune diseases, which indicates that the inflammasome acts as a first line of defence against cell stress<sup>51,52</sup>. Signals from the cell-stress-activated inflammasome might cooperate with signals from TLRs and other PRRs, which are activated by endogenous or exogenous ligands, in regulating inflammatory responses.

Another example of the stimulation of both TLRs and NLRs by pathogens is the ability of flagellin to stimulate both TLR5, which is expressed on the cell membrane, and the NLR ICE-protease-activating factor (**IPAF**; also known as CARD12 and CLAN) in the cytoplasm of *Salmonella*-infected macrophages<sup>53,54</sup>. IPAF is required for both caspase-1 activation and IL-1 $\beta$  production in response to infection<sup>53,54</sup>, and the ability of the CARD-containing IPAF to activate caspase-1, unlike the NALPs, is partially independent of the adaptor ASC<sup>54</sup>. The recognition of pathogens by different intracellular receptors, and in some cases the capacity of the same microbial molecules to activate both TLRs and cytoplasmic receptors, highlights a new and interesting perspective in our understanding of the cooperative effect of the various PRRs.

The ability of NLRs to cooperate with TLR-induced signalling pathways has not yet been analysed in detail,

#### Crohn's disease

A form of chronic inflammatory bowel disease that can affect the entire gastrointestinal tract, but is most common in the colon and terminal ileum. It is characterized by transmural inflammation and granuloma formation, and it is thought to result from an abnormal T-cell-mediated immune response to commensal bacteria.

#### Caspases

A family of cytosolic proteases that contain a cysteine residue in the active site and that cleave their substrate after an aspartic-acid residue. They can be divided into pro-inflammatory caspases (caspase-1, -4, -5 and -11), which cleave and activate pro-inflammatory cytokines, and pro-apoptotic caspases, which cleave and activate pro-apoptotic substrates.

#### Contact hypersensitivity

The initial reaction that occurs after the first exposure to a 'sensitizer' hapten or antigen. This step requires dendritic-cell migration to lymph nodes to prime contact-antigen-specific T cells.

owing to the difficulty in studying RLR ligands in isolation without also activating other receptors, particularly TLRs. For example, polyI:C has been shown to activate both TLR3 and MDA5, resulting in the production of different cytokines<sup>55,56</sup>. In particular, activation of TLR3 by polyI:C was mainly responsible for the induction of pro-inflammatory cytokines other than type I IFNs, whereas mice genetically deficient for *Mda5* were unable to produce type I IFNs in response to polyI:C. However, because the signalling pathways downstream of the TLR3-associated adaptor TRIF and of RLRs are in part overlapping, it will be of interest to investigate the possibility that signalling through RLRs might synergize with ligands for the TLRs that use MyD88.

PRRs other than the TLRs, NLRs and RLRs have been implicated in the recognition of fungal pathogens in particular. Receptors that recognize mannans and  $\beta$ -glucans have been shown to have an important role in the recognition and phagocytosis of zymosan particles<sup>57</sup> and in the recognition of *Mycobacterium tuberculosis*<sup>58,59</sup>. The interaction of these receptors with TLR signalling, particularly that of TLR2, results in either synergy or inhibition of pro-inflammatory cytokine production<sup>58,59</sup>. In particular, the mouse  $\beta$ -glucan receptor, dectin-1, has been shown in DCs to cooperate with various TLRs to increase the production of pro-inflammatory cytokines, such as TNF and IL-12 p40 (REF. 60). This study also showed that dectin-1 can induce synthesis of the anti-inflammatory cytokine IL-10 and have an anti-inflammatory effect through a spleen tyrosine kinase (SYK)-dependent, TLR-independent signalling pathway. By contrast, the activation of IRAK-M by the polysaccharide antigen lipoarabinomannan from *M. tuberculosis*, probably through the mannose receptor, negatively regulates TLR-dependent IL-12 p40 production by macrophages in an IL-10-independent manner<sup>58</sup>.

The triggering of immunoreceptor tyrosine-based activation motif (ITAM)-associated cell-surface receptors has been reported to have a variable effect on TLR signalling. **TREM1** (triggering receptor expressed on myeloid cells 1) and **TREM2** activate myeloid cells by signalling through the adaptor protein DAP12 (DNAX activation protein 12), which contains an ITAM in its cytoplasmic tail. TREM1 amplifies TLR- and NLR-mediated signalling in neutrophils<sup>61,62</sup>, whereas TREM2 inhibits TLR-induced cytokine production by macrophages<sup>63,64</sup>. Among the Fc receptor  $\gamma$ -chain-coupled receptors (which also contain ITAMs), the crosslinking of Fc receptors specifically inhibits IL-12 production by monocytes in response to TLR ligands<sup>65</sup>. By contrast, signalling through the human osteoclast-associated receptor (OSCAR) increases the pro-inflammatory responses of human monocytes and neutrophils to TLR ligands<sup>66</sup>. Because the exogenous and/or endogenous ligands of these ITAM-associated receptors are still unknown, with the exception of the Fc receptors, the biological relevance of the above findings is unclear. Nevertheless, the published data clearly indicate that these receptors are closely involved in the regulation of the inflammatory response.

