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REVIEW ARTICLE

Pathogen Recognition Receptors: Ligands and Signaling Pathways by Toll-Like Receptors

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Toll-like receptors (TLRs) play critical roles in host defense against microbes. In the past decade, growing numbers of *in vitro*, *in vivo* and *in silico* studies have been performed and revealed the physiological significance and structural basis of their ligands and signal transduction, which involves various extracellular, membrane-bound, cytoplasmic and nuclear signaling molecules for the activation of TLR signaling. However, negative regulation of TLR-mediated responses is also essential for the prevention of autoimmunity and is mediated by a number of molecules. In this review, we will introduce recent advances in the understanding of TLR biology in terms of their ligands and signaling pathways.

Keywords: cell signaling, host defense, innate immunity, pathogen recognition receptors, TLR

Recognition of microbes by the host innate immune system is fundamental to host defense against infection. It is now widely accepted that induction of innate immunity immediately after microbial infection is important for the establishment of adaptive immune responses mediated by antigenic receptors expressed by B and T cells. However, compared with antigenic receptors that theoretically recognize more than 10^{11} different molecules and which are generated by combination and junctional diversity in VDJ recombination, receptors functioning in innate immunity are germline encoded and recognize much smaller numbers of nonspecific molecules, and therefore have been considered nonessential.

The discovery of toll-like receptors (TLRs) in the mid-1990s demonstrated that innate immune receptors such as TLRs recognize specific patterns of microbes called pathogen-associated molecular patterns (PAMPs) [1–4]. TLRs consist of 10 or 13 members in humans and mice, respectively. TLRs1–10 are conserved between humans and mice, although the murine TLR10 locus is disrupted by the insertion of retrovirus, resulting in the loss of functionality [5]. TLR11, TLR12 and TLR13 are deleted in the human genome [6]. *In vitro* and *in vivo* studies have demonstrated that TLRs differentially recognize various PAMPs by the N-terminal outer membranous domain called leucine-rich repeat (LRR) [7]. Compared to the antigen presenting receptors such as B cell receptor or T cell receptor that mainly recognize peptide or sugar chain moieties,

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TABLE 1. Ligands of each TLR.

TLR1/2	Triacyl lipoproteins (Pam3CSK4 and OspA), peptidoglycan (cell wall component of gram-positive bacteria)
TLR2/6	Diacyl lipoproteins (MALP2 from mycobacteria), zymosan (fungi)
TLR2	tGPI-mucin (a protozoan parasite)
TLR3	Double-stranded RNA (dsRNA), polyinosinic-polycytidylic acid [poly(I:C)]
TLR4	Lipopolysaccharide (bacteria)
TLR5	Flagellin (bacteria)
TLR7	Single-stranded form of RNA (virus, bacteria), imidazoquinoline (imiquimod, resiquimod (R-848)), CL075)
TLR8	Single-stranded form of RNA (virus, bacteria), synthetic compounds (CL075, VTX-1463)
TLR9	Unmethylated CpG DNA (bacteria, viruses, parasites), hemozoin (parasites)

ligands of TLRs have been shown to be lipoproteins, lipids and nucleic [8]. The recognition of ligands by TLRs defines the specific reaction of TLR-mediated immune responses such as production of proinflammatory cytokines and type I interferons.

The C-terminus of TLRs is essential for receptor signal transduction and is called the Toll–interleukin 1 receptor (TIR) domain, to which various adaptor molecules are recruited to positively transmit an activation signal downstream to the cell nucleus. Negative regulators as well as positive ones are also required for adequate control of TLR-mediated immune responses *in vivo*. Moreover, cell-type-specific TLR-mediated roles have recently been demonstrated. In this review, we summarize the recent advances of TLR-mediated innate immune responses in terms of ligand recognition and their signaling pathways.

LIGAND RECOGNITION BY TLRS

According to ligand recognition, TLRs can be divided into cell surface type and endosome (or endolysosome) type. In humans, the former group includes TLR1, TLR2, TLR5 and TLR6 and the latter group is composed of TLR3, TLR7, TLR8 and TLR9. TLR4 is shared by both the groups. The cell-surface type TLRs mainly recognize microbial components located on the surface or outer/inner membranes of various bacteria such as lipoproteins, lipids and proteins (see Table 1) [7]. The endosome/endolysosome type TLRs mostly sense nucleic acids from pathogens (see Figure 1).

TLR2 recognizes various components of microbes including bacteria, viruses, fungi and parasites. The most characterized ligands for TLR2 are lipoproteins [9]. TLR2 together with TLR1 or TLR6 is essential for the recognition of triacyl and diacyl lipoproteins, respectively. Genetic evidence demonstrates that TLR2 forms heterodimers with either TLR1 or TLR6 to differentially recognize the lipoproteins. Although TLR2-deficient mice are hyporesponsive to both lipoproteins, TLR1-deficient mice exhibit defective responses to triacyl lipoproteins such as Pam3CSK4 and OspA, and can induce normal responses to a diacyl lipoprotein, MALP-2, which is derived from mycoplasma. Meanwhile MALP-2-mediated responses, but not triacyl lipoprotein-mediated responses, are abolished in TLR6-deficient mice [10]. The crystal structures of extracellular LRRs of TLR1, TLR2 and TLR6 have been resolved, and the structural basis of differential recognition of lipoproteins by TLR1/TLR2 and TLR6/TLR2 heterodimers has been proposed [11, 12]. Both heterodimers form an M-shaped structure to generate internal pockets, which are essential parts of the interaction with lipoproteins [11]. For both heterodimers, two lipid chains from lipoproteins interact with TLR2. In the TLR1/TLR2 heterodimeric complex, the hydrophobic channel of TLR1 offers an additional link to the third chain of the lipoprotein. However, the

