Newly described pattern recognition receptors team up against intracellular pathogens

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Abstract | Recognizing the presence of invading pathogens is key to mounting an effective innate immune response. Mammalian cells express different classes of germline-encoded pattern recognition receptors that monitor the extracellular and intracellular compartments of host cells for signs of infection and that activate several conserved signalling pathways. An efficient immune response often requires the sequential detection of a pathogen by different receptors in different subcellular compartments, which results in a complex interplay of downstream signalling pathways. In this Review, we discuss the recent identification of previously unknown pattern recognition receptors and how they complement the repertoire of established receptors.

Cyclic dinucleotides
Small bacterial or host-derived nucleic acids — such as cyclic diguanylate monophosphate (c-di-GMP), cyclic diadenylate monophosphate (c-di-AMP) or cyclic GMP–AMP — that function as secondary messengers and that can induce an innate immune response when present in the cytosol.

Leaderless cytokines
Cytokines that lack a classical amino-terminal secretion signal sequence (also referred to as leader peptide or leader sequence) and that are thought to be secreted by an endoplasmic reticulum- and Golgi-independent mechanism.

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The mammalian innate immune system provides a first line of defence against microbial attack through phagocytosis and the induction of inflammation. These responses are stimulated by several classes of germline-encoded pattern recognition receptors (PRRs) that primarily recognize conserved microbial molecules termed pathogen-associated molecular patterns (PAMPs) but that also recognize host-derived danger signals, which are released in response to stress, tissue damage and necrotic cell death[1]. Bacterial PAMPs are diverse and include various molecules ranging from lipoproteins, lipopolysaccharide (LPS), flagellin and peptidoglycan to unique bacterial nucleic acid structures, such as cyclic dinucleotides (CDNs). By contrast, viruses are mainly recognized through viral fusion glycoproteins and through unique nucleic acids, such as double-stranded RNA (dsRNA), uncapped single-stranded RNA (ssRNA) and viral DNA. Comparably little is known about the recognition of intracellular parasites. However, similar to other microorganisms, parasite recognition is dependent on the detection of unique molecules[2].

PRRs initiate antimicrobial defence mechanisms through several conserved signalling pathways. The activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon-regulatory factors (IRFs) promotes the production of inflammatory cytokines and type I interferons (IFNs), respectively. Other PRRs initiate the assembly of cytoplasmic signalling complexes, termed inflammasomes, which activate inflammatory caspases[3,4]. Active caspase 1 controls the maturation and the secretion of leaderless cytokines such as interleukin-1β (IL-1β) and IL-18, and induces pyroptosis, which is a lytic form of cell death that can restrict pathogen replication[5]. The inflammatory response that is induced by PRR activation recruits and activates circulating immune cells and is essential for priming adaptive immune responses.

Two main classes of PRRs have been described in mammalian cells: membrane-bound receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and cytoplasmic sensors, including NOD-like receptors (NLRs), pyrin and HIN domain-containing (PYHIN) family members, RIG-I-like receptors (RLRs) and an increasing range of cytosolic nucleic acid sensors. TLRs were the first group of PRRs to be characterized and they recognize PAMPs in the extracellular compartment or within endosomes[6]. Following ligation with their ligands, TLRs interact with different combinations of the adaptor proteins TIR domain-containing adaptor protein (TIRAP; also known as MAL), myeloid differentiation primary-response protein 88 (MYD88), TIR-domain-containing adaptor protein inducing IFNβ (TRIF; also known as TICAM1) and TRIF-related adaptor molecule (TRAM; also known as TICAM2)[7]. The MYD88-dependent pathway controls the activation of mitogen-activated protein kinases (MAPKs) and the transcription factor NF-κB, whereas the TRIF-dependent pathway mainly mediates type I IFN production. Plasmacytoid dendritic cells (pDCs) have an unusual network of signalling pathways that links MYD88 to IRF7 and that enables these cells to produce large quantities of IFNα in response to TLR7 and TLR9 ligands[8].

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Box 1 | **Necroptosis — a new innate immune pathway?**

Programmed cell death is an important part of innate immunity; for example, caspase 1-dependent pyroptosis is known to restrict pathogen replication and results in the re-exposure of intracellular bacteria to extracellular immune responses[5]. Although caspases are a major trigger of cell death, caspase-independent pathways also exist. Among these, necroptosis, which is a form of programmed necrosis, has recently attracted attention as a possible innate immune mechanism.

Necroptosis is a lytic type of cell death and requires the kinase activities of receptor-interacting protein 1 (RIP1) and RIP3. The discovery of necroptosis was prompted by the observation that tumour necrosis factor (TNF) treatment induces a necrotic type of cell death when caspase 8 activity is compromised[101]. Under these conditions TNF receptor 1 recruits RIP1, which then dissociates from the receptor and forms a cytosolic complex with RIP3, called the necrosome or complex IIb[102]. The signalling pathways downstream of RIP3 are unclear, but seem to involve at least two other proteins: mixed lineage kinase domain-like protein (MLKL)[103] and the mitochondrial serine/threonine phosphatase PGAM5 (REF. 104). Necroptosis can also be induced following the stimulation of PRRs, such as TLR3 and TLR4, or following the induction of genotoxic stress (characterized by inhibitor of apoptosis (IAP) degradation)[105,106]. In this case, the high-molecular mass complex formed by RIP1 and RIP3 is called a ripoptosome.

The antimicrobial function of necroptotic death has been mainly studied in the context of viral infections. As viruses are heavily dependent on the host cell, they have evolved a variety of cell death suppressors, such as caspase 8 inhibitors[102]. As inhibition of caspase 8 drives necroptosis, there must also be viral inhibitors of necroptosis. Indeed, the murine cytomegalovirus protein vIRA (viral inhibitor of RIP activation) has recently been reported to block the interaction between RIP1 and RIP3 (REF. 107). Interestingly, these studies have also shown a role for the cytosolic DNA sensor DAI (DNA-dependent activator of IFN-regulatory factors) in inducing RIP1-independent necroptosis through its direct interactions with RIP3 (REF. 18) (FIG. 2). Necroptosis was also recently shown to be induced in macrophages infected with *S.* Typhimurium. In contrast to the viral studies, the authors propose that necroptosis functions as a bacterial strategy to eliminate immune cells[108]. The receptors and the pathways that are involved in initiating necroptosis in response to *Salmonella* spp. or to other bacteria remain to be determined, but type I interferon production seems to have an essential role[108].

NLRs constitute the largest group of cytoplasmic receptors. The first members of this group that were identified — nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 — recognize peptidoglycan fragments and initiate both NF-κB activation and IFNβ production[9]. Some reports have also linked NOD2 to the recognition of RNA[10]. In addition, other members of the NLR family drive the assembly of inflammasome complexes in response to various danger signals and PAMPs[4].

Nucleic acids and their derivatives are one of the most important groups of PAMPs, particularly in the innate immune response against viruses that otherwise present few conserved PAMPs[11]. Microbial nucleic acids can be discriminated from self nucleic acids using various parameters, such as their sequence, their tertiary structure, their molecular modifications and their localization. In addition, mislocalized DNA and RNA can be an indicator of cellular damage and infection. Different classes of PRRs recognize cytoplasmic nucleic acids and initiate several distinct immune responses. RLRs — comprising retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and LGP2 (also known as DHX58) — detect several different ssRNA and dsRNA viruses and induce type I IFN production through mitochondrial antiviral signalling protein (MAVS) and IRF3 (REFS 12–17). The response to cytoplasmic DNA is not as well characterized, but can lead to type I IFN production, to inflammasome activation and even to the induction of a newly described cell death pathway that is linked to immunity, termed necroptosis[18] (BOX 1). A surprisingly large number of cytoplasmic DNA receptors have been identified in recent years, but many still await definitive validation.

Pyroptosis
A lytic pro-inflammatory form of programmed cell death that is initiated by the activation of inflammatory caspases.

Plasmacytoid dendritic cells
(pDCs). A dendritic cell subset that morphologically resembles a plasmablast. pDCs produce large amounts of type I interferons in response to viral infection.

Necroptosis
A form of programmed necrosis that is initiated by the kinases receptor-interacting protein 1 (RIP1) and RIP3 in response to external signals, in conditions in which caspase 8 activity is compromised.

In this Review, we discuss recently identified PRRs, their ligands, their modes of signalling and their interactions with other mammalian PRRs.

New functions for orphan TLRs

TLRs are arguably the best-studied group of PRRs. So far, 10 members of the TLR family have been identified in humans and 12 in mice, and a number of genetic studies have revealed their respective ligands and their modes of signalling (for a review see REFS 6,19). TLR-mediated recognition of PAMPs can occur at the plasma membrane or at endosomal and endolysosomal membranes. TLR1, TLR2, TLR4, TLR5 and TLR6 primarily, but not exclusively, localize to the plasma membrane and recognize microbial components such as lipids, lipoproteins, LPS and proteins. Conversely, TLR3, TLR7, TLR8 and TLR9 localize to intracellular vesicular compartments and are involved in the recognition of nucleic acids (TABLE 1). Nevertheless, the ligands for several TLRs including TLR10, which is only found in humans, and TLR11, TLR12 and TLR13, which are present in mice but not humans, have remained unknown so far (FIG. 1).