### Evaluating TLR cooperation in vivo

Mice deficient in the TLR and IL-1R adaptor molecule MyD88 are highly susceptible to a broad range of bacterial, viral, fungal and protozoan pathogens, which indicates that this family of PRRs might have an important role in host defence *in vivo*. Although it has been argued that MyD88 deficiency can lead to TLR-independent developmental defects in macrophage or DC function<sup>67,68</sup>, there is as yet no compelling evidence of a role for such nonspecific defects in the impaired resistance of *Myd88*<sup>-/-</sup> mice to infection *in vivo*. By contrast, knockout mice lacking a single TLR have, in most cases, either normal control of infection or more resistance to infection than *Myd88*<sup>-/-</sup> mice<sup>1,5</sup>. In such cases, and in particular when a well-defined TLR–ligand interaction has been determined *in vitro*, the absence of an *in vivo* phenotype can be explained by redundancy in TLR function or by the cooperation of multiple TLR and/or IL-1R signals in host resistance. However, there is no formal way to distinguish between these two possibilities. It is of interest that for several bacterial and protozoan pathogens, the susceptibility of single-TLR-deficient mice to infection is increased under conditions of high-dose challenge. For example, whereas *Tlr2*<sup>-/-</sup> or *Tlr9*<sup>-/-</sup> mice have nearly normal resistance to conventional, low-dose aerosol challenge with *M. tuberculosis*, at high doses these mice are clearly more susceptible to infection than are their wild-type counterparts<sup>29,69</sup>. This indicates that under high levels of infectious stress, the function of each TLR involved in pathogen recognition becomes more crucial for microbial control, an interpretation that is consistent with TLR cooperation in host defence.

A further consideration in evaluating the function of TLRs in host resistance to infection *in vivo* is that for certain pathogens, the activation of TLRs might actually promote infection and/or pathology, such that TLR deficiency leads to increased, rather than decreased, host resistance. For example, West Nile virus seems to use its interaction with TLR3 as a mechanism to induce a local inflammatory response that results in the disruption of the blood–brain barrier, thereby allowing the virus to infect the central nervous system (CNS). Consequently, *Tlr3*<sup>-/-</sup> mice are less susceptible to virus-induced CNS pathology than are wild-type mice<sup>70</sup>. Similarly, *Tlr2*<sup>-/-</sup> mice have decreased mortality when infected with herpes simplex virus 1 (HSV1), a phenotype that is attributed to the role of TLR2 in virus-induced encephalitis<sup>71</sup>.

Finally, in considering the outcome of TLR deficiency *in vivo*, it is important to take into account the possible role of TLRs in the negative regulation of anti-microbial effector responses. For example, *Tlr2*<sup>-/-</sup> mice have been reported to be deficient in CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (T<sub>Reg</sub>) cells and ligation of TLR2 on adoptively transferred T<sub>Reg</sub> cells impairs their suppressive function *in vivo*, resulting in increased resistance to *Candida albicans* infection<sup>72</sup>. Therefore, the effect (or lack of effect) of TLR deficiency on the control of pathogenic infection might not necessarily reflect the direct consequences of TLR signalling on the effector functions driven by antigen-presenting cells, as was originally thought.



### TLRs in host defence against pathogens

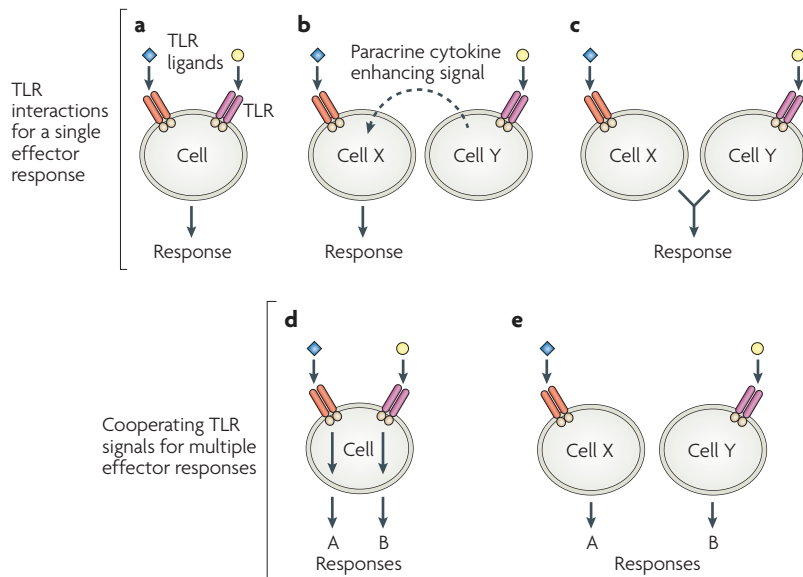
Despite the above complexities, there are many situations where it has been possible to establish a role for multiple TLRs in host defence *in vivo* and in several cases to investigate TLR cooperation directly using mice with genetic deletions of these receptors. For example, both TLR3-deficient and TLR9-deficient (or mutated) mice have decreased resistance to infection with murine cytomegalovirus (MCMV). In both cases, this decreased resistance was associated with decreased type I IFN production and IL-12 synthesis<sup>73</sup>. In addition, TLR2 is required for resistance to MCMV infection and to control the production of IL-18 and other cytokines, as well as NK-cell activation<sup>74</sup>. These data indicate that the expression of at least three TLRs (TLR2, TLR3 and TLR9) is required for the control of MCMV infection and that deficiency of any of these three receptors differentially affects distinct effector mechanisms and patterns of cytokine secretion, resulting in decreased resistance to infection. Nevertheless, mice deficient for the adaptor molecule MyD88 were much more susceptible to MCMV infection than were mice deficient for either TLR2, TLR3 or TLR9 (REF. 75). Therefore, in the case of MCMV, different TLRs seem to activate complementary defence mechanisms, as well as to synergize for the induction of anti-viral cytokines.

