

Review

Intrinsic danger: activation of Toll-like receptors in rheumatoid arthritis

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Abstract

RA is a debilitating disorder that manifests as chronic localized synovial and systemic inflammation leading to progressive joint destruction. Recent advances in the molecular basis of RA highlight the role of both the innate and adaptive immune system in disease pathogenesis. Specifically, data obtained from *in vivo* animal models and *ex vivo* human tissue explants models has confirmed the central role of Toll-like receptors (TLRs) in RA. TLRs are pattern recognition receptors (PRRs) that constitute one of the primary host defence mechanisms against infectious and non-infectious insult. This receptor family is activated by pathogen-associated molecular patterns (PAMPs) and by damage-associated molecular patterns (DAMPs). DAMPs are host-encoded proteins released during tissue injury and cell death that activate TLRs during sterile inflammation. DAMPs are also proposed to drive aberrant stimulation of TLRs in the RA joint resulting in increased expression of cytokines, chemokines and proteases, perpetuating a vicious inflammatory cycle that constitutes the hallmark chronic inflammation of RA. In this review, we discuss the signalling mechanisms of TLRs, the central function of TLRs in the pathogenesis of RA, the role of endogenous danger signals in driving TLR activation within the context of RA and the current preclinical and clinical strategies available to date in therapeutic targeting of TLRs in RA.

Key words: Toll-like receptors, rheumatoid arthritis, damage-associated molecular patterns, therapies

RA

RA is a chronic autoimmune disease that can affect multiple joints of the hands and feet simultaneously. It results in pain, stiffness and progressive destruction of bone and cartilage, leading to significant disability and deformity, often with extra-articular manifestations such as vasculitis and scleritis [1]. RA is prevalent in ~1% of the population and its association with increased mortality and shortened lifespan imposes vast socio-economic costs [2].

RA is characterized by persistent inflammation of the synovial membrane. Fibroblast proliferation and infiltration of inflammatory cells including T cells, B cells and macrophages [3] lead to pannus development, which invades joint tissue [4, 5]. These cell types release

pro-inflammatory cytokines and chemokines that induce degrading enzymes such as matrix metalloproteinases (MMPs) and osteoclast activation, resulting in further cartilage degradation and bone erosion [6]. Biological agents targeting some of these cytokines such as TNF- α , IL-1 β and IL-6 show significant clinical benefit and halt radiographic progression, confirming the key role of these innate inflammatory mediators in RA [3, 7]. In addition, adaptive immunity is also pivotal in RA pathogenesis. Abundant activated T cells exist in rheumatoid synovium [8]. Disruption of T-cell co-stimulation using soluble cytotoxic T-lymphocyte antigen (CTLA)-4 fused with immunoglobulin improves clinical outcomes in RA patients [9]. IL-17-producing Th cells have also been detected in RA synovium, and targeting this cytokine using soluble antibodies has proven beneficial [10, 11]. Additionally, B-cell therapy utilizing mAbs targeting cluster of differentiation 20 (CD20) has been efficacious in treating RA patients [12]. However, despite the success of these treatments, only a proportion of RA patients respond well. Adverse side effects are associated with global suppression of immunity, and most patients suffer disease recurrence [13]. One of the major obstacles to finding a cure for RA is that the factors that drive persistent cellular activation and inflammatory mediator synthesis remain elusive.

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Although there have been great advances in understanding the cellular and molecular mechanisms that drive inflammation in RA, the aetiology of this disease is still a mystery. RA is a multifactorial disease in which genetic and environmental factors have been implicated. Since the identification of *HLA-DRB1* as a susceptible gene in RA, more than 30 other loci have been shown to exhibit genetic variants that predispose to the disease, including *PADI4*, *PTPN22* and *FCRL3* (reviewed in [14]). Epidemiological studies also propose cigarette smoking as a major environmental trigger for RA, especially in genetically predisposed individuals [15], which is supported by data from animal models [16, 17]. In addition, infection and tissue injury have also been implicated in triggering inflammation in RA. Over the past decade, Toll-like receptors (TLRs), central players in sensing infection and injury, have been proposed to drive inflammation in RA. Here we review the evidence surrounding the emergence of these pattern recognition receptors (PRRs) in RA disease pathogenesis.