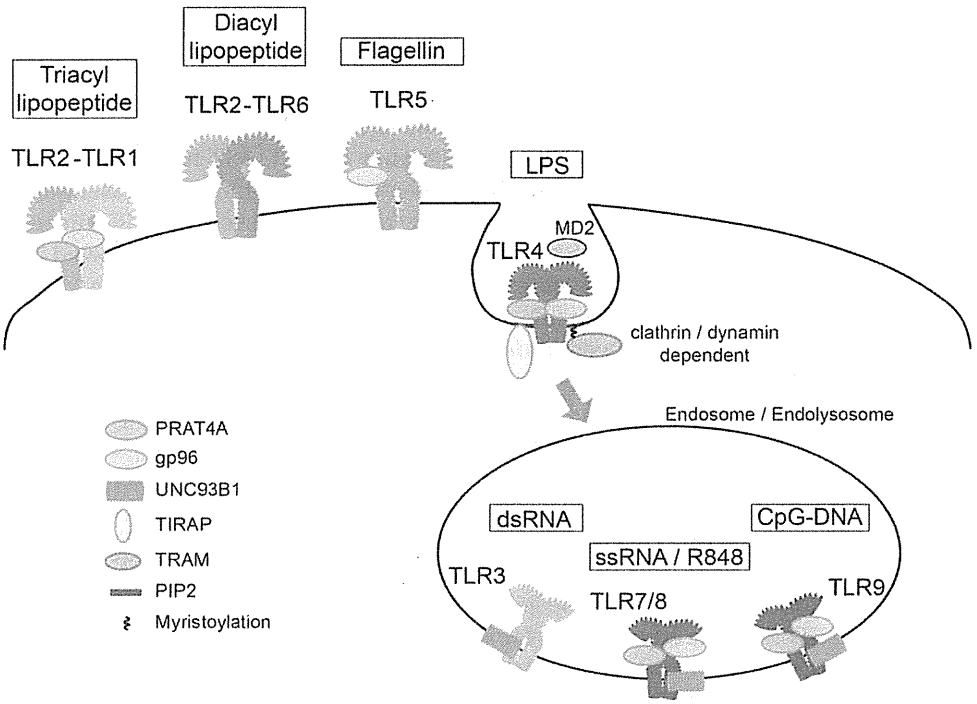

FIGURE 1. Recognition of PAMPs by human Toll-like receptors. *Note.* TLR family members are divided into two groups by cellular location. One group is expressed on the cell surface, and the other exists in an intracellular compartment. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, and TLR6. TLR1 and TLR2 form a heterodimer that recognizes triacyl lipopeptide. The TLR2 and TLR6 heterodimers recognize diacyl lipopeptide. TLR5 recognizes flagellin which forms the bacterial flagella. TLR4 forms a complex with MD2 to recognize LPS. One of the adaptor proteins for TLR4 singling "TIRAP" binds to phosphatidylinositol 4, 5-bisphosphate (PIP2) that is present on the plasma membrane. Another adaptor protein for TLR4, "TRAM' is myristoylated and anchored on the membrane. Once TLR4 is activated, it relocalizes intracellularly. The internalization of TLR4 is clathrin/dynamin-dependent. All of the TLRs that recognize nucleic acid are localized in intracellular compartments. TLR3 recognizes dsRNA derived from virus-infected cells. In plasmacytoid dendritic cells, TLR7 and TLR8 recognize ssRNA produced by the RNA virus. In addition to TLR3, TLR7, and TLR8, TLR9 recognizes microbial DNA derived from either virus or bacteria. Cleavage and locational changes of the intracellular TLRs are necessary to facilitate their signaling. UNC93B1 binds to intracellular TLRs and is essential for their trafficking to the endolysosome from which the intracellular TLRs signal.

TLR6/TLR2 complex fails to form the hydrophobic channel, resulting in the recognition of lipoproteins with two lipid chains. In addition to lipoproteins, TLR2 senses peptidoglycan (derived from a cell wall component of gram-positive bacteria), zymosan (fungi), glycosylphosphatidylinositol-anchored mucin-like glycoproteins from Trypanosoma cruzi trypomastigotes tGPI-mucin (a protozoan parasite) and lipoarabinomannan (from mycobacteria) [7, 13, 14]. Moreover, to enhance the TLR2-mediated recognition of PAMPs, a C-type lectin called dectin-1 and a scavenger receptor CD36 facilitate the internalization of TLR2 agonists to induce activation of the downstream signaling pathways [15, 16].

An early *in vitro* study using HEK293 cells revealed that TLR5 recognizes a bacterial flagella-derived protein called flagellin [17]. However, the physiological function of the receptor has been an enigma because of the unique cell-type-specific *in vivo* expression of TLR5, which is different from other TLRs that are highly expressed

by peritoneal macrophages and bone-marrow-derived dendritic cells. TLR5 is highly expressed on intestinal lamina propria dendritic cells that are important in the differentiation of CD4 T helper (Th) cell lineage cells to Th17 cells and naive B cells to IgA-producing plasma cells in the intestine [18]. In mice, TLR11 and TLR12 are structurally similar to TLR5 at the amino acid sequence level. TLR11 recognizes components of uropathogenic bacteria or profiling-like molecule, which is an essential protein for *Toxoplasma gondii*, a protozoan parasite, to invade host cells [19, 20]. Recently, it has been reported that TLR12 forms heterodimer with TLR11 and is involved in the recognition of profiling-like molecules on *T. gondii* [21]. TLR11 was shown to recognize Salmonella-derived flagellin [22].

TLR4 is essential in the recognition of lipopolysaccharide (LPS), the main component of cell walls on gram-negative bacteria and the greatest causative agent of lethal septic shock [7]. LPS is widely used as a mitogen to stimulate lymphocyte proliferation and proinflammatory cytokine production. A GPI-anchored surface protein CD14 was the first identified receptor of LPS, but it has no intracellular domain [23]. Therefore, an LPS receptor that could activate cellular signaling pathways was searched for over a long time. A forward genetic study using a mouse line C3H/HeJ, which is hyporesponsive to LPS, revealed a point mutation in the intracellular domain of TLR4 [24]. A subsequent reverse genetic study using TLR4-deficient mice demonstrated that TLR4 is the key receptor for signal transduction. CD14 together with LPS-binding protein (LBP), a soluble plasma protein, associates with LPS to deliver an LPS–LBP complex to TLR4 [25]. MD2 is also required for the recognition of LPS by forming a complex with TLR4. The structural basis of TLR4–MD2 complex to sense LPS is that phosphate groups of LPS interact with positively charged residues of TLR4 and lipid chains of LPS bind with the hydrophobic portion of MD2, resulting in the symmetrical homodimerization of the LPS–TLR4–MD2 complex [26, 27]. In addition to LPS, TLR4 also recognizes viral envelope or fusion proteins derived from viruses such as mouse mammary tumor virus or respiratory syncytial virus, respectively [28, 29].