LPS-hyporesponsive mice (for example, C3H/HeJ mice) have been known for many years to be highly susceptible to infection with *Salmonella typhimurium*<sup>76</sup>, a phenotype that is now attributed to defective TLR4 function<sup>77</sup>. *S. typhimurium* also contains potent TLR2 ligands, which trigger cytokine production during infection of macrophages. After either intraperitoneal or oral *S. typhimurium* infection, whereas *Tlr4*<sup>-/-</sup> mice are more susceptible than wild-type mice to infection (as expected), *Tlr2* and *Tlr4* double-knockout mice are more susceptible to infection than either of the *Tlr2*<sup>-/-</sup> or *Tlr4*<sup>-/-</sup> parental strains<sup>78</sup>. Moreover, in the case of oral infection, the mortality rate and infection load in *Tlr2*<sup>-/-</sup>*Tlr4*<sup>-/-</sup> mice were similar to those observed in *Myd88*<sup>-/-</sup> mice. No increase in susceptibility to infection over wild-type mice was observed for the two single-knockout strains or the double-knockout mice when given a two-log lower challenge dose. This finding was interpreted by the authors as being the result of the lack of a requirement in these conditions to respond to the acute phase of infection normally accompanying high-dose challenge. On the basis of *in vitro* studies with macrophages exposed to bacterial TLR ligands, they proposed a model of sequential TLR interaction and TNF induction to explain these *in vivo* data. In this model, both TLRs can stimulate TNF production in response to either endotoxin (TLR4) or bacterial lipoprotein (TLR2) from the pathogen, but TLR4 and TLR2 are triggered at different times after infection. The expression of TLR2 is regulated as a consequence of the ligation of TLR4, which, by contrast, is basally expressed on resting macrophages; this provides an explanation for the stronger phenotype observed in TLR4-deficient mice than in TLR2-deficient mice<sup>78</sup>.

Another example of TLR cooperation that involves the dual activation of TLR2 and TLR9 has been documented for infection with *M. tuberculosis* or *Trypanosoma cruzi* in mice<sup>29,30</sup>. Both pathogens contain TLR2 ligands that stimulate the production of TNF by macrophages. In addition, TLR9 has an important role in regulating the production of IL-12 p40 by DCs in response to the live pathogens *in vitro* (presumably owing to the activation of this TLR by CpG motifs present in the pathogen DNA). DCs from *Tlr2* and *Tlr9* double-knockout mice produced even lower levels of IL-12 p40 than did cells from single TLR9-deficient mice. Importantly, double-knockout mice had increased susceptibility to infection and increased pathogen loads compared with either wild-type or single TLR2- or TLR9-deficient mice after challenge with either of the microbial agents. Indeed, during *T. cruzi* infection, the peripheral-blood parasitaemia in *Tlr2*<sup>-/-</sup>*Tlr9*<sup>-/-</sup> mice was equivalent to that seen in *Myd88*<sup>-/-</sup> mice. Nevertheless, in terms of both mortality and, in the case of *M. tuberculosis* infection, bacterial load, the double-knockout mice were clearly less susceptible to infection than *Myd88*<sup>-/-</sup> mice, therefore indicating that other MyD88-dependent signalling events, in addition to TLR2 and TLR9 signalling, are involved in host resistance to these pathogens.

The synergy between TLR2 and TLR9 is also evident in the induction of IL-12 p40 and other cytokines by the live pathogens *in vitro*. These effects were also observed when the purified glycosylphosphatidylinositol (GPI) anchor and DNA from *T. cruzi* were used as TLR ligands. Deficiency of TLR9, but not of TLR2, selectively affected IFN $\gamma$  production by CD4<sup>+</sup> T cells *in vivo* (presumably through defective production of IL-12), whereas TNF secretion by macrophages was shown to be regulated mainly by TLR2 in both infection models<sup>29,30</sup>. The explanation for the failure of TLR2 deficiency to affect T<sub>H</sub>1-cell responses is not clear, but it might involve the selective activation of TLR9 on DCs by the pathogens *in vivo*. Together, the above data indicate that the observed synergy between TLR2 and TLR9 in the control of *M. tuberculosis* or *T. cruzi* infection stems from their regulation of two distinct effector arms of the immune response (that is, TNF and IFN $\gamma$  production) that are necessary for the control of infection. This mechanism contrasts with that emerging from the studies of MCMV<sup>73</sup> and *S. typhimurium*<sup>78</sup> infection, in which TLRs that interact to activate the host defence system do so by cooperating for the production of the same pro-inflammatory cytokines.