TLRs

TLRs are a family of evolutionarily conserved single-span transmembrane receptors that exhibit high homology with the Toll gene in *Drosophila melanogaster* [18]. With the addition of newly identified murine TLR13 [19], 14 mammalian TLRs have been reported, with 10 subtypes in humans. TLRs are expressed on many different cell types, including myeloid cells, fibroblasts, epithelial and endothelial cells. TLRs sense pathogen-associated molecular patterns (PAMPs) derived from viruses, bacteria, protozoa and fungi, with each TLR recognizing a specific subset of ligands (reviewed in [20, 21]). TLR4, the receptor for bacterial lipopolysaccharide (LPS), was the first human TLR identified [22] and remains the best characterized to date. It utilizes myeloid differentiation protein 2 (MD2), CD14 and LPS-binding protein (LBP) as accessory molecules for LPS recognition [23]. TLR1, TLR2 and TLR6 detect bacterial lipoproteins and dimerization of TLR2 with either TLR1 or TLR6 confers ligand specificity. TLR5 senses flagellin, TLR9 recognizes unmethylated C-phosphate-G (CpG) bacterial DNA and TLR3, TLR7 and TLR8 bind to viral nucleic acids. The subcellular localization of TLRs corresponds well to the nature of their pathogenic ligands; TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface and detect pathogenic surface elements, whereas TLR3 and TLR7–9 are found primarily in the intracellular endosome and detect internalized ligands. The exact function of TLR10 remains largely unexplored due to the fact that it is not present in mice, though a recent study indicates that TLR10 might cooperate with TLR2 in detecting lipoproteins [24, 25].

More recently, TLRs have also been found to sense endogenous danger signals or damage-associated molecular patterns (DAMPs) (Table 1). These include intracellular molecules released during cell necrosis [26], such as cytosolic HSPs and nuclear high mobility group box 1 (HMGB1). DAMPs also comprise extracellular matrix (ECM) molecules such as versican, biglycan, fibrinogen, fibronectin extra-domain A (EDA) and tenascin-C that are

specifically up-regulated in response to tissue injury, and low molecular weight fragments of the ECM, such as HA and heparin sulphate, that are generated by tissue damage. TLRs also exhibit specificity for DAMPs, with cell surface family members detecting protein, proteoglycan and fatty acids and endosomal TLRs sensing endogenous nucleic acids. DAMPs are vital for the initiation of inflammation during tissue injury in the absence of infection, but have also been implicated in various pathophysiological conditions that are characterized by sterile chronic inflammation (Fig. 1) such as tumorigenesis, stroke, cardiovascular and autoimmune diseases (reviewed in [27–31]).

TLR-mediated signalling pathways

Each TLR consists of amino-terminal leucine-rich repeats (LRRs) that comprise the ligand-sensing domain and a carboxy-terminal Toll/IL-1 receptor (TIR) signalling domain (reviewed in [82]). The overall sequence homology among the TLRs is low, but the TIR domain that is essential for downstream signalling is highly conserved [83, 84]. Ligand binding induces receptor conformational changes and results in the recruitment and/or activation of adaptor molecules that initiate a cascade of inflammatory signalling events. These are reviewed extensively elsewhere [20, 21, 85–87] and are summarized in Fig. 2.

PAMPs vs DAMPs: mechanism of ligand recognition and downstream signalling

Recent crystallographic structures of PAMP-TLR ectodomain complexes have disclosed a common m-shape configuration of TLR dimers [90–94]. Pam₃CSK₄ binding to TLR1–TLR2 facilitates receptor heterodimerization via hydrophobic interactions [90] and two phenylalanine residues in the TLR2–TLR6 heterodimer are important for lipopeptide docking and binding specificity [91]. Crystal structures of several pathogen-derived TLR2 agonists have also been reported, including PorB [95], NapA [96], lipoprotein A [97] and LprG [98]. TLR3 interacts with dsRNA through the cationic surface of the receptor, while the receptor dimer is held in place by intermolecular forces within TLR3 [93]. In contrast, LPS binding to MD2 results in the formation of a multimer composing two units of the TLR4–MD2–LPS complex [92, 94]; multifaceted interactions within this complex mediate hydrophobic and hydrophilic bonding between TLR4 and MD2, as well as to the overhanging sixth acyl chain of LPS [94]. Thus, although TLRs display similar scaffold structures, distinct binding mechanisms exist to facilitate recognition of ligands derived from diverse origins.