TLR4 senses ligands both on cell surfaces and in endosomes to produce type I interferons, as discussed later. The endosome and endolysosome are important cellular sites for nucleic acid-sensing TLRs including TLR3, TLR7, TLR8 and TLR9 that mediate antiviral immune responses. TLR3 recognizes double-stranded RNA (dsRNA), which is considered to be generated during virus replication. Indeed, TLR3 senses dsRNA produced by RNA viruses such as West Nile virus, respiratory syncytial virus and encephalomyocarditis virus [7, 30]. Moreover, TLR3-deficient mice and TLR3-mutated humans are highly susceptible to mouse cytomegalovirus and herpes simplex virus type 1 (HSV-1), respectively [6, 31]. *In vitro* and structural studies for TLR3 recognition of dsRNA have been performed using a synthetic dsRNA analog, polyinosinic–polycytidylic acid, poly(I:C) [32]. Stimulation by poly(I:C) strongly induces the production of type I interferon in macrophages or dendritic cells by a TLR3 dependently. However type I interferon induction through poly(I:C) stimuli is also regulated by a TLR-independent mechanism. TLR3-independent pathways are mainly regulated by cytosolic RNA sensors, which consist of retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). These recognize viral RNA in cytosol and induce type I interferon through IRF3 activation [33, 34]. The molecular mechanism and signaling pathway on cytosolic RNA sensors would refer to other great reviews [35, 36]. Analysis of the crystal structure of the extracellular domain of TLR3 and dsRNA revealed that the N-terminal domain of TLR3 is a horseshoe-like shape that is considered to increase the surface area for interactions with dsRNA [37, 38].

A single-stranded form of RNA (ssRNA) is also recognized by TLRs such as TLR7 and TLR8. Single-stranded RNA from human immunodeficiency virus, influenza virus and vesicular stomatitis virus are ligands for TLR7 [7, 39, 40]. In addition, synthetic poly(U) RNA also stimulates TLR7-mediated signaling [41]. The first identified TLR7 ligands were derivatives of a nucleic acid analog, imidazoquinoline, such as imiquimod and resiquimod (R-848) [42]. Stimulation of TLR7 by ssRNA or imidazoquinoline induces proinflammatory cytokine production in macrophages and conventional dendritic cells. In addition, interferon-α, a type I interferon, is strongly induced by TLR7 ligands during *in vivo* treatment. Subsequent studies demonstrated that plasmacytoid dendritic cells (pDCs) are largely responsible for the production of type I interferon by TLR7 stimulation [43]; pDCs also play an important role in the production of antiviral interferon-α responses to RNA virus infection *in vivo* [44]. TLR7 recognizes ssRNA of some RNA viruses in a replication-independent manner in pDCs [45–48]. Furthermore, autophagy is the catabolic mechanisms that make up for energy by dissolving their own compartments under nutrient deficiency. An autophagy-related molecule, Atg5, is the essential molecule to induce autophagy, and it also performs the exposure of viral RNA in the endolysosome, where it is sensed by TLR7, by inducing autophagosome formation [48]. Although the physiological role of TLR8 in mice remains uncertain, human TLR8 is involved in the recognition of ssRNA and R-848. TLR7 and TLR8 are tandemly aligned and share highly similar amino acid sequences. TLR8 is known to be expressed in monocytes, macrophages and conventional dendritic cells (cDCs). TLR13 is specific in mouse and was recently shown that it is important to recognize 23S ribosomal RNA from gram-negative and gram-positive bacteria [49]. Detailed analysis regarding TLR13-mediated immune responses against bacteria will be analyzed in future studies.

TLR3, TLR7/TLR8 and TLR13 recognize RNA. In contrast, TLR9 recognizes unmethylated CpG DNA, which is abundantly present in bacteria, viruses and parasites, but is not frequent in mammalian cells [50]. A synthetic oligonucleotide harboring a 2′-deoxyribo CpG DNA motif stimulates the production of proinflammatory cytokines in macrophages and cDCs and type I interferons in pDCs, similar to TLR7 ligands [7]. DNA and a crystalline metabolite are produced by malaria parasites and hemozoin that is a residual product of heme-detoxification by malaria infection, and are ligands recognized by TLR9. Hemozoin directly associates with TLR9 and stimulates antigen-specific immune response to the parasite without DNA, resulting in the induction of adaptive immune responses to malaria infection [51]. However, another report showed that hemozoin is neither TLR9 ligands nor carrier of DNA into cells. Protein–DNA complex from malaria activates innate immunity through TLR9 [52]. These observations are likely controversial; however, the former work focused on vaccine development *in vivo*. On the other hand, the latter work focused on the activation of innate immunity *in vitro*. Thus, the differential points of view may possibly result in the discrepancy of those studies. Therefore, further studies to clarify whether the crystal of hemozoin or its DNA is responsible for TLR9-mediated responses are required. Recently, granulin, an unusual cysteine-rich protein, was shown to interact with CpG DNA and facilitate the binding of CpG DNA with TLR9. Thus, granulin functions as a cofactor of TLR9 [53].

TIGHTLY REGULATED CELLULAR LOCALIZATION OF TLRS

TLR3, TLR7 and TLR9 are localized at the endosome or endolysosome when they recognize their cognate ligands [54]. The endosomal localization of TLR9 is determined by its transmembrane region, since the chimeric receptor harboring the TLR9-derived

ectodomain and TLR4-derived transmembrane plus intracellular domain is localized to cell surfaces. Furthermore, the TLR9-TLR4 chimeric receptor elicits proinflammatory cytokine production in response to non-unmethylated CpG self-derived DNA, independently of endosomal acidification [55]. Thus, the localization of TLR9 plays an important role in the prevention of autoimmunity. Furthermore, although TLR7 and TLR9 are localized at the endoplasmic reticulum (ER) in nonstimulated cells, both receptors are relocalized upon ligand stimulation. Re-localization requires an ER-localizing transmembrane protein with 12 membrane-spanning domains called UNC93B1, whose physiological function was revealed using a chemically mutagenized mouse line, Triple D (3d) [56]; 3d mice harbor a missense mutation in the coding region of UNC93B1 and exhibit severe defects in TLR3-, TLR7- and TLR9-dependent responses [57]. Furthermore, UNC93B1-deficient humans are prone to HSV-1-mediated encephalitis and are also defective for responses to TLR3, TLR7 and TLR9 agonists [58]. UNC93B1 associates with the transmembrane domains of TLR3, TLR7 and TLR9 and facilitates these TLRs to move from the ER to endosomes or endolysosomes, where they sense their ligands [59]. In addition, mice bearing an amino acid substitution (D 34 to A) in the N-terminal portion of UNC93B1 show a TLR7-hyperreactive and TLR9-hyporeactive phenotype and eventually develop autoimmunity. This suggests that UNC93B1 can regulate the localization of TLR7 and TLR9, as well as controlling the homeostatic balance of TLR7/TLR9 activation [60, 61]. Other ER localization proteins such as gp96 and PRAT4A are also required for the appropriate localization of TLRs [62]. PRAT4A is involved in the trafficking of TLR4 and TLR9 to the endolysosome [63]. In addition to PRAT4A, gp96 functions as an ER chaperone for both cell-surface- and endosome/endolysosome-type TLRs. Mice deficient in gp96 show hyporesponsiveness to stimulations by most of TLRs except for TLR3 and TLR8. [64]. Thus, ER proteins regulate the localization of TLRs and play a pivotal role in immune responses. To recognize the ligand, the N-terminus of TLR9 is proteolytically cleaved by intracellular peptidases such as cathepsin family members and asparagine endopeptidases in endolysosomes [65–69]. However, DNA recognition of TLR9 is abolished by *in vitro* deletion of the N-terminal LRR, which is required for its interactions with CpG DNA. Cleavage of TLR9 likely enhances its activation since it initiates binding with its signaling molecules strongly. Furthermore, other intracellular TLRs (TLR3 and TLR7) also have shown their cleaved forms, indicating that cleavage of the N-terminal region is a positive regulator of its signaling among intracellular TLRs. Future studies are necessary to understand why such a tight regulations exist in signaling intracellular TLRs.