Evidence for yet another mechanism of TLR cooperation in host defence comes from a recent report comparing the roles of the MyD88-dependent and TRIF-dependent signalling pathways in host resistance to *T. cruzi*<sup>79</sup>. Macrophages and DCs from TRIF-deficient mice had normal control of intracellular infection. By contrast, cells from *Myd88* and *Trif* double-knockout mice were highly susceptible to infection, and were even more susceptible than the same cell populations from *Myd88*<sup>-/-</sup> mice. The TRIF-mediated signalling that is required for optimal *in vitro* control of infection was shown to involve the activation of IFN $\beta$ . Indeed,



**Figure 4 | Summary of the basic mechanisms by which TLR signals cooperate for the generation of host resistance to infection.** The signalling pathways that activate innate resistance to pathogens involve multiple Toll-like receptor (TLR)–ligand interactions, which function together to generate a single effector response (for example, interleukin-12 production), as shown in the upper panels, or involve the cooperation of distinct TLR signals for the generation of multiple effector responses, as shown in the lower panels. **a** | Synergy between multiple TLR signals in one responding cell to generate a single effector response. **b** | Enhancement of one TLR-mediated effector response by a paracrine cytokine signal induced by a different TLR ligand on a separate cell type. **c** | Additive responses generated by distinct TLR signals on different cell types. **d** | Independent activation of different effector responses by distinct TLR signals in the same cell. **e** | Triggering of different effector responses on separate cell types, which together cooperate to mediate host resistance. Although not considered here, similar schemes might regulate the interactions between TLRs and other pattern-recognition receptors.

cells from MyD88- and IFNAR-deficient mice had similar defects in the control of intracellular infection to *Myd88*<sup>-/-</sup>*Trif*<sup>-/-</sup> cells. Importantly, studies of *in vivo* infection confirmed these *in vitro* findings, with both *Myd88*<sup>-/-</sup>*Trif*<sup>-/-</sup> and *Myd88*<sup>-/-</sup>*Ifnar*<sup>-/-</sup> mice having higher levels of peripheral-blood parasitaemia and accelerated mortality compared with either *MyD88*<sup>-/-</sup> or *Trif*<sup>-/-</sup> mice. Additional studies<sup>79</sup> indicated that the effects of IFN $\beta$  on parasite growth are due to the induction of IFN-inducible gene 47 (IRG47), a p47 GTPase that has previously been implicated in intracellular host defence<sup>80</sup>. Although the specific TLR that is involved in TRIF-dependent IFN $\beta$  induction has not been formally identified, TLR4 is a probable candidate given the presence of known ligands for this PRR in *T. cruzi*<sup>81</sup>. Taken together with the findings of Bafica *et al.*<sup>30</sup>, these observations indicate that TLR2, TLR9 and possibly TLR4 cooperate for the control of *T. cruzi* infection, with each TLR triggering a distinct effector pathway.

Given their crucial role in detecting bacteria and viruses that gain entry into the cytoplasm and their important function in caspase activation, NLRs are likely to work with TLRs to provide innate immune resistance to these pathogens. As already noted, NOD-receptor and TLR agonists from bacteria can strongly synergize in the induction of pro-inflammatory cytokine production

and therefore it is expected that in future studies, mice with combined deficiencies of TLRs and NOD receptors or other NLRs will have marked susceptibility to several infectious agents.

**Evolutionary and therapeutic implications**

The findings discussed in this Review emphasize the importance of multiple TLR signalling events in the generation of host resistance to infection and they also show that TLR cooperation and synergy can arise through different mechanisms. Hypothetically, TLRs might interact at the level of the same cell or at the level of multiple cell types (FIG. 4). The end result of the triggering of multiple receptors can be either the enhancement of a single effector function<sup>73,78</sup> or the coordinated induction of distinct responses<sup>29,30,79</sup>, which together mediate more effective control of pathogen growth. It is important to remember that TLRs and other PRRs are widely distributed on haematopoietic and non-haematopoietic cells, with each cell type expressing a typical pattern of PRRs. This pattern of expression is further modulated or modified by the activation, maturation or differentiation of the cells. Also, the same cell types, for example epithelial cells or DCs, might express different patterns of PRRs in different tissues. Although the distribution and regulation of PRR expression by different cell types in different locations is outside the scope of this Review, it is important to keep this fact in mind when analysing the role of PRR cooperation in host resistance to infection.

The evolution of such a multi-pronged, integrated system for sensing pathogens has obvious advantages for the host in protecting itself against infectious agents that might delete or mutate crucial ligands for TLRs or other PRRs. It also has advantages for protecting against mutations in host PRRs or associated downstream signalling molecules that would cripple the protective immune response. The quantitatively limited and often pathogen-selective effects on host resistance of single-nucleotide polymorphisms in human TLRs<sup>82,83</sup> are consistent with this concept.