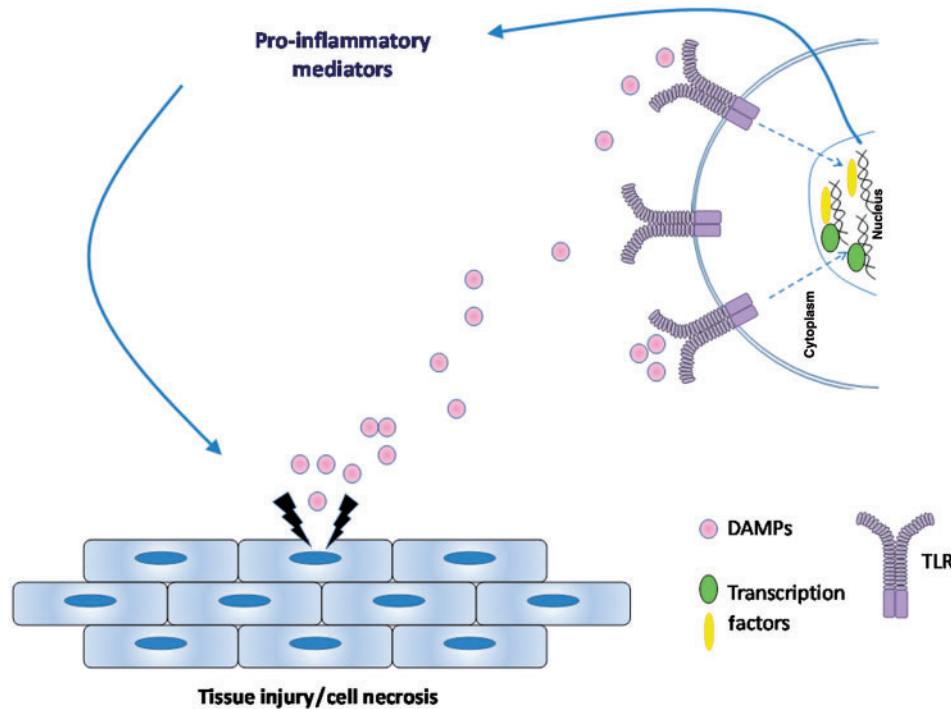
While the mode of PAMP activation of TLRs is being elucidated, very little is known about the mode of TLR activation by DAMPs. To date, no direct interaction of a DAMP with its cognate TLR has been described. Little structural information about endogenous TLR ligands exists, except a recent study that described the crystal structure of pancreatic adenocarcinoma up-regulated factor (PAUF) and confirmed its role as a novel endogenous TLR2 and TLR4 agonist [44]. Accumulating evidence has

TABLE 1 Endogenous TLR activators

Class of activator	TLR1	TLR2	TLR4	TLR3	TLR7	TLR8	TLR9
Proteins/peptides	β -defensin 3 [32]	HSP60, 70, GP96 [33–35] HMGB1 [36, 37] HMGB1-nucleosome complexes [38] β -defensin 3 [32] Surfactant proteins A, D [39, 40] Neurotoxin [41] aPLs [42] Cardiac myosin [43] PAUF [44]	HMGB1 [36, 37] Fibronectin EDA [45] Fibrinogen [46] Tenascin-C (FBG) [47] Surfactant proteins A, D [40, 48] β -defensin 2 [49] HSP90, 70, 72, 22, GP96 [33–35, 50–53] S100A8 (MRP8) [54, 55] S100A9 (MRP14) [54, 55] NE [56] aPLs [57, 58] Lactoferrin [59] Resistin [60] PAUF [44] Serum amyloid A [66, 67] Oxidized LDL [68–70] Saturated fatty acids [71, 72] Biglycan [73] Heparin sulphate fragments [76] HA fragments [75] Monosodium urate crystals [77]		aPLs [61]	aPLs [61, 62] Cardiac myosin [43]	
Fatty acids/lipoproteins		Serum amyloid A [63, 64] CEP [65]					
Proteoglycans/glycosaminoglycans		Biglycan [73] Versican [74] HA fragments [75]					
Organic molecules		Monosodium urate crystals [77, 78]					
Nucleic acids/protein-nucleic acids complexes				mRNA [79]	ssRNA [80]	ssRNA [80]	IgG-chromatin complexes [81]

CEP: omega-(2 carboxyethyl)pyrrole; IgG: immunoglobulin G; LDL: low-density lipoprotein; NE: neutrophil elastase; PAUF: pancreatic adenocarcinoma up-regulated factor; ssRNA: single-stranded RNA.