Activation of TLR4 signaling pathways results in the production of proinflammatory cytokines and another type I interferon, interferon-β. After ligation of LPS to TLR4 on cell surfaces, the TLR4/LPS complex is internalized and traffics to early endosomes and induces the production of interferon-β, suggesting the tight regulation of TLR4 localization and responses. The internalization of TLR4 is mediated by a clathrin-/dynamin-dependent mechanism [70]. TIR-domain-containing adaptor molecules TIRAP and TRAM participate differentially in TLR4-mediated pathways. TIRAP contains a phosphatidylinositol 4,5-bisphosphate (PIP2)-binding domain, which recruits an essential adaptor protein, MyD88, to the TLR4 complex to activate signaling at cell surfaces [71]. In addition, TRAM harbors a myristoylation site at the N-terminus, which is required for the targeting of TRAM to plasma membranes. A mutation in the myristoylation site in TRAM abolishes responsiveness to LPS, suggesting the importance of TRAM myristoylation [72]. Furthermore, TRAM harbors a bipartite sorting signal that regulates its trafficking between cell surfaces and endosomes. Thus, TLR4 activates downstream signaling pathways in plasma membranes and endosomes by virtue of TIRAP and TRAM.

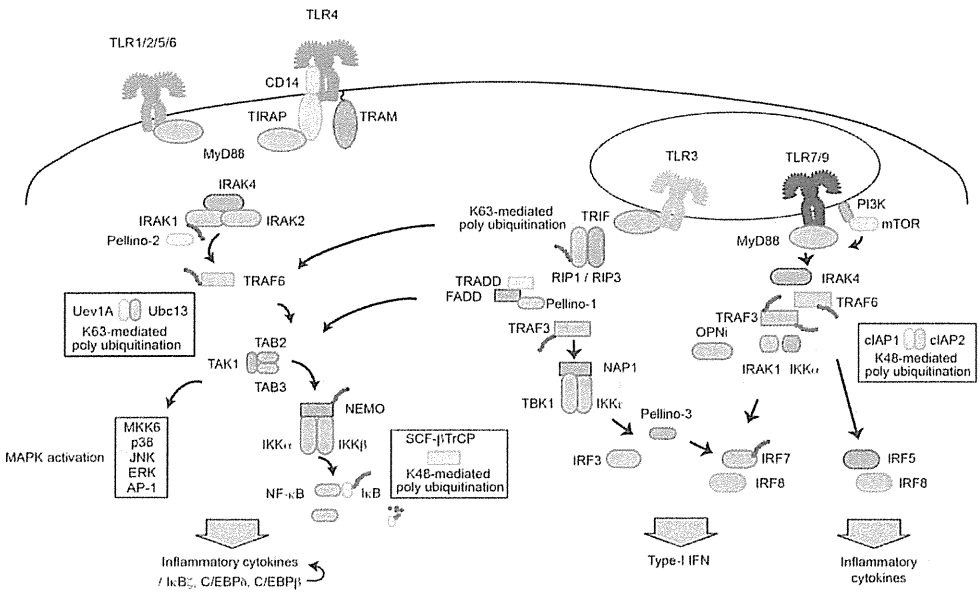

FIGURE 2. Positive regulators for TLR signaling. *Note.* TLR-mediated cytokine production is mainly regulated by five TIR domain containing adaptor molecules. Most TLRs use the signaling adaptor protein MyD88, except TLR3 that utilizes TRIF as a signaling adapter. TLR4 can utilize both MyD88 and TRIF as signaling adaptors. MyD88 binds to IRAK4 through their common death domains. IRAK4 forms a complex with IRAK1 and IRAK2. The IRAK complex is modulated by K63-linked polyubiquitination to induce activation. TRAF6 functions as the E3 ligase that transfers the K63-ubiquitin chain to IRAK1, to activate it. Activated IRAK1 induces activation of the TAK1/TAB2/TAB3 complex. This complex then turns on theMAPK pathway and the NF-κB pathway, resulting in the induction of inflammatory cytokines. MyD88 forms a unique signaling complex in different cell types. In plasmacytoid dendritic cells, TLR7- and TLR9-mediated type I IFN induction is MyD88-dependent. Meanwhile, TLR3 cytokine production is TRIF-dependent, and can lead to type I IFN induction through the TRADD/FADD/TAK complex or inflammatory cytokine production through the RIP1/RIP3/TRAF6 complex.

TLR-MEDIATED SIGNALING PATHWAYS BY TIR-DOMAIN-CONTAINING ADAPTORS

TLRs differentially induce unique immune responses by stimulus-specific and cell-type-specific mechanisms. In terms of macrophages, TLR1/TLR2 or TLR2/TLR6 ligands mainly stimulate the production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) or IL-12. In contrast, TLR3 induces interferon-β rather than proinflammatory cytokines. In addition, TLR4 signaling activates the production of both proinflammatory cytokines and interferon-β (see Figure 2). After ligation of TLRs by ligands, activation signaling pathways are elicited from the TIR domain located in the intracellular C-terminus of TLRs [7]. To date, five family members of TIR-domain-containing cytoplasmic adaptors have been identified [73, 74]. MyD88 was the first identified adaptor that was used by all TLRs except TLR3. MyD88 contains a C-terminal TIR domain and an N-terminal death domain, which is critically required for downstream signaling pathways to activate the transcription factors NF-κB and AP-1, culminating in the production of proinflammatory cytokines. TIRAP (also known as Mal) was the second identified member and connects TLR2 and TLR4 to MyD88, thus functioning as a sorting adaptor in MyD88-dependent pathways. TRIF (also known as TICAM-1) was the third identified adaptor and has a pivotal role in the induction of type I interferon by