TLR11: a new flagellin receptor. Previous work had shown that TLR11 recognizes profilin[20], which is a protein from the apicomplexan parasite *Toxoplasma gondii*[20] and is an unknown proteinaceous component of uropathogenic *Escherichia coli* (UPEC)[21]. TLR11 is highly expressed in the intestinal epithelium and therefore its role in the recognition of enteropathogenic bacteria has recently been investigated[22]. TLR11-knockout mice infected with *Salmonella enterica* subsp. *enterica* serovar Typhimurium showed signs of increased intestinal invasion and enhanced bacterial dissemination to systemic organs, which indicates that TLR11 detects an *S.* Typhimurium ligand. Fractionation of heat-killed *S.* Typhimurium and UPEC extracts showed that TLR11 recognized flagellin[21], which is also a TLR5 ligand[23]. Further analysis showed that TLR11 induces immune responses independently of TLR5 and that TLR5-knockout mice had higher levels of expression of TLR11 (REF. 22), which might be an explanation for the previously reported increased resistance of *Tlr5*$^{-/-}$ animals to *S.* Typhimurium infection[24].

Although both TLR5 and TLR11 recognize flagellin, they function in different subcellular compartments. TLR5 is reportedly localized at the cell membrane, whereas TLR11 probably localizes to the endolysosomes as its function requires protein unc-93 homolog B1 (UNC93B1) (REF. 25), which is a protein that is necessary for the trafficking of TLRs from the endoplasmic reticulum (ER) to the endosomes[26]. Interestingly, the presence of TLR11 correlates with the resistance of mice to

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Table 1 | **TLRs: localization, species, typical ligands and recognized pathogens**

TLR	Localization	Species	Natural ligands	Synthetic ligands	Recognized pathogens	Tissue-specific and cell type-specific expression
TLR1	Extracellular	Humans and mice	Triacyl lipopeptides	Pam3CSK4	Bacteria	• Ubiquitous tissue expression • Monocytes, macrophages, DCs, leukocytes, B cells, T cells and NK cells
TLR2	Extracellular	Humans and mice	Lipoproteins, peptidoglycan, LTA, zymosan and mannan	Pam3CSK4	Bacteria	• Brain, heart, lungs and spleen • Macrophages, DCs and granulocytes
TLR3	Endolysosomal compartment	Humans and mice	dsRNA	polyI:C and polyU	dsRNA	• Placenta and pancreas • DCs, T cells and NK cells
TLR4	Extracellular and endolysosomal compartment	Humans and mice	LPS, RSV and MMTV fusion protein, mannans, and glyco-inositolphosphate from *Trypanosoma* spp.	Lipid A derivatives	Gram-negative bacteria and viruses	• Spleen • PBLs, B cells, DCs, monocytes, macrophages, granulocytes and T cells
TLR5	Extracellular	Humans and mice	Flagellin	ND	Bacteria	• Ovaries and prostate • PBLs and monocytes
TLR6	Extracellular	Humans and mice	Diacylipopetides, LTA and zymosan	MALP2	Bacteria	• Thymus, spleen and lungs • B cells and monocytes
TLR7	Endolysosomal compartment	Humans and mice	GU-rich ssRNA and short dsRNA	Imidazoquinolines and guanosine analogues	Viruses and bacteria	• Lung, placenta, spleen, lymph nodes and tonsils • Monocytes, B cells and DCs
TLR8	Endolysosomal compartment	Humans and mice	GU-rich ssRNA, short dsRNA and bacterial RNA*	Imidazoquinolines and guanosine analogues	Viruses and bacteria	• Lungs, placenta, spleen, lymph nodes and bone marrow • PBLs and endothelial cells
TLR9	Endolysosomal compartment	Humans and mice	CpG DNA and hemozoin from *Plasmodium* spp.	CpG ODNs	Bacteria, viruses and protozoan parasites	• Spleen, lymph nodes and bone marrow • PBLs, B cells and DCs
TLR10	ND	Humans	ND	ND	ND	• Spleen, lymph nodes, thymus and tonsils • B cells
TLR11	Endolysosomal compartment	Mice	Profilin and flagellin	ND	Apicomplexan parasites and bacteria (including *Salmonella* spp. and UPEC)	• Spleen, kidney, liver and small intestines • Epithelium, DCs and macrophages
TLR12	Endolysosomal compartment	Mice	Profilin	ND	Apicomplexan parasites	• Small intestines and spleen • DCs and macrophages
TLR13	Endolysosomal compartment	Mice	Bacterial 23S rRNA with CGGAAAGACC motif	ND	Gram-negative and Gram-positive bacteria	• Spleen • DCs and macrophages

DC, dendritic cell; dsRNA, double-stranded RNA; LPS, lipopolysaccharide; LTA, lipotechoic acid; MALP2, macrophage-activating lipopeptide 2; MMTV, mouse mammary tumour virus; ND, not defined; NK, natural killer; ODN, oligodeoxynucleotide; Pam3CSK4, Pam3Cys-Ser-Lys4-trihydrochloride; PBLs, peripheral blood leukocytes; polyI:C, polyinosinic–polycytidylic acid; polyU, poly-uridine; rRNA, ribosomal RNA; RSV, respiratory syncytial virus; ssRNA, single-stranded RNA; TLR, Toll-like receptor; UPEC, uropathogenic *Escherichia coli*. *For human TLR8.

Salmonella enterica subsp. *enterica* serovar Typhi infection, which has in the past hindered the development of a small-animal model of typhoid fever. Mathur *et al.*[22] found that *Tlr11*$^{-/-}$ mice were susceptible to *S.* Typhi infections, that they showed signs of febrile illness with features of human typhoid fever and that they could be efficiently immunized against *S.* Typhi. Thus, TLR11 alone mediates the resistance of mice to *S.* Typhi infections, which suggests that *Tlr11*$^{-/-}$ mice might be a suitable mouse model of human typhoid fever.

However, several questions remain unanswered; for example, why can *S.* Typhimurium overcome the intestinal TLR11 barrier whereas *S.* Typhi cannot? A reduced arsenal of virulence factors in *S.* Typhi might be a possible explanation. Alternatively, motility might be involved: it seems to be important for *S.* Typhi infections, as aflagellated *S.* Typhi are non-virulent, whereas flagella are less important for *S.* Typhimurium pathogenesis[22]. *S.* Typhi infection of *Tlr11*$^{-/-}$ mice does not fully recapitulate the pathological and immunological features of human typhoid fever. In particular, the severe intestinal destruction and IL-12 production seen in these mice are not typically seen in *S.* Typhi-infected humans[22]. Nevertheless, the availability of a small-animal model for the study of *S.* Typhi infections is an important step in understanding typhoid infections and might also contribute to the study of other human enteric diseases.

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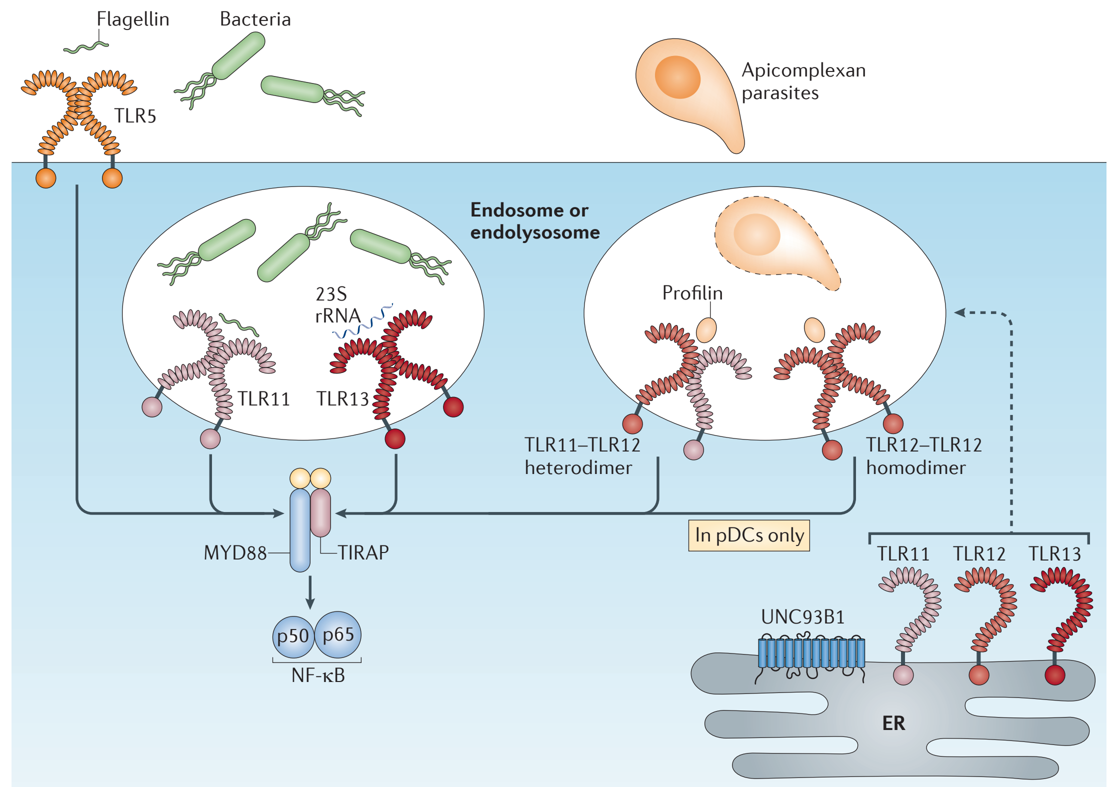

Figure 1 | **New functions for orphan TLRs in mice.** Two Toll-like receptors (TLRs) — TLR5 and TLR11 — recognize bacterial flagellin and induce nuclear factor-κB (NF-κB) signalling through the adaptor molecules myeloid differentiation primary-response protein 88 (MYD88) and TIR domain-containing adaptor protein (TIRAP; also known as MAL). TLR5 localizes to the cell membrane, whereas TLR11 is thought to localize from the endoplasmic reticulum (ER) to the endolysosomal compartments (indicated by the dashed arrow), as it requires protein unc-93 homolog B1 (UNC93B1; a protein that is necessary for the ER–endosome trafficking of TLRs) for its function. TLR11 also forms a heterodimer in endosomes with TLR12, which recognizes profilin-like proteins from apicomplexan parasites, such as *Toxoplasma gondii* and *Plasmodium falciparum*. The correct function of TLR12 also requires UNC93B1. In plasmacytoid dendritic cells (pDCs), TLR12 has the ability to form homodimers that recognize profilin and that induce MYD88 signalling. Finally, mouse TLR13 recognizes the CGGAAAGACC motif of bacterial 23S ribosomal RNA (rRNA) in the endosomal compartment and induces NF-κB signalling via MYD88.