Fig. 1 Release of DAMPs during tissue injury and cell damage activate TLRs—a vicious inflammatory cycle. Binding of DAMPs to TLRs leads to receptor activation and downstream signalling stimulation, resulting in translocation of transcription factors into nucleus and up-regulation of pro-inflammatory mediators such as cytokines, chemokines and MMPs. This in turn perpetuates the inflammatory responses and results in chronic inflammation.



suggested significant disparity in the ligand recognition mechanism for PAMPs and DAMPs (reviewed in [99]). Endogenous ligands might occupy or bind different sites on TLR. For example, mutation of asp299 and/or thr399 in TLR4, which are important for receptor dimerization, rendered the receptor unresponsive to LPS, but stimulated fibrinogen-mediated TLR4 activation [100]. Additionally, DAMPs appear to employ distinctive combinations of co-receptors and accessory proteins to mediate receptor activation (Table 2). Interestingly, certain DAMPs seem to be involved in novel signalling paradigms; for example, biglycan stimulates the NLRP3/ASC inflammasome via TLR2/TLR4 and purinergic P2X₄/P2X₇ receptors [101], and PAUF signals through a TLR2/CXC chemokine receptor 4 (CXCR4) complex [44].

It seems logical to envisage that the signalling outcomes of DAMPs might be distinct from PAMP. This is supported by several lines of evidence. For example, gene expression profiles induced by hyaluronan and tenascin-C are significantly different from that induced by LPS [47, 103, 111]. Genes such as MMP13 and suppressor of cytokine signalling 3 (SOCS3) were specifically induced by hyaluronan but not LPS [103]. Tenascin-C did not induce IL-8 in synovial fibroblasts, contrary to the effect of LPS [47]. Interestingly, PAUF-stimulated mitogen-activated protein kinase (MAPK) signalling pathways, in contrast to LPS, did not activate nuclear factor kappa B (NF- κ B), but instead cooperate with CXCR4 to inhibit TLR2-mediated NF- κ B activation [44].