TLR3 and TLR4. In addition to the C-terminal TIR domain, TRIF harbors a large N-terminal domain, which activates a critical transcription factor for the induction of type I interferon, IRF3 [75, 76]. TRAM was the fourth member of the TIR adaptors to be identified and connects TLR4 to TRIF and allows TLR4 to traffic to endosomes [77–79]. Sterile α and Armadillo motif (SARM) containing protein was the fifth member of the TIR adaptor. In contrast to the other four members that show ubiquitous distributions, SARM is largely expressed in neurons of mice and humans. Although *in vitro* studies using a human cell line demonstrated that SARM functions as a negative regulator of the TLR3 pathway, it remains unclear in mice [80]. In contrast, SARM-deficient mice are modestly susceptible to West Nile virus infection and show impaired TNF-α production in the central nervous system [81], suggesting a positive role in antiviral immune responses. It remains to be uncertain whether SARM functions in TLR-mediated immune responses. Together, the TLR-mediated signaling pathways are roughly divided into two major pathways: the MyD88- and the TRIF-dependent pathways.

THE MYD88-DEPENDENT PATHWAYS

The death domain of MyD88 is required for the activity of a death-domain-containing kinase, IRAK4. The kinase activity of IRAK4 is essential for the sequential activation of the IRAK family members, IRAK1 and IRAK2 [7]. Recently, death domains of MyD88, IRAK4 and IRAK2 were coexpressed and crystallized, revealing that the MyD88–IRAK4–IRAK2 death domain complex (Mydssome) forms a tour-shaped structure containing approximately four layers with IRAK2 at the top layer, IRAK4 in the middle layer and MyD88 in the bottom two layers [82]. The formation of the Mydssome then induces interactions with an E3 ubiquitin ligase, TRAF6, which transfers lysine 63 (K63)-linked polyubiquitin chains to upstream and downstream target proteins such as IRAK1 and TRAF6 itself. In contrast to lysine 48 (K48)-linked polyubiquitination that plays an important role in proteasome-dependent protein degradation, K63-linked polyubiquitination was originally shown to be involved in DNA damage responses, where E2 ubiquitin conjugates enzymes such as Ubc13 and Mms2 in the synthesis of K63-linked polyubiquitin chains. In TLR-mediated signaling pathways, Ubc13 and another E2 conjugating enzyme, Uev1A, are involved in the generation of K63-polyubiquitin chains, resulting in autoubiquitination of TRAF6. The K63-linked polyubiquitin further connects TRAF6 with downstream adaptor proteins harboring a novel-type zinc finger and ubiquitin-binding domains, TAB2 and TAB3. TRAF6-dependent ubiquitin binding of TAB2 and TAB3 induces their oligomerization, culminating in the activation of a MAP kinase kinase kinase, TAK1. This kinase was identified as a mediator in the transforming growth factor-β (TGF-β)-dependent signaling pathway. In addition to TAB2 and TAB3, TRAF6 transfers K63-linked polyubiquitin to the regulatory subunit of IκB kinase (IKK) complex called NEMO (also known as IKKγ) at the lysine 392 residue. In the complex containing TRAF6/TAK1/NEMO, IKKβ, which is a critical kinase for the activation of NF-κB, is phosphorylated by TAK1, since IKKβ is also included in the complex through interactions with NEMO. IKKβ phosphorylates IκB proteins, which are bound to NF-κB subunits, and prevents their nuclear translocation [83]. Phosphorylated IκB proteins undergo K48-linked polyubiquitination by the SCF-βTrCP E3 ubiquitin ligase complex and are eventually degraded. Liberated NF-κB subunits consisting of RelA (also known as p65), c-Rel and p50 translocate to the nucleus and participate in the transcription of genes for proinflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-12 p40 [84]. In addition to NF-κB, the transcription factor complex AP-1 is also activated in the MyD88-dependent pathway. TAK1 activates the IKK complex

and MAP kinase kinases (MKKs) including MKK6. Activated MKKs subsequently phosphorylate MAP kinases such as p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). JNK further phosphorylates c-Jun, culminating in interactions with c-Fos to form the complete AP-1 complex [83].

The timing of individual gene expression for proinflammatory cytokines is differentially regulated by MyD88-dependent pathways. For instance, mRNAs for TNF-α and IL-1β are rapidly induced after TLR stimulation. In contrast, mRNAs for IL-6 and IL-12 p40 are upregulated at later time points. Therefore, MyD88-dependent gene expression is regulated by at least two steps. The early inducible genes include a nuclear protein called IκBζ, which contains ankyrin repeats and is structurally similar to cytoplasmic IκB proteins. IκBζ associates with the p50 subunit of NF-κB and induces the robust expression of a subset of late inducible genes including IL-6 and IL-12 p40 mRNAs [85]. IκBζ is recruited to the promoter of IκBζ-regulated genes in association with p50 and selectively regulates H3K4 trimethylation and assembly of preinitiation complex after nuclear remodeling [86]. Furthermore, Controlled Amino Acid Therapy (CAAT)/Enhancer Binding Protein (C/EBP) family members of transcription factors, C/EBPβ and C/EBPδ, are induced in a MyD88-dependent manner and play a role in the maximal production of IL-6 and TNF-α. Taken together, the MyD88-dependent positive feedback loop is required for the adequate activation of TLR-mediated immune responses [87].

TLR7 and TLR9 ligands stimulate interferon-α production in a MyD88-dependent fashion in pDCs [88]. MyD88 directly associates with the transcription factor IRF7, which is constitutively expressed and essential to induce type I interferon in pDCs. Subsequently, IRF7 is activated by a complex containing IRAK1, IRAK4, TRAF3, TRAF6 and IKKα [30]. Phosphorylation of IRF7 is mediated by IKKα or/and IRAK1. TRAF6 also induces Ubc13-dependent ubiquitination, resulting in the maximal activation of IRF7 [89]. pDC-specific MyD88-dependent interferon-α production involves other unique signaling molecules such as a precursor of osteopontin (OPNi) and phosphoinositol 3-OH kinase (PI3K)/mammalian target of rapamycin (mTOR) pathways. OPNi is included in the MyD88/IRF7 complex and positively regulates TLR9-mediated interferon-α production [90, 91]. In addition, the PI3K/mTOR pathway also regulates the interaction of MyD88 with TLR9. IRF transcription factor members are involved in the productions of type I interferon and proinflammatory cytokines under TLRs signaling [92]. Like IRF7, IRF5 also associates with MyD88, but it is specifically involved in the MyD88-dependent production of IL-6 and IL-12 in all TLR-mediated responses [93]. In contrast, IRF4 competes with IRF5 binding to MyD88 and limits the production of proinflammatory cytokines [94, 95]. Moreover, IRF1 also interacts with MyD88 and mediates production of type I interferon in cDCs, especially those induced by TLR9 responses [96]. IRF8 functions in pDCs to stimulate type I interferons and proinflammatory cytokines in cooperation with NF-κB. Thus, IRF family members play an important role in MyD88-dependent signaling pathways.