The concept that multiple TLR–ligand interactions are required for the induction of effective host resistance to pathogens has important implications for the design of improved strategies for vaccination and immunotherapy against infectious diseases. Individual TLR7, TLR8 and TLR9 agonists have already been used successfully as adjuvants to boost CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses to candidate microbial vaccine antigens. These agonists seem to be particularly effective when they are covalently conjugated to the immunogens<sup>84–86</sup>. Several studies have convincingly shown the improved efficacy of treatment with multiple TLR ligands compared with single TLR ligands in stimulating cellular immune responses *in vivo*. For example, co-administration of polyI:C and CpG ODNs increases serum cytokine production and the expression of nitric-oxide synthase 2 and MHC class I molecules by immune cells, and this treatment controls pulmonary metastases in a mouse tumour model when compared with administration of either of the ligands alone<sup>16</sup>. Bone-marrow-derived DCs loaded with antigen and exposed to both polyI:C

**Adjuvant**  
An agent mixed with an antigen that increases the immune response to that antigen after immunization.

and the synthetic TLR7 ligand R848 induced markedly increased cytotoxic T-lymphocyte responses compared with DCs exposed to either TLR ligand alone<sup>87</sup>. This study highlights the importance of direct DC targeting for the adjuvant effects of such regimens involving multiple TLR ligands. The relevance of combined TLR stimulation in the induction of immunity to pathogens is underscored by the finding that the live, attenuated yellow fever vaccine, which confers highly efficient and long-lasting immunity against viral challenge,

simultaneously and potently activates TLR2, TLR7, TLR8 and TLR9 on different DC subsets<sup>88</sup>. The stage is now set for a major research effort to examine the protective efficacy of multiple TLR- and PRR-ligand combinations in vaccines against infectious diseases. The *in vitro* and *in vivo* studies discussed here have provided an important conceptual foundation for this work, as well as a starting point to unravel the complex interaction of PRR signals that initiate the induction of host resistance to pathogens.

- Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783–801 (2006).  
**A comprehensive review of TLR biology.**
- Lemaitre, B. The road to Toll. *Nature Rev. Immunol.* **4**, 521–527 (2004).
- Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A. Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* **388**, 394–397 (1997).  
**A pioneering report describing Toll-like signalling molecules in a higher vertebrate.**
- Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* **86**, 973–983 (1996).  
**Characterization of the role of Toll in resistance to pathogens of *D. melanogaster*.**
- Fritz, J. H. & Girardin, S. E. How Toll-like receptors and Nod-like receptors contribute to innate immunity in mammals. *J. Endotoxin Res.* **11**, 390–394 (2005).
- Takeda, K., Kaisho, T. & Akira, S. Toll-like receptors. *Annu. Rev. Immunol.* **21**, 335–376 (2003).
- Rifkin, I. R., Leadbetter, E. A., Busconi, L., Viglianti, G. & Marshak-Rothstein, A. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunol. Rev.* **204**, 27–42 (2005).
- McGreal, E. P., Miller, J. L. & Gordon, S. Ligand recognition by antigen-presenting cell C-type lectin receptors. *Curr. Opin. Immunol.* **17**, 18–24 (2005).
- Brown, G. D. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nature Rev. Immunol.* **6**, 33–43 (2006).
- Creagh, E. M. & O'Neill, L. A. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol.* **27**, 352–357 (2006).
- Iwasaki, A. & Medzhitov, R. Toll-like receptor control of the adaptive immune responses. *Nature Immunol.* **5**, 987–995 (2004).  
**This paper describes the differential expression of TLRs by different antigen-presenting cells.**
- Uematsu, S. *et al.* Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c<sup>+</sup> lamina propria cells. *Nature Immunol.* **7**, 868–874 (2006).
- Beutler, E., Gelbart, T. & West, C. Synergy between TLR2 and TLR4: a safety mechanism. *Blood Cells Mol. Dis.* **27**, 728–730 (2001).
- Sato, S. *et al.* Synergy and cross-tolerance between toll-like receptor (TLR)2- and TLR4-mediated signaling pathways. *J. Immunol.* **165**, 7096–7101 (2000).  
**References 13 and 14 are early reports of cooperation between TLRs.**
- Girardin, S. E. *et al.* Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* **278**, 8869–8872 (2003).
- Whitmore, M. M. *et al.* Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced antitumor activity. *Cancer Res.* **64**, 5850–5860 (2004).  
**The first study to describe the use of multiple TLR ligands to increase host resistance.**
- Gautier, G. *et al.* A type I interferon autocrine–paracrine loop is involved in Toll-like receptor-induced interleukin-12 p70 secretion by dendritic cells. *J. Exp. Med.* **201**, 1435–1446 (2005).
- Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F. & Lanzavecchia, A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nature Immunol.* **6**, 769–776 (2005).  
**References 17 and 18 are the first reports to describe the strong synergistic effects of TLR ligands on IL-12 p70 production by DCs.**
- Re, F. & Strominger, J. L. IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of T<sub>H</sub>1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. *J. Immunol.* **173**, 7548–7555 (2004).  
**One of the early studies showing differential and antagonistic functional consequences of the activation of different TLRs.**
- Roelofs, M. F. *et al.* The expression of Toll-like receptors 3 and 7 in rheumatoid arthritis synovium is increased and costimulation of Toll-like receptors 3, 4, and 7/8 results in synergistic cytokine production by dendritic cells. *Arthritis Rheum.* **52**, 2313–2322 (2005).
- Ma, X. *et al.* The interleukin 12 p40 gene promoter is primed by interferon- $\gamma$  in monocytic cells. *J. Exp. Med.* **183**, 147–157 (1996).
- Edwards, A. D. *et al.* Microbial recognition via Toll-like receptor-dependent and -independent pathways determines the cytokine response of murine dendritic cell subsets to CD40 triggering. *J. Immunol.* **169**, 3652–3660 (2002).
- Abdi, K., Singh, N. & Matzinger, P. T-cell control of IL-12p75 production. *Scand. J. Immunol.* **64**, 83–92 (2006).
- Bekeredjian-Ding, I. *et al.* T cell-independent, TLR-induced IL-12p70 production in primary human monocytes. *J. Immunol.* **176**, 7438–7446 (2006).
- Liu, J., Guan, X., Tamura, T., Ozato, K. & Ma, X. Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein. *J. Biol. Chem.* **279**, 55609–55617 (2004).
- Hasan, U. A., Trinchieri, G. & Vlach, J. Toll-like receptor signaling stimulates cell cycle entry and progression in fibroblasts. *J. Biol. Chem.* **280**, 20620–20627 (2005).
- Schulz, O. *et al.* CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells *in vivo* requires a microbial priming signal. *Immunity* **13**, 453–462 (2000).
- Bagchi, A. *et al.* MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J. Immunol.* **178**, 1164–1171 (2007).  
**A recent report documenting synergy between MyD88- and TRIF-dependent signalling by TLR agonists.**
- Bafica, A. *et al.* TLR9 regulates T<sub>H</sub>1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*. *J. Exp. Med.* **202**, 1715–1724 (2005).
- Bafica, A. *et al.* Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in *Trypanosoma cruzi* infection. *J. Immunol.* **177**, 3515–3519 (2006).  
**The first description of TLR cooperation in host defence against a protozoan pathogen.**
- Kagan, J. C. & Medzhitov, R. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* **125**, 943–955 (2006).
- Rhee, S. H., Jones, B. W., Toshchakov, V., Vogel, S. N. & Fenton, M. J. Toll-like receptors 2 and 4 activate STAT1 serine phosphorylation by distinct mechanisms in macrophages. *J. Biol. Chem.* **278**, 22506–22512 (2003).
- Dahlberg, A., Auble, M. R. & Petro, T. M. Reduced expression of IL-12 p35 by SJL/J macrophages responding to Theiler's virus infection is associated with constitutive activation of IRF-3. *Virology* **353**, 422–432 (2006).
- Honda, K. *et al.* Spatiotemporal regulation of MyD88–IRF-7 signalling for robust type-I interferon induction. *Nature* **434**, 1035–1040 (2005).
- Liew, F. Y., Xu, D., Brint, E. K. & O'Neill, L. A. Negative regulation of Toll-like receptor-mediated immune responses. *Nature Rev. Immunol.* **5**, 446–458 (2005).
- van Heel, D. A. *et al.* Synergistic enhancement of Toll-like receptor responses by NOD1 activation. *Eur. J. Immunol.* **35**, 2471–2476 (2005).
- Fritz, J. H. *et al.* Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur. J. Immunol.* **35**, 2459–2470 (2005).
- Uehara, A. *et al.* Muramyl dipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. *Cell. Microbiol.* **7**, 53–61 (2005).
- Tada, H., Aiba, S., Shibata, K., Ohteki, T. & Takada, H. Synergistic effect of Nod1 and Nod2 agonists with Toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. *Infect. Immun.* **73**, 7967–7976 (2005).
- Watanabe, T., Kitani, A., Murray, P. J. & Strober, W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nature Immunol.* **5**, 800–808 (2004).
- Kobayashi, K. S. *et al.* Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* **307**, 731–734 (2005).
- Watanabe, T., Kitani, A. & Strober, W. NOD2 regulation of Toll-like receptor responses and the pathogenesis of Crohn's disease. *Gut* **54**, 1515–1518 (2005).
- van Heel, D. A. *et al.* Synergy between TLR9 and NOD2 innate immune responses is lost in genetic Crohn's disease. *Gut* **54**, 1553–1557 (2005).
- Strober, W., Murray, P. J., Kitani, A. & Watanabe, T. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nature Rev. Immunol.* **6**, 9–20 (2006).
- Mariathasan, S. *et al.* Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* **430**, 213–218 (2004).
- Agostini, L. *et al.* NALP3 forms an IL-1 $\beta$ -processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* **20**, 319–325 (2004).
- Dinarello, C. A. Unraveling the NALP-3/IL-1 $\beta$  inflammasome: a big lesson from a small mutation. *Immunity* **20**, 243–244 (2004).
- Kanneganti, T. D. *et al.* Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* **281**, 36560–36568 (2006).
- Sutterwala, F. S. *et al.* Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* **24**, 317–327 (2006).
- Mariathasan, S. *et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440**, 228–232 (2006).
- Ogura, Y., Sutterwala, F. S. & Flavell, R. A. The inflammasome: first line of the immune response to cell stress. *Cell* **126**, 659–662 (2006).



52. Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237–241 (2006).

53. Franchi, L. *et al.* Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin-1 $\beta$  in salmonella-infected macrophages. *Nature Immunol.* **7**, 576–582 (2006).

54. Miao, E. A. *et al.* Cytoplasmic flagellin activates caspase-1 and secretion of interleukin-1 $\beta$  via Ipaf. *Nature Immunol.* **7**, 569–575 (2006).

55. Kato, H. *et al.* Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**, 101–105 (2006).

56. Gitlin, L. *et al.* Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc. Natl Acad. Sci. USA* **103**, 8459–8464 (2006).

57. Underhill, D. M. & Ozinsky, A. Phagocytosis of microbes: complexity in action. *Annu. Rev. Immunol.* **20**, 825–852 (2002).

58. Pathak, S. K. *et al.* *Mycobacterium tuberculosis* lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin-12 p40 production in macrophages. *J. Biol. Chem.* **280**, 42794–42800 (2005).

59. Yadav, M. & Schorey, J. S. The  $\beta$ -glucan receptor Dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. *Blood* **108**, 3168–3175 (2006).

60. Rogers, N. C. *et al.* Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C-type lectins. *Immunity* **22**, 507–517 (2005).

61. Netea, M. G. *et al.* Triggering receptor expressed on myeloid cells-1 (TREM-1) amplifies the signals induced by the NACHT-LRR (NLR) pattern recognition receptors. *J. Leukocyte Biol.* **80**, 1454–1461 (2006).

62. Radsak, M. P., Salih, H. R., Rammensee, H. G. & Schild, H. Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: differential regulation of activation and survival. *J. Immunol.* **172**, 4956–4963 (2004).

63. Turnbull, I. R. *et al.* Cutting edge: TREM-2 attenuates macrophage activation. *J. Immunol.* **177**, 3520–3524 (2006).

64. Hamerman, J. A. *et al.* Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. *J. Immunol.* **177**, 2051–2055 (2006).

65. Cappiello, M. G., Sutterwala, F. S., Trinchieri, G., Mosser, D. M. & Ma, X. Suppression of IL-12 transcription in macrophages following Fc $\gamma$  receptor ligation. *J. Immunol.* **166**, 4498–4506 (2001).

66. Merck, E. *et al.* Ligation of the FcR $\gamma$  chain-associated human osteoclast-associated receptor enhances the proinflammatory responses of human monocytes and neutrophils. *J. Immunol.* **176**, 3149–3156 (2006).

67. Palliser, D., Ploegh, H. & Boes, M. Myeloid differentiation factor 88 is required for cross-priming *in vivo*. *J. Immunol.* **172**, 3415–3421 (2004).

68. Sun, D. & Ding, A. MyD88-mediated stabilization of interferon- $\gamma$ -induced cytokine and chemokine mRNA. *Nature Immunol.* **7**, 375–381 (2006).

69. Reiling, N. *et al.* Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. *J. Immunol.* **169**, 3480–3484 (2002).

70. Wang, T. *et al.* Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nature Med.* **10**, 1366–1373 (2004).

71. Kurt-Jones, E. A. *et al.* Herpes simplex virus 1 interaction with Toll-like receptor 2 contributes to lethal encephalitis. *Proc. Natl Acad. Sci. USA* **101**, 1315–1320 (2004).

**References 70 and 71 describe the detrimental effects of specific TLR signals in viral infection.**

72. Suttmuller, R. P. *et al.* Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Invest.* **116**, 485–494 (2006).

**This study describes how TLR signalling can negatively regulate immune responses through the induction of regulatory T cells.**

73. Tabela, K. *et al.* Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc. Natl Acad. Sci. USA* **101**, 3516–3521 (2004).

74. Szomolanyi-Tsuda, E., Liang, X., Welsh, R. M., Kurt-Jones, E. A. & Finberg, R. W. Role for TLR2 in NK cell-mediated control of murine cytomegalovirus *in vivo*. *J. Virol.* **80**, 4286–4291 (2006).

75. Delale, T. *et al.* MyD88-dependent and -independent murine cytomegalovirus sensing for IFN- $\alpha$  release and initiation of immune responses *in vivo*. *J. Immunol.* **175**, 6723–6732 (2005).

76. O'Brien, A. D. *et al.* Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. *J. Immunol.* **124**, 20–24 (1980).

77. Beutler, B. TLR4 as the mammalian endotoxin sensor. *Curr. Top. Microbiol. Immunol.* **270**, 109–120 (2002).

78. Weiss, D. S., Raupach, B., Takeda, K., Akira, S. & Zychlinsky, A. Toll-like receptors are temporally involved in host defense. *J. Immunol.* **172**, 4463–4469 (2004).

**An early paper describing TLR cooperation in the control of bacterial infection.**

79. Koga, R. *et al.* TLR-dependent induction of IFN- $\beta$  mediates host defense against *Trypanosoma cruzi*. *J. Immunol.* **177**, 7059–7066 (2006).