Recognition of apicomplexan parasites by TLR12. *T. gondii* is an obligate intracellular apicomplexan parasite that infects a wide range of warm-blooded hosts. In infected mice, survival requires IL-12 production, which is dependent on MYD88 (REF. 27). However, although TLR2, TLR4 and TLR11 induce a cytokine response following the recognition of *T. gondii* glycosylphosphatidylinositol (GPI)-anchored proteins and *T. gondii* profilin, deletions of these TLRs do not recapitulate the lethality observed in MYD88-deficient mice, which indicates that additional TLRs recognize *T. gondii*[20,27,28].

Koblansky *et al.*[29] have now shown that TLR12 has a crucial role in the control of *T. gondii* infections. TLR12 is highly homologous to TLR11 and, although their expression overlaps in macrophages and DCs, TLR12 is predominantly expressed in myeloid cells, whereas TLR11 is mostly expressed in epithelial tissue[29]. Macrophages and conventional DCs deficient for both TLR11 and TLR12 failed to respond to *T. gondii* profilin, which indicates that these TLRs function as a heterodimer in these cell types. Interestingly, TLR11 and TLR12 also recognize profilin from *Plasmodium falciparum*, but only TLR11 responds to UPEC flagellin[29]. Nevertheless, unlike $Tlr11^{-/-}$ animals, $Tlr12^{-/-}$ mice rapidly succumb to *T. gondii* infection. A possible explanation for this was provided by the observation that TLR12 alone was necessary and sufficient to induce IL-12 and IFNα expression in pDCs in response to profilin, which leads to the production of IFNγ by natural killer (NK) cells and to host resistance against *T. gondii* infection[29]. Thus, in addition to TLR11, TLR12 is involved in the recognition of profilin from apicomplexan parasites. TLR12 can function either alone or as a heterodimer with TLR11. It is unclear why mice and rats but not humans have evolved such an efficient system to recognize apicomplexan parasites, but it is possible that resistance to these parasites might be more important in rodents, as they are an intermediate host in the *T. gondii* life cycle.

Mouse TLR13 and human TLR8 recognize bacterial RNA. TLR2 is generally thought to be a central detector of Gram-positive bacteria. However, the activation of host immune responses by group A streptococcus was shown to occur by a MYD88-dependent but TLR2-, TLR4- and TLR9-independent pathway[30], which

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suggests that other TLRs might be important for the recognition of this pathogen. Mice lacking UNC93B1 were similarly unresponsive[31]. Surprisingly, macrophages lacking multiple TLRs (specifically TLR2, TLR3, TLR4, TLR7 and TLR9) were still responsive to heat-inactivated *Streptococcus aureus*, but not if the preparations had been treated with ribonuclease A (RNase A)[32], which suggests that an RNA-sensing pathway is involved.

Using DC subsets with distinct TLR expression profiles, a recent study showed that the TLR2-independent sensing of heat-inactivated *S. aureus* only requires TLR13. Large bacterial ribosomal RNAs (rRNAs) — specifically the conserved CGGAAAGACC motif of 23S rRNA — were identified as the ligand for TLR13. Notably, this immunostimulatory sequence is targeted by the macrolide, lincosamide and streptogramin B (MLS) group of antibiotics. Importantly, the modification of 23S rRNA in certain MLS-resistant clinical isolates of *S. aureus* abolished their immunostimulatory activity[32]. This motif is highly conserved among Gram-negative and Gram-positive bacteria: the 23S rRNA of *E. coli* was also shown to induce a transcriptional response that was dependent on TLR13, which resulted in the induction of pro-IL-1β[33]. Finally, a third study confirmed the importance of these results, showing that both live and heat-killed *Streptococcus pyogenes* are recognized by this TLR13-dependent pathway[34]. Given the importance of TLR13 in sensing bacteria, it is surprising that this TLR is not present in humans. It is possible that a related RNA-sensing PRR has evolved in humans to recognize species of bacteria that have modified their 23S rRNA.

Although the studies mentioned above excluded an involvement of mouse TLR8 in the recognition of bacterial RNA[32], human TLR8 has a different specificity to both physiological and synthetic TLR8 ligands. Indeed, several reports indicate that human TLR8 responds to total bacterial RNA[35], as well as to infections with several bacterial pathogens, by inducing the expression of pro-inflammatory cytokines and type I IFNs[36]. However, the exact ligands for TLR8 and the possible redundancies between TLR8 and TLR13 still need to be determined. In addition, links and cooperations between RNA sensing by endosomal TLRs and by cytoplasmic RNA receptors remain uncharacterized.

Sensing cytosolic DNA and CDNs

The cytosolic responses to RNA and RNA viruses have been fairly extensively characterized. Members of the DExD/H box helicase (Asp–Glu–x–Asp/His box) family — RIG-I, MDA5 and LGP2 — are involved in the recognition of cytosolic ssRNA and dsRNA and signal through MAVS, which activates IRF1, IRF3, IRF7 and NF-κB; this ultimately leads to the expression of type I IFNs and pro-inflammatory cytokines[11] (FIG. 2).

By contrast, the response to cytosolic DNA, which leads to the induction of type I IFNs and/or inflammasome activation, has not been characterized to the same extent. The first cytosolic DNA sensors to be identified were DNA-dependent activator of IFN regulatory factors (DAI; also known as ZBP1)[37] and absent in melanoma 2 (AIM2)[38,39].

However, subsequent studies have shown that DAI is not essential for the IFN response to DNA[40] and instead DAI was linked to the recognition of murine cytomegalovirus and to the induction of necroptosis[18] (BOX 1). In addition, AIM2 assembles inflammasome complexes and does not promote an IFN response[38,39,41]. So, how is IFN induced by cytosolic DNA? RNA polymerase III can also function as a sensor of B-form DNA (poly(dA:dT)) by converting it into dsRNA that is recognized by RIG-I (REFS 42,43). However, as other forms of DNA induce type I IFN independently of RNA polymerase III, there must be additional cytoplasmic DNA sensors. The search for these elusive receptors has led to the recent identification of several different candidate proteins that seem to be involved in cytosolic nucleic acid sensing, either as receptors or as signalling adaptors (TABLE 2).

STING: a PRR and a signalling adaptor protein? A role for stimulator of IFN genes protein (STING) in the cytosolic response to nucleic acids was independently reported by several groups that screened for proteins that activate the IFNβ promoter[44,45]. STING was subsequently found to predominantly reside in the ER and to have a crucial role in the response to transfected dsDNA, as well as to viral, bacterial and eukaryotic intracellular pathogens in bone marrow-derived macrophages (BMDMs) and in bone marrow-derived DCs (BMDCs)[46]. Further analysis indicated that STING was also essential for the IFN response to CDNs[47]. However, for both the sensing of DNA and CDNs, STING was thought to function as an adaptor protein, linking upstream PRRs to IRF3 activation.

In an attempt to identify host components that are upstream of STING in the CDN-sensing pathway, Burdette *et al.*[48] found that the expression of STING alone is sufficient to reconstitute IFNβ production following cyclic diguanylate monophosphate (c-di-GMP) and cyclic diadenylate monophosphate (c-di-AMP) treatment of HEK293T cells, which lack endogenous STING expression. STING directly binds to CDNs, and an Arg231Ala mutant of STING does not respond to CDNs but still responds to DNA when expressed in *Sting*$^{-/-}$ BMDMs. These findings, taken together with the observation that STING alone is not sufficient to restore the responsiveness of HEK293T cells to transfected DNA, indicate that STING can function both as a direct sensor of CDNs and as a signalling adaptor molecule in response to cytosolic DNA[48].

Five groups have recently published the crystal structure of STING alone or in a complex with c-di-GMP[49–53]. These studies show that the cytoplasmic domain (CTD) of STING adopts a new α/β-fold that has some structural similarity to the RAS family of small G proteins. In addition, these studies suggest that the STING CTD forms a V-shaped dimer, even when it is not bound to its ligand, and that it binds to one molecule of c-di-GMP at the interface of the dimer. This indicates that ligand-induced dimerization is not the mechanism by which STING is activated. As only one part of STING has been crystallized, it remains to be investigated how ligand binding affects other domains of STING, which could be involved in downstream signalling.

Ribonuclease A
(RNase A). An endoribonuclease that specifically cleaves single-stranded RNA and that is often used to remove RNA from samples.

Macrolide, lincosamide and streptogramin B
(MLS). A group of antibiotics that function as translational inhibitors by targeting the 50S ribosomal subunit, which contains 23S ribosomal RNA.