THE TRIF-DEPENDENT PATHWAYS

Another IRF family member, IRF3, is required for interferon-β production in TRIF-dependent pathways mediated by TLR3 and TLR4 [92]. TRIF recruits IKKε (also known as IKK-*i*) and TANK (TRAF family member-associated NF-κB activator)-binding kinase 1 (TBK1), which directly phosphorylate IRF3, resulting in the nuclear translocation of IRF3 to strongly activate the interferon-β promoter [97]. TRIF is involved in the activation of NF-κB. TLR3-mediated NF-κB activation is critically dependent on TRIF. However, MyD88 and TRIF are both required for TLR4-mediated NF-κB activation. MyD88 and TRIF participate in NF-κB activation during the early

and late phases, respectively. TRIF harbors a receptor-interacting protein (RIP) homotypic binding motif (RHIM) in the C-terminal portion, to which RIP1 and RIP3 bind, and TRAF-binding motifs in the N-terminus, through which TRAF3 and TRAF6 associate. TRAF6 and RIP1/RIP3 are involved in NF-κB activation [98]. In TLR3-mediated signaling, RIP1 undergoes K63-linked polyubiquitination, which is dependent on TRADD and FADD, essential adaptors in the TNF receptor signaling pathway. Pellino-1 is an E3 ubiquitin ligase that mediates TLR3-dependent ubiquitination of RIP1. Mice deficient in Pellino-1 are hyporesponsive to TLR3 and TLR4 ligands. Pellino family members are differentially involved in TLR-mediated signaling pathways [99]. Pellino-2 is critical for TLR4-mediated K63-linked ubiquitination of IRAK1 [100]. Pellino-3 was originally identified as a strong activator of p38 MAPK. Furthermore, Pellino-3 was recently shown to target the IRF7 pathway and facilitate the autoregulation of TLR3-mediated expression of type I interferons [101].

Like TRAF6, TRAF3 functions as an E3 ubiquitin ligase that catalyzes K63-linked polyubiquitination. In response to TLR3, TRAF3 undergoes autoubiquitination, which results in the activation of TBK1 and IKKε [102]. TRAF3 also plays an important role in the TLR7/TLR9-mediated production of type I interferon, suggesting a general role in TLR-mediated production of type I interferons, in which TRAF3 is recruited to the MyD88 complex in TLR4, TLR7 and TLR9-mediated pathways [103]. The incorporation of TRAF3 in the Mydssome results in K48-linked ubiquitination and its own degradation by cIAP1 and cIAP2. The proteasome-dependent degradation of TRAF3 is an essential event in the activation of MAP kinase and eventual proinflammatory cytokine production by the MyD88-dependent pathway, since it induces TAK1 activation because of the translocation of the membrane-proximal signaling complex to the cytoplasm [104]. Thus, TRAF3 is involved in MyD88- and TRIF-dependent responses.

NEGATIVE REGULATION OF TLR-MEDIATED SIGNALING PATHWAYS

Since the termination of TLR signaling pathways is important for the prevention of autoimmunity, TLR activation is negatively regulated in both MyD88- and TRIF-dependent pathways at the receptor, adaptor, transcription and post-transcriptional levels (see Table 2).

At the receptor level, a soluble form of TLR9 inhibits MyD88-dependent signaling. Although TLR9 is processed by peptidases to the active form, soluble TLR9 is generated by cathepsin S cleavage in endosomes [105]. SIGIRR (also known as TIR8) is a TIR member that is important in gut homeostasis, intestinal inflammation and colitis-associated tumiorigenesis [106]. In the TLR4 signaling pathway, the TIR domain of SIGIRR is required for attenuation of the recruitment of the Mydssome to the receptor [107]. In addition to the TLR2 pathway, another member of the TIR family, ST2, inhibits the formation of the TLR2/MyD88/IRAK immunocomplex [108]. Thus, an unusual form of TLR and members of TIR function as negative regulators at the receptor level. In addition, a RING (really interesting new gene) finger protein, Triad3A, acts as an E3 ubiquitin ligase and mediates polyubiquitination of TLR4 and TLR9, but not TLR2, resulting in the degradation of TLR4 and TLR9 [109].

In the cytoplasm, a splice variant of the short form of MyD88, MyD88s, limits TLR4-mediated responses. MyD88s lacks the intermediate domain separating the death domain and TIR domain and thus fails to interact with IRAK4 [110]. TRAM also has a splicing variant called TAG, which harbors a GOLD domain involved in Golgi dynamics. TAG inhibits TLR4-mediated IRF3 activation in late endosomes [111]. In addition, another GOLD-domain-containing transmembrane protein, TMED7, colocalizes with TRAM and negatively regulates TLR4-mediated chemokine production [112]. TIRAP also inhibits JNK activation and limits IL-6 production in the TLR3 pathway [113].

TABLE 2. Negative regulators for TLR signaling.

Target signals/target molecules	Negative regulator	Effects
TLRs	SIGIRR	Binding competition
	ABIN-3	Enhance the effects of A20
TLR9	Soluble TLR9	Binding competition
TLR4/TLR9	Triad3A	Accelerate degradation
TLR2/MyD88/IRAK complex	ST2	Disruption of signaling complex
TLR3 pathway	TIRAP	Inhibit MAPK activation
TLR2	CYLD	Acelete deubiquitination
TLR4	NF-κB1	Inhibit MAPK activation
	IκBNS	Inhibit cytokine production
	ATF-3	Inhibit cytokine production
	TPL-2	Inhibit MAPK activation
TRIF pathway	TRIM38	Accelerate degradation
IRAKI / IRAK4	IRAK-M	Disruption of signaling complex
TRIF/TBK1 complex	SHIP-1	Disruption of signaling complex
TRAF6	A20	Accelerate degradation
	TANK	Inhibit TRAF6 autoubiquitination
IKKα / IKKβ	NLRX1	Inhibit phosphorylations
MyD88	IRF4	Binding competition with IRF5
	MyD88s	Binding competition
TRAM	TAG	Binding competition
	TMED7	Binding competition
IRAKI	IRAKIc	Binding competition
IL-6 / IL12p40 mRNA	Regnase-1	Enhance mRNA degradation
TNF-αmRNA	TTP	Enhance mRNA degradation

Together, TIR-domain-containing adaptors are used not only in the activation, but also in the negative regulation of TLR signaling pathways.