**The first report of cooperation of TRIF- and MyD88-dependent signalling pathways in host defence in vivo.**

80. Taylor, G. A., Feng, C. G. & Sher, A. p47 GTPases: regulators of immunity to intracellular pathogens. *Nature Rev. Immunol.* **4**, 100–109 (2004).

81. Oliveira, A. C. *et al.* Expression of functional TLR4 confers proinflammatory responsiveness to *Trypanosoma cruzi* glycoinositolphospholipids and higher resistance to infection with *T. cruzi*. *J. Immunol.* **173**, 5688–5696 (2004).

82. Turvey, S. E. & Hawn, T. R. Towards subtlety: understanding the role of Toll-like receptor signaling in susceptibility to human infections. *Clin. Immunol.* **120**, 1–9 (2006).

**A recent review of the effect of human TLR polymorphisms on host resistance to infectious diseases.**

83. Ku, C. L. *et al.* Inherited disorders of human Toll-like receptor signaling: immunological implications. *Immunol. Rev.* **203**, 10–20 (2005).

84. Krieg, A. M. Therapeutic potential of Toll-like receptor 9 activation. *Nature Rev. Drug Discov.* **5**, 471–484 (2006).

85. Wille-Reece, U. *et al.* HIV GAG protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of T $_H$ 1 and CD8 $^+$  T cell responses in nonhuman primates. *Proc. Natl Acad. Sci. USA* **102**, 15190–15194 (2005).

**This study describes the effects of coupling a TLR ligand to antigen on the immune response induced to a vaccine antigen in a primate model.**

86. Yarovinsky, F., Kanzler, H., Hiency, S., Coffman, R. L. & Sher, A. Toll-like receptor recognition regulates immunodominance in an antimicrobial CD4 $^+$  T cell response. *Immunity* **25**, 655–664 (2006).

87. Warger, T. *et al.* Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses *in vivo*. *Blood* **108**, 544–550 (2006).

88. Querec, T. *et al.* Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J. Exp. Med.* **203**, 413–424 (2006).

89. Sato, S. *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.* **171**, 4304–4310 (2003).

90. Levy, D. E., Marie, I., Smith, E. & Prakash, A. Enhancement and diversification of IFN induction by IRF-7-mediated positive feedback. *J. Interferon Cytokine Res.* **22**, 87–93 (2002).

91. Tailor, P., Tamura, T. & Ozato, K. IRF family proteins and type I interferon induction in dendritic cells. *Cell Res.* **16**, 134–140 (2006).

92. Honda, K. *et al.* Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proc. Natl Acad. Sci. USA* **101**, 15416–15421 (2004).

93. Kawai, T. *et al.* Interferon- $\alpha$  induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nature Immunol.* **5**, 1061–1068 (2004).

94. Barnes, B. J., Moore, P. A. & Pitha, P. M. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon- $\alpha$  genes. *J. Biol. Chem.* **276**, 23382–23390 (2001).

95. Guiducci, C. *et al.* Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. *J. Exp. Med.* **203**, 1999–2008 (2006).

96. Netea, M. G. *et al.* Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Invest.* **116**, 1642–1650 (2006).

97. Cambi, A., Koopman, M. & Figdor, C. G. How C-type lectins detect pathogens. *Cell. Microbiol.* **7**, 481–488 (2005).

98. Nimmerjahn, F. & Ravetch, J. V. Fc $\gamma$  receptors: old friends and new family members. *Immunity* **24**, 19–28 (2006).

99. Bouchon, A., Faccchetti, F., Weigand, M. A. & Colonna, M. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* **410**, 1103–1107 (2001).

100. Wysocka, M. *et al.* Interleukin-12 is required for interferon- $\gamma$  production and lethality in lipopolysaccharide-induced shock in mice. *Eur. J. Immunol.* **25**, 672–676 (1995).

101. Asselin-Paturel, C. *et al.* Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nature Immunol.* **2**, 1144–1150 (2001).

102. Byrnes, A. A. *et al.* Type I interferons and IL-12: convergence and cross-regulation among mediators of cellular immunity. *Eur. J. Immunol.* **31**, 2026–2034 (2001).

103. Gerosa, F. *et al.* Reciprocal activating interaction between natural killer cells and dendritic cells. *J. Exp. Med.* **195**, 327–333 (2002).

104. Gerosa, F. *et al.* The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *J. Immunol.* **174**, 727–734 (2005).

105. D'Andrea, A., Ma, X., Aste-Amezaga, M., Paganini, C. & Trinchieri, G. Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor  $\alpha$  production. *J. Exp. Med.* **181**, 537–546 (1995).

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**Competing interests statement**

The authors declare no competing financial interests.

**DATABASES**

The following terms in this article are linked online to: Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene> ASC | caspase-1 | dectin-1 | IL-12 p35 | IL-12 p40 | IPAF | MDA5 | MyD88 | NALP3 | NOD1 | NOD2 | RIG-I | TIRAP | TLR2 | TLR3 | TLR4 | TLR5 | TLR7 | TLR8 | TLR9 | Toll | TRAM | TREM1 | TREM2 | TRIF

**FURTHER INFORMATION**

Alan Sher's homepage: <http://www3.niaid.nih.gov/labs/aboutlabs/lpd/immunobiologysection/sher.htm> Access to this links box is available online.