DExD/H box helicase
An enzyme that can unwind double-stranded RNA using energy derived from ATP hydrolysis. The DExD/H box is a characteristic amino acid signature motif of many RNA-binding proteins.

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Recent reports have confirmed the central role of STING in the type I IFN response to DNA and CDNs. Sensing of the bacterial secondary messengers c-di-GMP and c-di-AMP by STING was initially thought to be a mechanism for the detection of intracellular bacteria, such as *Listeria monocytogenes*, *Legionella pneumophila* and *Pseudomonas aeruginosa*[54], but recently it has been shown that CDNs could also be an endogenous secondary messenger or a danger signal[55,56]. A first study showed that cyclic GMP–AMP (cGAMP) was synthesized from

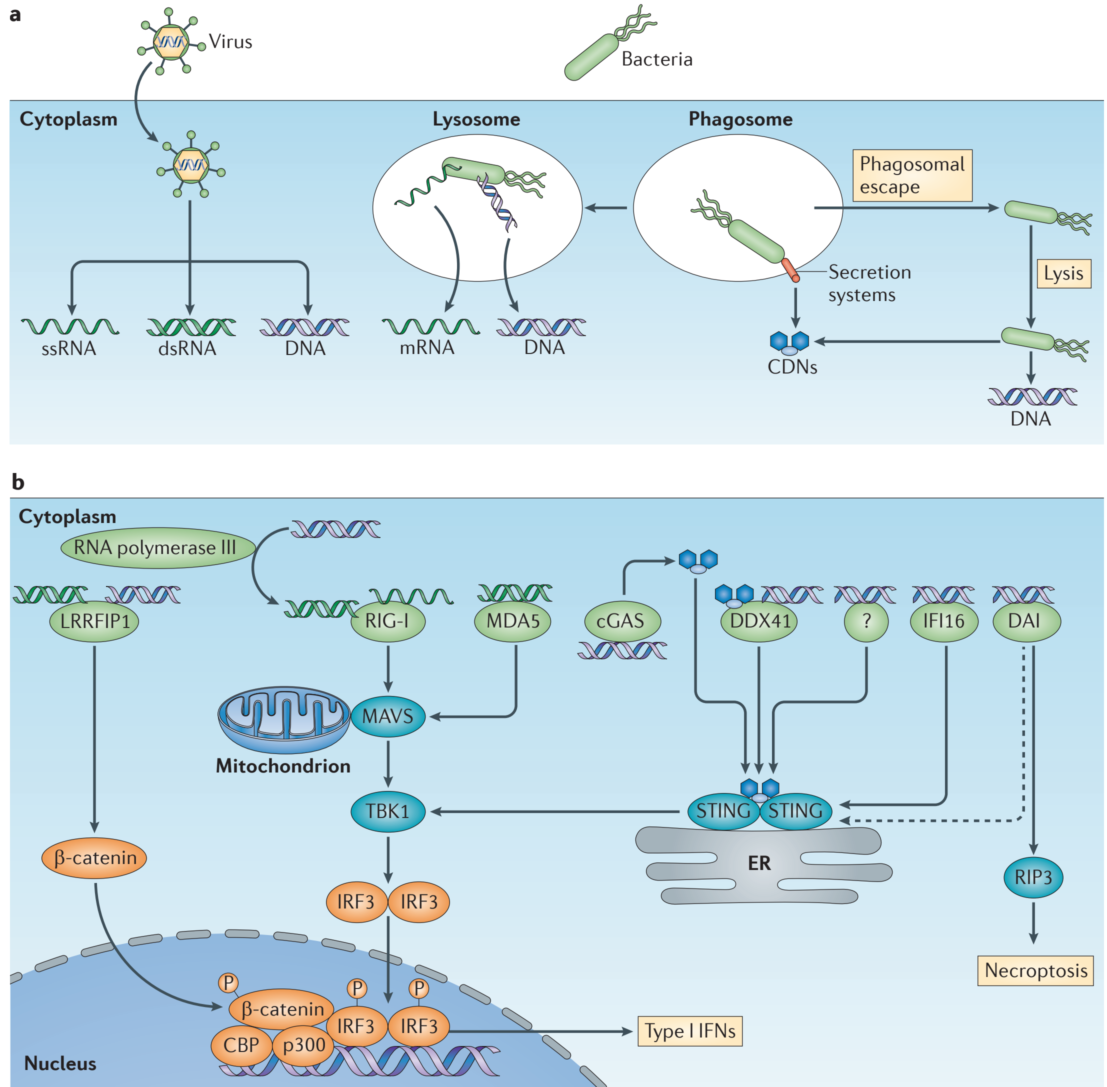

Figure 2 | **The cytosolic type I IFN response to nucleic acids.** **a** | Microbial pathogens release different types of nucleic acids into the cytosol. Viruses are mainly detected by their single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) or DNA. In addition, virulence-associated secretion systems might leak cyclic-dinucleotides (CDNs) into the host cell cytosol that can be sensed by pattern recognition receptors. The lysis of bacteria in the cytosol can release DNA and CDNs, as has been shown for *Francisella novicida* and *Listeria monocytogenes*. Bacterial RNA is thought to reach the cytosol by leakage from the lysosomes and endolysosomes. **b** | Various cytosolic receptors detect these different types of nucleic acids. Retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) detect ssRNA and dsRNA and induce type I interferons (IFNs) through mitochondrial antiviral-signalling protein (MAVS), TANK-binding kinase 1 (TBK1) and IFN-regulatory factor 3 (IRF3). RNA polymerase III can convert AT-rich DNA into dsRNA that is recognized by RIG-I. DNA is detected by DDX41, IFNγ-inducible protein 16 (IFI16) and maybe another as yet unknown receptor (depicted as a question mark), and all of these receptors signal through the endoplasmic reticulum (ER)-resident protein stimulator of IFN genes protein (STING), which acts upstream of TBK1. DNA-dependent activator of IFN-regulatory factors (DAI) is another sensor that has been proposed to interact with STING (dashed arrow), but which has recently been implicated in the induction of necroptosis through its direct interaction with receptor-interacting protein 3 (RIP3). STING not only functions as a signalling adaptor for the cytosolic DNA response but has also been shown to directly bind to CDNs and to activate type I IFN signalling. DDX41 also detects CDNs and activates STING. In response to DNA, cyclic GMP–AMP synthase (cGAS) synthesizes CDNs, which function as endogenous danger signals. A dsRNA-sensing and DNA-sensing pathway that is controlled by leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) functions as a co-activator of type I IFN signalling by activating β-catenin, which enhances IFNβ production. CBP, CREB-binding protein.

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GTP and ATP in cytosolic extracts treated with DNA, or in cells transfected with DNA or infected with a DNA virus[56]. Interestingly, cGAMP could activate IRF3 by binding to STING, indicating that it might be a danger signal[56]. A second study identified cGAMP synthase (cGAS) as a cytosolic DNA sensor[55]. Knockdown of cGAS reduced IRF3 activation and IFNβ induction in response to DNA and to a DNA virus[55]. Purified cGAS catalysed the synthesis of cGAMP in the presence of different forms of DNA, but not RNA, which indicates that cGAS activity is only stimulated by DNA[55]. In addition, cGAS was shown to directly bind immunostimulatory DNA but not RNA. Finally, subcellular fractionation and immunofluorescence studies confirmed that cGAS primarily localizes to the cytosol and not to the nucleus, where it can sense cytosolic DNA[55].

In conclusion, these studies have established STING as an essential component of cytosolic nucleic acid sensing, functioning as a PRR as well as a signalling adaptor protein. In addition, CDNs (either host-derived or from intracellular bacterial pathogens) are important immunostimulatory compounds that might be valuable as immunotherapeutics or as adjuvants[57,58].

DDX41 recognizes DNA and CDNs. Several reports suggest the existence of at least one putative DNA sensor upstream of STING. One possible candidate is DDX41, which is a member of the DExD/H box helicases. DDX41 was identified in a small interfering RNA screen in a mouse DC line (the D2SC cell line), and knockdown of DDX41 affected the IFN response to B-form DNA (poly(dA:dT)), to Z-form DNA (poly(dG:dC)) and to the DNA virus herpes simplex virus 1 (HSV1)[59]. Interestingly, despite its homology to other DExD/H box helicases, the knockdown of *Ddx41* does not affect the response to polyinosinic–polycytidylic acid (polyI:C) or to RNA viruses. Similar effects of *Ddx41* knockdown were observed in BMDCs and in the THP1 monocytic cell line[59]. Biochemical analysis indicates that DDX41 specifically binds DNA via its DExD/H box domain, that it interacts with endogenous STING and TANK-binding kinase 1 (TBK1) and that it colocalizes with STING in the ER. These results suggest that DDX41 is a cytosolic DNA sensor, but it remains to be definitively shown whether DDX41 activates STING to induce an IFN response. Although these knockdown studies show a role for DDX41 in sensing cytosolic DNA, it remains possible that there are additional cytosolic DNA sensors that recognize other forms of DNA and that induce IFN.

In addition, DDX41 might have a role in the recognition of CDNs. Knockdown of DDX41 by short hairpin RNA (shRNA) in D2SC cells and THP1 cells abolishes the IFN response to CDN transfection and *L. monocytogenes*[60], which is a bacterial pathogen that is known to induce IFN via the release of c-di-AMP[54]. Binding assays using biotinylated c-di-GMP showed that the central DExD/H box domain, but not the helicase domain, of DDX41 is required for CDN binding[60]. The mechanism of DDX41 signalling remains unclear, but CDN transfection led to the co-immunoprecipitation of DDX41 with STING, and the CDN-dependent STING–TBK1 association was reduced in the presence of DDX41-specific shRNA. This indicates that DDX41 and STING might form a CDN-sensing complex, in which STING functions downstream of DDX41 or as a cofactor. This model is supported by results showing that DDX41 has a higher affinity for c-di-GMP than STING does[60] and that *Ddx41* knockdown reduces the association of STING with c-di-GMP[60]. Further crystallographic analysis of DDX41 in complex with c-di-GMP, and ideally also with STING, will be necessary to fully understand the mechanism underlying CDN recognition.