An IRAK family member, IRAK-M, is rapidly induced upon TLR stimulation and prevents dissociation of IRAK1 and IRAK4 from MyD88. IRAK-M-deficient mice are highly susceptible to LPS-induced septic shock [114]. IRAK-M expression is partly controlled by a transcription factor SMAD4, which also regulates SHIP-1 [115]. SHIP-1 is a SH2 domain containing inositol-5-phosphate that negatively regulates formation of the TBK1 and TRIF complex in the TLR3 pathway. SHIP-1-deficient cells display enhanced TLR3-mediated interferon-β production [116]. IRAK1 has a splicing variant, IRAK1c, which lacks a kinase domain and is highly expressed in the brain. IRAK1c suppresses TLR signaling by interacting with MyD88 and IRAK2, and inhibits their dissociation [117].

TRAF6 is also targeted by various negative regulators. A20 (also known as TNFAIP3) is a deubiquitinating enzyme, which removes polyubiquitin chains from TRAF6. A20-deficient mice exhibit spontaneous inflammation [118]. Furthermore, A20-binding inhibitor of NF-κB activation (ABIN)-3 facilitates A20-induced suppression of TLR-mediated responses [119]. Moreover, TANK is involved in the suppression of auto-ubiquitination of TRAF6. Although TANK was originally identified as a positive regulator of IRF3 and NF-κB activation [120], TANK-deficient mice display normal IRF3 activation and enhanced NF-κB activation due to increased TRAF6 ubiquitination, resulting in spontaneous fatal glomerulonephritis [121]. In addition, a NOD-like receptor family member, NLRX1, interacts with TRAF6. Upon stimulation, NLRX1 dissociates from TRAF6 and binds to IKK to inhibit the phosphorylation of IKKα and IKKβ [122]. TLR2 signaling is inhibited by another deubiquitinating enzyme, CYLD [123]. TRIM38 is also an E3 ubiquitin ligase, which inhibits the TLR3/TLR4-mediated production of interferon-β [124]. NAP1 is a positive regulator of the TRIF-dependent pathway, resulting in K48-linked ubiquitination by TRIM38

and proteasome-dependent degradation [125, 126]. Thus, the mode of ubiquitination for various molecules in the TLR signaling pathways is tightly regulated by numerous negative regulators to prevent dysregulated autoinflammation.

NF-κB1 (also known as p105), a precursor of the NF-κB p50 subunit, inhibits the induction of TLR4-mediated interferon-β production [127]. NF-κB1-deficient mice exhibit high levels of LPS-induced interferon-β production because of the loss of ERK activation. LPS-induced activation of ERK is regulated by an MAP kinase kinase kinase family member, TPL-2, and NF-κB1 regulates the stabilization of TPL-2 by the C-terminal ankyrin repeat domain. TPL-2-dependent ERK activation induces a transcriptional regulator, c-Fos, that inhibits interferon-β production in dendritic cells [128]. Nuclear proteins participate in the negative regulation of TLR-mediated responses. A family member of nuclear IκB proteins, IκBNS, is upregulated by TLR stimulation and limits the production of proinflammatory cytokines such as IL-6 and IL-12, which are induced at a later phase by IκBζ [129, 130]. Furthermore, TLR-mediated production of IL-6 and IL-12 is inhibited by ATF-3, which is also rapidly induced by LPS stimulation [131]. Thus, TLR-mediated responses are downregulated at the transcriptional level.

Genes rapidly induced upon TLR stimulation include genes required for secondary transcription and regulatory RNA-binding proteins with a CCCH-type zinc finger domain such as regnase-1 (also known as Zc3h12a) and tristetraprolin (TTP). Regnase-1 targets 3′ untranslated regions of IL-6 and IL-12 p40 mRNA. Moreover, regnase-1-deficient mice show higher concentrations of serum immunoglobulin and autoantibodies [132]. Similarly, TTP acts as a deadenylase and associates with AU-rich 3′ untranslated regions of TNF-α mRNA to remove the poly(A) tail, resulting in the degradation of TNF-α mRNA [133]. Together, regnase-1 and TTP affect mRNA stability and cause the downregulation of proinflammatory cytokine production in TLR-mediated responses.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this review, we have focused on the roles of TLRs in terms of ligands and their signaling pathways. During initial studies, the specificity of ligands and TLRs is mainly determined by genetic means. Recent structural studies have established the molecular basis of ligand recognition by heterodimers and homodimers of TLRs. Furthermore, a number of *in vitro* studies have revealed the involvement of various activating signaling molecules by protein modifications including phosphorylation and ubiquitination, and inactivation, leading to regulation of the activity of transcription factors NF-κB, AP-1 and IRFs. Insufficient activation of TLRs results in the impairment of host innate and adaptive immune responses to microbes. However, overactivation of TLRs leads to autoinflammation and eventually autoimmunity. Therefore, the balance that determines the activation and inactivation of TLR-mediated immune responses is tightly regulated at multiple steps by various factors, and an understanding of the process is ongoing. Multidisciplinary approaches including *in vitro*, *in vivo* and *in silico* studies will increase our precise growing knowledge and greatly facilitate the future development of treatments for detrimental infectious diseases and autoimmunity.

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Declaration of Interest

The authors have no conflicting financial interests to declare. The authors alone are responsible for the content and writing of the article.

ABBREVIATIONS

TLRs:	Toll-like receptor;
TIR:	Toll–interleukin 1 receptor;
cDCs:	conventional dendritic cells;
pDCs:	plasmacytoid dendritic cells;
MyD88:	myeloid differentiation factor 88;
TIRAP:	Toll–interleukin 1 receptor domain-containing adaptor protein;
TRIF:	TIR-domain-containing adaptor-inducing interferon-β;
TRAM:	TRIF-related adaptor molecule;
IRAK:	interleukin receptor-associated kinase;
TRAF:	tumor necrosis factor-associated factor;
IRF:	interferon regulatory factor;
MAPK:	mitogen-activated protein kinase;
IKK:	IκB kinase;
TRADD:	Tornado Research and Defense Development;
TRIM:	tripartite motif;
FADD:	Fas-associated death domain;
SIGIRR:	single IG IL-1-related receptor;
TMED7:	transmembrane emp24 domain-containing protein 7;
SHIP-1:	Src homology 2 domain-containing inositol polyphosphate phosphatase;
CYLD:	cylindromatosis;
NAP1:	NF-κB-activating kinase-associated protein 1;
ATF-3:	activating transcription facto

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LETTER

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Critical role of Trib1 in differentiation of tissue-resident M2-like macrophages

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Macrophages consist of at least two subgroups, M1 and M2 (refs 1–3). Whereas M1 macrophages are proinflammatory and have a central role in host defence against bacterial and viral infections[4,5], M2 macrophages are associated with responses to anti-inflammatory reactions, helminth infection, tissue remodelling, fibrosis and tumour progression[6]. Trib1 is an adaptor protein involved in protein degradation by interacting with COP1 ubiquitin ligase[7]. Genome-wide association studies in humans have implicated TRIB1 in lipid metabolism[8–10]. Here we show that Trib1 is critical for the differentiation of F4/80$^+$MR$^+$ tissue-resident macrophages—that share characteristics with M2 macrophages (which we term M2-like macrophages)—and eosinophils but not for the differentiation of M1 myeloid cells. Trib1 deficiency results in a severe reduction of M2-like macrophages in various organs, including bone marrow, spleen, lung and adipose tissues. Aberrant expression of C/EBPα in Trib1-deficient bone marrow cells is responsible for the defects in macrophage differentiation. Unexpectedly, mice lacking Trib1 in haematopoietic cells show diminished adipose tissue mass accompanied by evidence of increased lipolysis, even when fed a normal diet. Supplementation of M2-like macrophages rescues the pathophysiology, indicating that a lack of these macrophages is the cause of lipolysis. In response to a high-fat diet, mice lacking Trib1 in haematopoietic cells develop hypertriglyceridaemia and insulin resistance, together with increased proinflammatory cytokine gene induction. Collectively, these results demonstrate that Trib1 is critical for adipose tissue maintenance and suppression of metabolic disorders by controlling the differentiation of tissue-resident M2-like macrophages.

Members of the tribble family are pseudokinase proteins that are conserved among species and implicated in various human diseases, such as leukaemia and metabolic disorders. Tribble proteins interact with an E3 ubiquitin ligase, COP1, for protein degradation. In thioglycollate-elicited macrophages, Trib1 is important for interleukin (IL)-12p40 production to lipopolysaccharide[11]. In addition, Trib1 and Trib2 have been implicated in acute myeloid leukaemia[12,13]. Trib3 can inhibit insulin signalling[14], although *Trib3*$^{-/-}$ mice do not show defects in insulin signalling or glucose homeostasis[15]. However, the roles of tribble proteins in haematopoietic cell development have not been clarified.

First, we examined the populations of tissue-resident macrophages in mice lacking tribble family genes in the spleen. Although the proportions of B cells, T cells, dendritic cells and Ly6C^{high}Mac1$^+$ inflammatory monocytes were not altered between wild-type and *Trib1*$^{-/-}$ splenocytes, F4/80$^+$Mac1$^+$ macrophages, which also expressed Mrc1 (also called MR), *Arg1* and *Fizz1* (also called *Retnla*), were markedly reduced and Siglec-F$^+$CCR3$^+$ eosinophils were absent in *Trib1*$^{-/-}$ spleens (Fig. 1a and Supplementary Figs 1 and 2). In contrast, the neutrophil population was increased in *Trib1*$^{-/-}$ spleens (Fig. 1a and Supplementary Fig. 3). Splenic F4/80$^+$ red pulp macrophages phagocytose aged red blood cells and accumulate iron[16,17]. Immunohistochemical staining of spleen sections confirmed the absence of red pulp macrophages in *Trib1*$^{-/-}$ spleen (Fig. 1b). Furthermore, Perl's Prussian blue staining of the spleen sections revealed that iron did not accumulate in *Trib1*$^{-/-}$ mice (Fig. 1c and Supplementary Fig. 4). In contrast, splenic metallophilic and marginal zone macrophages were normal in *Trib1*$^{-/-}$ mice (Supplementary Fig. 5). In addition to the spleen, tissue-resident macrophages in other tissues were severely decreased in *Trib1*$^{-/-}$ mice; however, peritoneal resident macrophages were comparable between wild-type and *Trib1*$^{-/-}$ mice (Supplementary Fig. 6). Newly generated *Trib2*$^{-/-}$ and *Trib3*$^{-/-}$ mice did not show any defects in myeloid and lymphoid cells in the spleen (Supplementary Figs 7 and 8). Because MR, Arg1 and Fizz1 expression is a hallmark characteristic of M2 macrophages, we termed this population M2-like macrophages. Thus, these findings indicate that Trib1 is critical for the differentiation of tissue-resident M2-like macrophages and eosinophils in the peripheral organs.

Differentiation of haematopoietic cells, including macrophages, occurs in the bone marrow, followed by their migration to peripheral tissues via the bloodstream. Consistent with the defects observed in peripheral organs, numbers of F4/80$^+$Mac1$^+$ cells and Siglec-F$^+$CCR3$^+$ eosinophils were severely decreased in the blood and bone marrow cells from *Trib1*$^{-/-}$ mice whereas numbers of Gr-1^{high} neutrophils were slightly increased (Fig. 1d, e). However, inflammatory monocytes were comparable between wild-type and *Trib1*$^{-/-}$ bone marrow cells (Supplementary Fig. 9). The adoptive transfer of *Trib1*$^{-/-}$ bone marrow cells to wild-type mice failed to increase F4/80$^+$ macrophage numbers in the bone marrow and spleen (data not shown). Furthermore, competitive transfer of CD45.1$^+$ wild-type and CD45.2$^+$ *Trib1*$^{-/-}$ bone marrow cells (1:1 ratio) to sublethal-irradiated wild-type mice led to severely impaired development of CD45.2$^+$ macrophages and eosinophils, and increased the population of neutrophils (Supplementary Fig. 10). These findings demonstrate that the defects observed in *Trib1*$^{-/-}$ mice are intrinsic to haematopoietic cells, and that Trib1 is critical for regulating the proper differentiation of myeloid cells in the bone marrow. To delineate the developmental competency of *Trib1*$^{-/-}$ bone marrow cells, we performed colony-forming assays. Whereas granulocyte/neutrophil colonies were increased, macrophage colonies were severely decreased and eosinophil colonies were not generated in *Trib1*$^{-/-}$ bone marrow cells compared with wild-type cells (Fig. 2a). We found that macrophage colonies could be morphologically classified into two subgroups, namely aggregated/small and diffused/large macrophages (Fig. 2b). Although most wild-type macrophage colonies were aggregated and small, the macrophage colonies obtained from *Trib1*$^{-/-}$ bone marrow cells were diffused and large (Fig. 2b). These results indicated that Trib1 is essential for the proper

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