IFI16: an unusual PYHIN member. IFNγ-inducible protein 16 (IFI16), which is a member of the PYHIN protein family, is another putative DNA sensor. Transfection of cells with either viral or synthetic DNA has long been known to induce a type I IFN response. Even though IFI16 is predominantly a nuclear protein, immunofluorescence analysis showed that IFI16 could colocalize with immunostimulatory DNA in the cytoplasm. Consistent with this observation, knockdown of *IFI16* reproducibly resulted in a reduced IFN response to cytosolic DNA or to HSV1 (REF. 61). The signalling mechanism of IFI16 is likely to involve STING, as STING was shown to co-immunoprecipitate with IFI16 from DNA-treated cells; however, it remains unclear whether this interaction is direct or whether it involves other proteins[61]. Interestingly, a recent report indicated that IFI16-mediated HSV1 sensing occurs in the nucleus[62], but the authors did not see a relocalization of IFI16 from the nucleus to the cytoplasm where STING is located. Thus, additional factors might mediate the interaction of IFI16 with STING[62]. Intriguingly, IFI16 might also be involved in the inflammasome response to Kaposi's sarcoma-associated herpesvirus and in the inflammasome response that restricts human cytomegalovirus replication independently of type I IFN[63]. In conclusion, more work is required to understand in which subcellular compartment IFI16 functions and how IFI16 can not only function as an activator of the STING signalling pathway but also as an initiator of inflammasome assembly.

LRRFIP1: a co-activator of the cytoplasmic DNA response. The leucine-rich repeat (LRR) protein LRRFIP1 (leucine-rich repeat flightless-interacting protein 1) was recently identified in a screen that investigated the role of LRR-containing and LRR-interacting proteins in the IFN response to *L. monocytogenes*[64]. Knockdown of *Lrrfip1* reduces IFNβ production in response to vesicular stomatitis virus (VSV), synthetic RNA and synthetic B-form and Z-form DNA, which confirms the previous reports that LRRFIP1 binds to both dsRNA and dsDNA[64]. Interestingly, *Lrrfip1* knockdown does not abolish the activation of IRF3, NF-κB and MAPKs in *L. monocytogenes*-infected cells, but rather it reduces the phosphorylation of β-catenin, which is thought to function as a transcriptional co-activator at the *Ifnb1* promoter[64]. Thus, these results have identified LRRFIP1 as an essential component of the type I IFN response to VSV and to *L. monocytogenes* infections.

Small interfering RNA
(siRNA). Short double-stranded RNAs of 19 to 23 nucleotides that induce RNA interference, which is a post-transcriptional process that leads to gene silencing in a sequence-specific manner.

Short hairpin RNA
(shRNA). A sequence of RNA that makes a tight hairpin turn, which can be used to silence target gene expression via RNA interference.

Leucine-rich repeat
(LRR). A protein structural motif composed of repeating stretches of 20 to 30 amino acids that are unusually rich in the hydrophobic amino acid leucine and that form an α/β-horseshoe fold. LRRs are found in many pattern recognition receptors, such as Toll-like receptors and NOD-like receptors, but also in many functionally unrelated proteins.

β-catenin
This protein functions both as a transcriptional activator and as a membrane–cytoskeleton linker protein by binding to E-cadherin. Following detachment from E-cadherin, β-catenin can relocate to the nucleus.

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As *L. monocytogenes* is known to induce the type I IFN response through CDNs[54], it will be interesting to determine the role of LRRFIP1 in CDN sensing. In addition, the generation of LRRFIP1-deficient mice is required to further understand the physiological role of LRRFIP1 *in vivo* and to delineate its role in the complex network of nucleic acid-sensing pathways.

Other cytoplasmic nucleic acid sensors. In addition to the sensors discussed above, a multitude of other proteins have been implicated in the sensing of cytoplasmic nucleic acids (TABLE 2); for example, NOD2 (REF. 10), NLRP3 (NOD-, LRR- and pyrin domain-containing 3)[65,66] and KU70 (also known as XRCC6; a DNA sensor that induces type III IFN)[67] can recognize nucleic acids.

Members of the DExD/H box helicases seem to have a very prominent role in RNA sensing. DDX3 was proposed to interact with RIG-I, MDA5 and MAVS to promote IFN production in response to viral RNA[68]. Another study proposed that DDX1, DDX21 and DDX36 form a complex that activates TRIF in response to polyI:C in DCs[69]. DDX60 associates with RIG-I, MDA5 and LGP2 and enhances type I IFN production in response to RNA and DNA viruses[70]. It has been suggested that DHX9 and DHX36 sense oligodeoxynucleotides and induce MYD88 signalling[71]. However, these results must be treated with caution, as the DExD/H box helicase family members also have an important role in RNA metabolism[72].

Another gene family that has a role in antiviral defence is the family of IFN-induced proteins with tetratricopeptide repeats (IFITs). Some of the IFITs recognize viral ssRNA that has a 5′-triphosphate group (PPP–ssRNA), which distinguishes it from the host RNA[73]. The crystal structures of IFIT5 and a fragment of IFIT1 showed there to be a previously unidentified domain with a positively charged cavity that specifically facilitates the binding of PPP–ssRNA, as well as providing the structural basis for the selective recognition of PPP–ssRNA that is distinct from the recognition of PPP–dsRNA by RIG-I (REF. 74). The mode of action of IFIT proteins is unclear, but it has been suggested to involve either the disruption of protein–protein interactions in the host translation-initiation machinery or the binding of viral RNA, thus preventing viral replication or packaging into new viral particles.

Recent progress has led to the identification of a surprisingly large variety of cytoplasmic nucleic acid sensors and has raised a number of questions. How do all of these newly identified PRRs cooperate in cytosolic nucleic acid sensing? Are there redundant pathways and cell type-specific differences? How do these sensors contribute to immunity, vaccination and autoimmune diseases *in vivo*? A better understanding will be gained from a rigorous validation of the function of these newly identified PRRs, as their identification and characterization has so far relied on gene knockdown and overexpression studies in non-physiological cell lines. Given the complexity of the cytoplasmic nucleic acid response, future studies need to move away from analysing each sensor and pathway individually and to take a more holistic approach, such as systems analysis, to understand the response as a whole.

Orphan NLRs assemble new inflammasomes

The recognition of intracellular pathogens is not restricted to the detection of nucleic acids but involves, in analogy to TLR sensing, the recognition of various PAMPs. This aspect of immune recognition is mainly carried out by the family of NLRs. The human genome encodes 23 NLR family members and more than 34 NLRs have been identified in mice. Some of the NLRs, such as NOD1, NOD2 and class II transactivator (CIITA), are involved in NF-κB signalling and transcriptional activation; however, the majority of these family members are thought to initiate the assembly of inflammasomes. Inflammasomes that are assembled by NLRs usually activate caspase 1 and are sometimes referred to as canonical inflammasomes. Other inflammasome complexes have been identified and are sometimes referred to as non-canonical inflammasomes because they activate other caspases that lead to pro-inflammatory cell death or to the release of pro-inflammatory cytokines (BOX 2). However, until recently only three NLRs — NLRC4 (NOD-, LRR- and CARD-containing 4), NLRP1 (or murine NLRP1B) and NLRP3 — were definitely known to initiate inflammasome assembly. In addition, the PYHIN member AIM2 was shown to assemble inflammasomes in response to cytoplasmic DNA[38,39,41]. The ligands and mode of signalling of these receptors have been extensively reviewed[4] and will not be discussed in this Review. As inflammasomes also induce pyroptosis of the infected cell, their activation is very tightly controlled; for example, the activation of TLRs and/or of the type I IFN response is required for the expression of pro-IL-1β, NLRP3, AIM2 and pro-caspase 1 (REFS 4,41,75,76), which shows the importance of PRR crosstalk in the response to intracellular pathogens. In the section below, we highlight recent work that has characterized the function of orphan NLRs in inflammasome signalling in response to intracellular pathogens (FIG. 3). Although there are some differences in the inflammasome recognition of certain pathogens between mice and humans[77], many of the mechanisms of inflammasome activation are similar.

The NAIP–NLRC4 inflammasome. One of the first NLRs that was shown to mediate the assembly of inflammasome complexes was NLRC4 (REF. 78). Previous work had shown that NLRC4 responds to both bacterial flagellin and to type III secretion system (T3SS) rod proteins from different bacterial pathogens[79–82], but it was unclear whether these proteins were directly sensed by NLRC4 or whether other factors were involved. Neuronal apoptosis inhibitory protein 5 (NAIP5) was implicated in NLRC4 activation in mouse macrophages in response to *L. pneumophila*, but NAIP5 only partially contributed to NLRC4 activation during *S.* Typhimurium and *P. aeruginosa* infections[83]. These puzzling observations were clarified by two recent studies that showed that NAIPs — four of which (NAIP1, NAIP2, NAIP5 and NAIP6) are expressed in C57BL/6 mice — function upstream of mouse NLRC4 as receptors for flagellin and T3SS rod subunits[84,85].

Type III secretion system
(T3SS). A virulence-associated specialized molecular machine present in some bacteria that facilitates the translocation of bacterial proteins into host cells.

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Table 2 | **Cytosolic nucleic acid-sensing PRRs: typical ligands, recognized pathogens, cell type and mode of signalling**

PRR	Alternative names	Protein family	Typical ligands	Recognized pathogens	Reported mode of signalling	Cell type (function or discovery)	Refs
RIG-I	DDX58	DExD/ H box helicases	PPP-ssRNA, RNA with base pairing and polyI:C	ssRNA viruses, DNA viruses, Flaviviridae, reovirus and bacteria	IPS1 or STING, TBK1, IRF1, IRF3, IRF7, NF-κB and NLRP3 inflammasome	Immune and non-immune cells	13,14
MDA5	IFIH1 and helicard	DExD/ H box helicases	Long dsRNA	Picornavirus, vaccinia virus, Flaviviridae, reovirus and bacteria	IPS1, TBK1, IRF1, IRF3, IRF7 and NF-κB	Immune and non-immune cells	12
LGP2	DHX58	DExD/ H box helicases	dsRNA	RNA viruses	Regulator of RIG-I and MDA5 activity	Immune and non-immune cells	15–17
DDX41	ND	DExD/ H box helicases	B-form DNA and CDNs	DNA viruses and *Legionella monocytogenes*	STING, TBK1 and NF-κB	D2SC cells	59,60
DHX9	DDX9 and NDHII	DExD/ H box helicases	DNA, RNA, CpG-A ODNs and CpG-B ODNs	ND	MYD88	Human pDCs	71
DDX3	DDX3X, FIN14	DExD/ H box helicases	Viral RNA	RNA viruses	RIG-I, MDA5 and LGP2	HEK293 or HeLa cells	68
DHX36	DDX36	DExD/ H box helicases	DNA, RNA, CpG-A ODNs and CpG-B ODNs	ND	MYD88	Human pDCs	71
DDX1–DDX21–DDX36	ND	DExD/ H box helicases	RNA and polyI:C	RNA viruses	TRIF	BMDCs	69
DDX60	ND	DExD/ H box helicases	ssRNA, dsRNA and dsDNA	RNA viruses and DNA viruses	RIG-I, MDA5 and LGP2	HeLa cells	70
KU70	XRCC6	ND	DNA	ND	Type III IFN	HEK293 and HeLa cells	67
cGAS	E330016A19	ND	DNA	DNA viruses	CDN synthesis	L929, Raw264.7, THP1 and HEK293 cells	55,56
STING	TMEM173, ERIS and MPYS	ND	CDNs (c-di-GMP and c-di-AMP)	Bacteria	TBK1	BMDMs	44–48
NOD2	CARD15 and NLRC2	NLRs	ssRNA	RNA viruses	IPS1, IRF3 and NF-κB	A539 and HEK293 cells	10
NLRP3	NALP3 and cryopyrin	NLRs	ssRNA, dsRNA, bacterial mRNA and oxidized mitochondrial DNA	RNA viruses, bacteria and cellular damage	Inflammasome assembly	THP1 cells, BMDCs, BMDMs and epithelial cells	65,66
AIM2	IFI210	PYHINs	DNA	DNA viruses and bacteria	Inflammasome assembly	BMDMs and BMDCs	38,39, 41
IFI16	p204	PYHINs	dsDNA	DNA viruses	STING, TBK1 and IRF3	Raw264.7 cells and MEFs	61
LRRFIP1	FLAP and FLIIAP1	ND	B-form DNA, Z-form DNA and dsRNA	VSV and *L. monocytogenes*	β-catenin	BMDMs and DCs	64
DAI	ZBP1 and DLM1	ND	DNA	MCMV	Necroptosis (via RIP3)	MEFs	18,37, 40
IFIT1,2,3 and 5	ND	IFITs	PPP-ssRNA	VSV, RVFV and parainfluenza virus type 5	Blocking viral replication	HeLa cells	73,74

AIM2, absent in melanoma 2; BMDC, bone marrow-derived dendritic cells; BMDM, bone marrow-derived macrophages; c-di-AMP, cyclic diadenylate monophosphate; c-di-GMP, cyclic diguanylate monophosphate; CDN, cyclic dinucleotide; cGAS, cyclic GMP–AMP synthase; DAI, DNA-dependent activator of IFN-regulatory factors; DC, dendritic cell; DExD/H box, Asp–Glu–x–Asp/His box; dsRNA, double-stranded RNA; IFI16, IFNγ-inducible protein 16; IFIT, IFN-induced proteins with tetratricopeptide repeats; IFN, interferon; IPS1, *IFNB* promoter stimulator 1; IRF, interferon-regulatory factor; LRRFIP1, leucine-rich repeat flightless-interacting protein 1; MCMV, mouse cytomegalovirus; MDA5, melanoma differentiation-associated protein 5; MEF, murine embryonic fibroblast; MYD88, myeloid differentiation primary-response protein 88; ND, not defined; NF-κB, nuclear factor-κB; NLR, NOD-like receptor; NLRP3, NOD-, LRR- and pyrin domain-containing 3; NOD2, nucleotide-binding oligomerization domain-containing protein 2; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; polyI:C, polyinosinic–polycytidylic acid; PPP-ssRNA, ssRNA with a 5′-triphosphate group; PRR, pattern recognition receptor; PYHIN, pyrin and HIN domain-containing; RIG-I, retinoic acid-inducible gene I; RIP3; receptor-interacting protein 3; RVFV, Rift Valley fever virus; ssRNA, single-stranded RNA; STING, stimulator of IFN genes; TBK1, TANK-binding kinase 1; TRIF, TIR-domain-containing adaptor protein inducing IFNβ; VSV, vesicular stomatitis virus.

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Box 2 | **Caspase 8 and caspase 11 inflammasomes**

Inflammasomes are generally defined as caspase 1-activating macromolecular platforms that control interleukin-1β (IL-1β) and IL-18 maturation and pyroptosis. However, recently other complexes have also been shown to induce these responses.

Caspase 11, which is the murine orthologue of human caspase 4 and caspase 5, is activated in response to a subset of triggers, including enteric bacteria (such as *Escherichia coli* and *Citrobacter rodentium*), and cholera toxin B with lipopolysaccharide (LPS)[109]. Interestingly, caspase 11 is sufficient to induce the lysis of macrophages as well as the release of high-mobility group protein B1 (HMGB1) and IL-1α, but it requires NLRP3 (NOD-, LRR- and pyrin domain-containing 3), the adaptor protein ASC and caspase 1 to promote IL-1β and IL-18 maturation[109]. Two follow-up studies have indicated that TIR-domain-containing adaptor protein inducing IFNβ (TRIF)-mediated type I interferon (IFN) production has an essential role in the activation of caspase 11 (REFS 75,76), which shows that these extracellular and intracellular pattern recognition pathways cooperate to mediate host defence against intracellular pathogens. The exact mechanism of caspase 11 activation is still being debated. One study reports that type I IFN is required to induce pro-caspase 11 expression and that caspase 11 auto-activates by intermolecular proteolytic cleavage when enough pro-caspase 11 has been produced. The second study shows that signalling via TRIF, IFN-regulatory factor 3, IFN-α/β receptor and signal transducer and activator of transcription 1 (STAT1) is required for the activation of the non-canonical caspase 11 inflammasome triggered by intracellular *Salmonella* spp.; however, this is not due to a lack of pro-caspase 11 induction[75]. Accordingly, Broz *et al.*[75] proposed a receptor-mediated or scaffold-mediated activation model for caspase 11, in which an IFN-stimulated gene (ISG) other than pro-caspase 11 functions as a cofactor for caspase 11 activation. This model was recently shown to also apply to *Legionella pneumophila*-induced caspase 11 activation[110]. Given that caspase 11 is the major determinant of LPS-induced lethality in a mouse model of septic shock, the nature of this ISG will surely be the subject of intense future research.

Caspase 8 was shown in several studies to process pro-IL-1β into its mature bioactive form, and distinct complexes activate caspase 8 in response to pathogens or other signals[111,112]. Fungal recognition by the C-type lectin receptor dectin 1 through spleen tyrosine kinase (SYK) induces the assembly of a complex containing caspase recruitment domain-containing protein 9 (CARD9), B cell lymphoma 10 and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) that activates caspase 8 to promote IL-1β maturation[111]. Vince *et al.* [112] show that ripoptosome formation after Toll-like receptor (TLR) priming and inhibitor of apoptosis (IAP) inhibition lead to the generation of bioactive IL-1β, which requires either the NLRP3 inflammasome or caspase 8. In addition, the complex containing the adaptor protein ASC and absent in melanoma 2 (AIM2) was also shown to activate caspase 8 and to induce apoptosis in the absence of caspase 1 (REF. 113). Future studies will probably identify additional inflammasomes.

These studies showed that NAIP2 binds to the *S.* Typhimurium rod protein PrgJ, which facilitates an NAIP2–NLRC4 interaction and results in the assembly of this inflammasome, and that NAIP5 and NAIP6 bind to flagellin. Interestingly, although *S.* Typhimurium is known to strongly induce caspase 1 activation in human cells, human NAIP does not bind flagellin or PrgJ[85]. Further analysis of human NAIP showed that it interacts with the T3SS needle subunit of *Citrobacterium violaceum* to activate NLRC4. Consistent with these findings, transfection of homologous subunits of enterohaemorrhagic *E. coli*, *Brucella thailandensis*, *P. aeruginosa*, *Shigella flexneri* and *S.* Typhimurium also activated the NAIP–NLRC4 inflammasome[85]. Thus, although human NAIP has a different substrate specificity to mouse NAIP2, NAIP5 and NAIP6, it can also recognize T3SS rod proteins and functions upstream of NLRC4.

The mechanism by which NAIPs activate NLRC4 remains to be investigated, but electron microscopic analysis of a complex containing an *S.* Typhimurium flagellin fragment, mouse NAIP5 and human NLRC4 showed there to be disc-shaped structures in which NAIP5 and NLRC4 occupied an equivalent position, which suggests that both proteins are part of a larger complex[86]. The generation of NAIP-deficient mice is still necessary to validate the above findings and to define the activation mechanism of NLRC4; this is particularly important given the recent observation that protein kinase Cδ (PKCδ)-mediated phosphorylation of NLRC4 is also required to 'license' the receptor for inflammasome activation during *S.* Typhimurium and *L. pneumophila* infection[87].

In the future it will be interesting to determine whether upstream sensors are also important for the activation of other inflammasomes, especially the NLRP3 inflammasome, which recognizes a panoply of chemically and structurally different stimuli.

NLRP6 inflammasome. Intestinal homeostasis depends on complex interactions between the microbiota, the intestinal epithelium and the host immune system. Previous studies have firmly established a role for the NLRP3 inflammasome in acute dextran sodium sulphate (DSS)-induced colitis, partly because of a defect in the repair of the intestinal mucosa in *Nlrp3*$^{-/-}$ mice[90]. Similarly, *Nlrp6*$^{-/-}$ mice are more susceptible to chemically induced colitis and colitis-induced tumourigenesis than wild-type mice[88,89]; this has been attributed to impaired self-renewal and proliferation of mucosal epithelial cells mediated by alterations in the intestinal stem cell niche[88], or alternatively to an impaired NLRP6 function in haematopoietic cells[89]. Consistent with these results, another study showed that *Nlrp6*$^{-/-}$ mice were characterized by spontaneous intestinal hyperplasia, by inflammatory cell recruitment and by an exacerbation of chemical colitis induced by exposure to DSS[90]. Surprisingly, 16S rRNA-based analysis of the faecal microbiota showed

Colitis
An inflammatory disease of the colon. In humans, colitis is most commonly classified as ulcerative colitis or as Crohn's disease, which are two inflammatory bowel diseases that have unknown aetiologies. Various hereditary and induced mouse models of human colitis have been developed.

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that *Nlrp6*$^{-/-}$ mice had an altered microbiota, which was characterized by an increased representation of bacteria from the phyla Bacteroidetes (the Prevotellaceae family) and TM7 (REF. 90). Further investigation showed that NLRP6 deficiency in colonic epithelial cells and reduced basal IL-18 secretion from epithelial cells caused this altered microbiota. Importantly, this study showed that co-housing wild-type mice with *Nlrp6*$^{-/-}$ mice increased the susceptibility of the wild-type mice to the development of DSS-induced colitis, which indicates that the microbiota that is associated with NLRP6 deficiency is colitogenic. The microbiota from *Nlrp6*$^{-/-}$ mice was associated with an increased production of CC-chemokine ligand 5 (CCL5), which might increase inflammation following epithelial damage by DSS, leading to the recruitment of immune cells, such as neutrophils, that induce a chronic inflammatory response and that exacerbate the DSS response.

IL-18 production is key to maintaining intestinal homeostasis and its loss is responsible for the increased severity of DSS-induced colitis in caspase 1-deficient mice[91]. The mechanism by which IL-18 exerts this protective effect is unclear, but the studies discussed above[88–90] suggest that IL-18 has a dual role in gut homeostasis, stimulating the release of antimicrobial peptides that control the gut microbiota[92] and controlling epithelial cell regeneration[93]. Thus, a deficiency in IL-18 production could result in increased severity of DSS-induced colitis through reduced epithelial repair[88] as well as through an altered colitogenic microbiota[90]. Whether the NLRP6 inflammasome functions in one or several intestinal cell types (epithelial or haematopoietic cells) remains to be determined. Nevertheless, several studies[88–90] have established a central role for the NLRP6 inflammasome in maintaining intestinal homeostasis. Further work is required to define to what extent the functions of the NLRP3 and NLRP6 inflammasomes in the gut overlap and to identify the bacterial or the host-derived ligands that are recognized by NLRP6.

The NLRP7 inflammasome detects bacterial lipopeptides. The characterization of the inflammasome response to *Mycoplasma* spp. led to the identification of an NLRP7 inflammasome in human macrophages and THP1 cells, which is induced in response to microbial acylated lipopeptides such as Pam3Cys-Ser-Lys4-trihydrochloride (Pam3CSK4)[94]. The activation of NLRP7 resulted in caspase 1 activation mediated by the adaptor protein ASC and the subsequent release of IL-1β and IL-18, but it did not result in pyroptosis[94]. Knockdown of *NLRP7* led to the increased replication of *S. aureus* and *L. monocytogenes* in THP1 cells[94], which was similar to *NLRP3* silencing[94]; this indicates that both inflammasomes restrict pathogen growth. Interestingly, this study shows that there must be important differences in the sensing of acylated lipopeptides between the human and the mouse systems, as NLRP7 is only found in humans and not in mice. Furthermore, Pam3CSK4 is generally used for inflammasome priming and the addition of exogenous ATP is required for a robust inflammasome response in BMDMs, whereas human cells respond to Pam3CSK4 even in the absence of exogenous ATP[94]. This observation shows that there is a fundamental and important difference in the requirement for inflammasome priming between the human and the mouse systems. Future studies are necessary to investigate whether there is a cytoplasmic acylated lipopeptide sensor in mice, as well as to define the ligand range and the binding mechanism of acylated lipopeptides to human NLRP7.

Triggering NLRP12. NLRP12 was initially identified as a negative regulator of non-canonical NF-κB signalling[95,96]; however, the role of NLRP12 in the inflammasome response to infections remained unclear. A recent report has linked NLRP12 to caspase 1 activation during *Yersinia* spp. infections[97]. Neutrophils and BMDMs from *Nlrp12*$^{-/-}$ mice infected with *Yersinia pestis* had partially reduced levels of active caspase 1 and mature IL-1β and IL-18 compared with cells from infected wild-type mice[97]. This phenotype was less severe than that of mice with a deficiency in ASC or caspase 1, which completely ablated cytokine maturation, but comparable to those with a deficiency in NLRP3. Activation of the NLRP12 inflammasome was also dependent on the *Y. pestis* T3SS and the effector molecule YopJ, which is similar to NLRP3 inflammasome activation during *Yersinia* spp. infections[98,99] and could indicate that NLRP12 functions in conjunction with NLRP3. Similarly to *Nlrp3* induction, NLRP12 expression was dependent on a preceding priming signal in the form of TLR4 signalling, which again shows the close connection between extracellular and intracellular pathogen recognition. Consistent with the *in vitro* data, *Nlrp12*$^{-/-}$ mice were more susceptible to *Y. pestis* infections and had reduced levels of IL-1β, IL-18 and IFNγ, which indicates that there is an important role for the NLRP12 inflammasome in host defence against *Yersinia* spp. infection.

Although the first evidence of a role for NLRP12 in inflammasome activation has now been shown[97], further work — particularly the generation of multigene-deficient mice — is necessary to clarify whether NLRP12 functions alone or together with NLRP3 and NLRC4, which are the two other NLRs known to be activated by *Yersinia* spp. infection[98,99]. In addition, it is unknown whether NLRP12 activation is restricted to *Yersinia* spp. infections or whether other pathogens also activate this pathway.

Analysis of the function of orphan NLRs has substantially increased our understanding of the inflammasome complex itself and its function in pathogen recognition as well as in tissue homeostasis. An important emerging theme is that several NLRs can engage in the assembly of the same inflammasome complex by providing specificity for different types of ligands, as has been elegantly shown for the NAIP–NLRC4 inflammasome. In addition, the discovery of the NLRP6 inflammasome has highlighted the importance of studying the role of inflammasomes in different cell types and not just in haematopoietic cells. Nevertheless, many questions regarding the new canonical and non-canonical inflammasomes remain unanswered; in particular, the nature of their ligands and their mode of activation will be active areas of research in the future.

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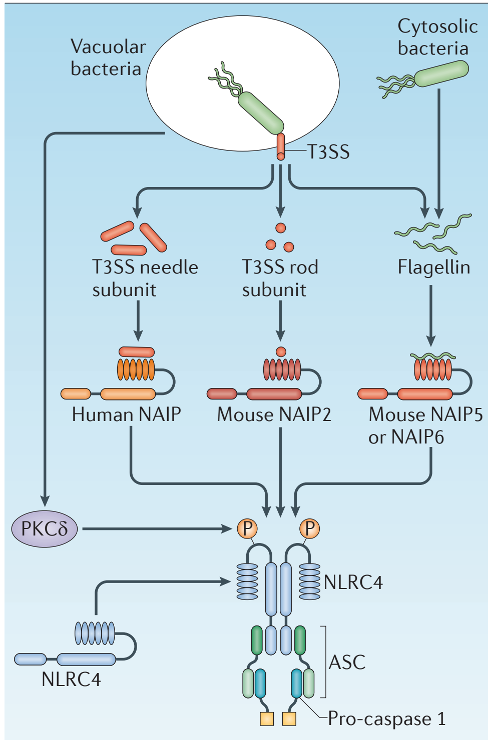

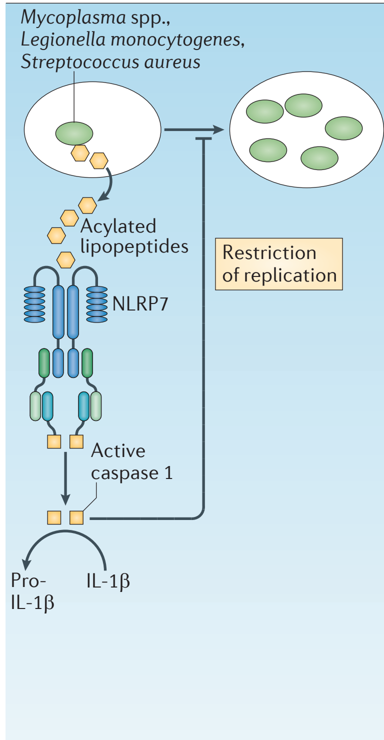

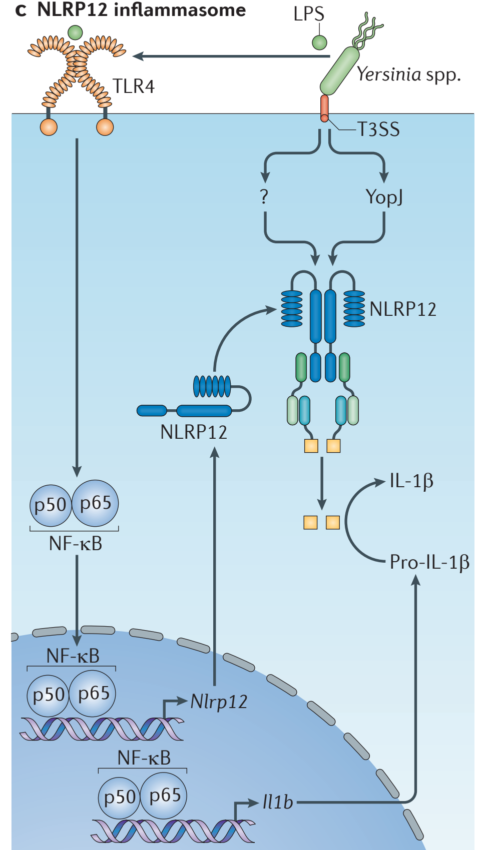

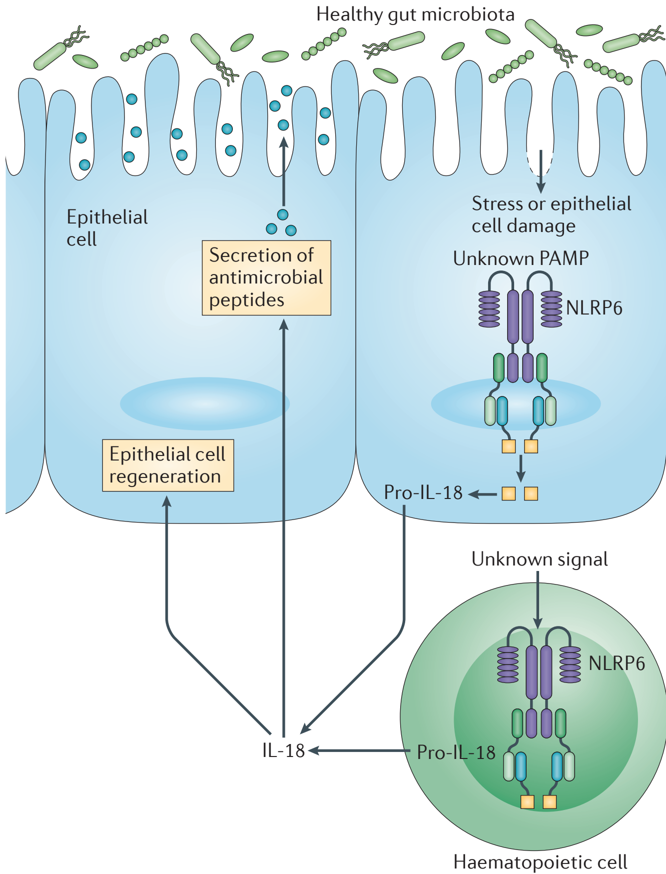

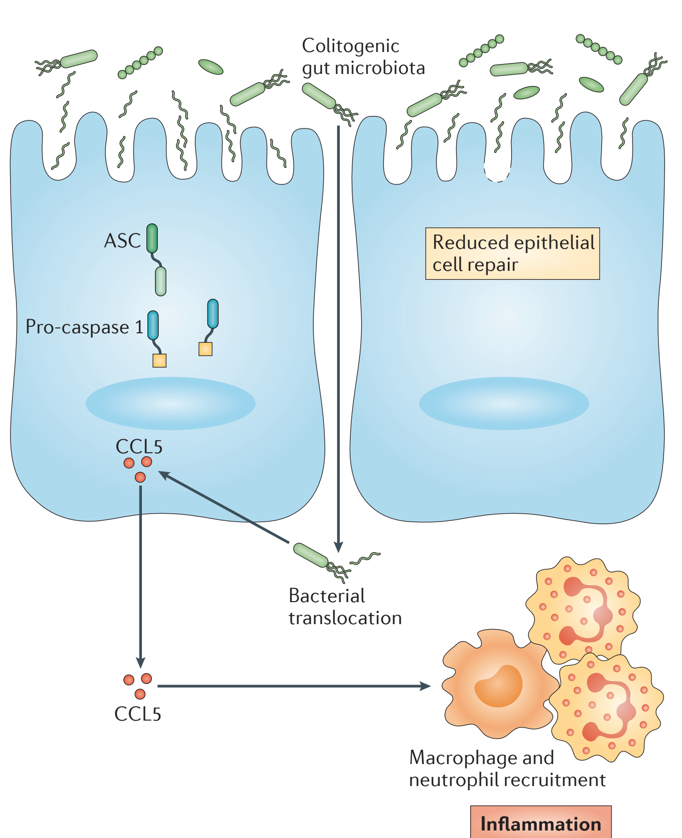

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◀ Figure 3 | **Orphan NLRs assemble new inflammasomes.** **a** | Neuronal apoptosis inhibitory proteins (NAIPs) function as upstream direct receptors for NLRC4 (NOD-, LRR- and CARD-containing 4). NAIP2 binds the type III secretion system (T3SS) rod subunit; NAIP5 or NAIP6 bind flagellin; and human NAIP binds the T3SS needle subunit. The binding of the ligand facilitates an interaction with NLRC4. NLRC4 function also requires the receptor to become phosphorylated, which is mediated by the protein kinase Cδ (PKCδ). **b** | Human NLRP7 (NOD-, LRR- and pyrin domain-containing 7) recognizes acylated lipopeptides from several bacteria. The activation of the NLRP7 inflammasome promotes cytokine maturation and restricts pathogen replication. **c** | Lipopolysaccharide (LPS) from *Yersinia* spp. induces NLRP12 and pro-interleukin-1β (pro-IL-1β) expression via Toll-like receptor 4 (TLR4). The activation of NLRP12 requires the T3SS of *Yersinia* spp., YopJ, and possibly the injection of an additional ligand (indicated by a question mark). **d** | In the presence of NLRP6, basal levels of IL-18 production maintain gut homeostasis and a normal gut microbiota. Possible mechanisms involve the production of antimicrobial peptides and epithelial cell regeneration. **e** | Absence of NLRP6 promotes an altered, colitogenic microbiota and reduced epithelial cell repair in response to damage. The colitogenic microbiota stimulates the secretion of CC-chemokine ligand 5 (CCL5) by epithelial cells, which results in the recruitment of immune cells, triggering a chronic inflammatory response. NF-κB, nuclear factor-κB; PAMP, pattern-associated molecular pattern.

Conclusions and perspectives

The concept of PRRs, as formulated by Charles Janeway Jr over two decades ago[1], has profoundly shaped our understanding of how pathogens are recognized and how innate immune responses are initiated. Research in the last couple of years has led to the identification and the characterization of an increasing number of extracellular and intracellular PRRs, including a growing number of cytoplasmic nucleic acid sensors. Interestingly, there seem to be several intracellular receptors for the same kind of ligands, as exemplified by cytoplasmic DNA sensors. A possible explanation for this could be that, depending on their source (bacterial, viral or endogenous), the ligands might have different modifications, thus enabling the host to use a range of receptors to specifically recognize these types of ligands and, accordingly, to tune the response to pathogens or to tissue damage. The compartmentalization and the activation kinetics of each of these receptors are probably different and might influence how they bind to and respond to ligands. In addition, there might be differences in terms of the tissue-specific and the cell type-specific expression, as well as the downstream signalling pathways, and this will require thorough validation. The generation of single-knockout and multi-knockout mice will be an essential tool to consolidate this wealth of information into broad models, in order to define the physiological significance of individual PRRs.

The mechanisms by which these pathways are regulated and whether there is crosstalk between PRRs that recognize the same PAMPs or pathogens will be an active area of research in the future. Only recently has it become apparent that these signalling pathways can interact to initiate appropriate and robust host responses, as exemplified by the strict requirement of prior NF-κB and type I IFN signalling for the activation of certain types of inflammasome complexes[41,75,76,100]. However, whether this cooperation extends further, for example, to the initiation of adaptive immune responses, remains to be determined. Finally, as research on pattern recognition continues, it is probable that the knowledge gained about these processes will yield new approaches for the selective therapeutic manipulation of innate immune signalling pathways during infectious and inflammatory diseases.

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Competing interests statement

The authors declare no competing financial interests.

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