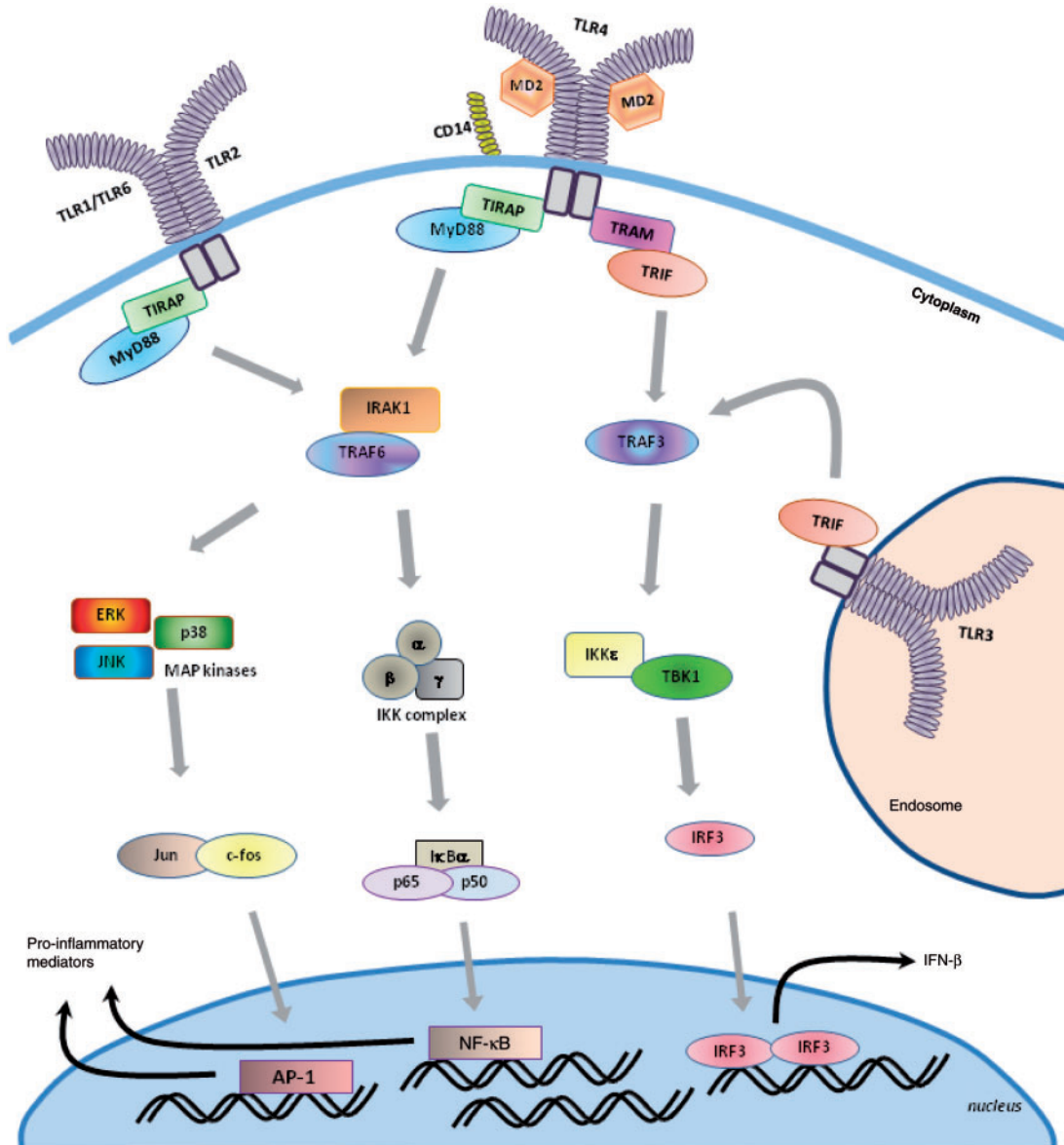
Evidence has emerged that some endogenous ligands such as HSPs, HMGB1 and surfactant protein A also possess the ability to bind LPS and/or sensitize the cells to LPS stimulation, raising the possibility that these molecules also behave as accessory proteins in LPS signalling (reviewed in [1]). For instance, HMGB1 mediates the transfer of LPS to CD14, thereby augmenting TLR4 activation [112, 113]. While low concentrations of Gp96 did not elicit any response on their own; co-incubation with TLR2 or TLR4 agonists resulted in potentiation of TLR response [114]. Taken together these data indicate that DAMPs exhibit intricate differences to PAMPs in terms of receptor activation and type of immune response induced, ranging from the utilization of specific co-receptors and, adaptor molecules to activation of signalling cascades and the resultant gene expression pattern.

The role of TLRs in RA

Elevated levels of TLR expression in human RA joints

A number of different studies provide compelling evidence for the presence of TLRs in human synovial tissues. TLR3 and TLR4 are highly expressed in human synovial fibroblasts, even in the early stages of RA [115]. Abundant expression of TLR2 was detected at sites of cartilage and bone erosion [116]. The expression of TLR2, TLR3 and TLR7 was significantly up-regulated in RA synovial fibroblasts (RASFs) compared with healthy controls or

Fig. 2 Overview of TLR signalling pathways. Cell surface TLRs are TLR1/6 with TLR2, TLR4 and TLR5 (not shown). Endosomal TLRs are TLR3 and TLR7–9 (not shown). MyD88 is an adaptor protein associated with all TLRs, with the exception of TLR3. TLR1, TLR2, TLR4 and TLR6 require an additional protein, TIRAP, for MyD88 recruitment, whereas TLR5, TLR7 and TLR9 can bind MyD88 directly. TLR3, together with TLR4, utilize TRIF to mediate signal transduction. TRIF-related adaptor molecule (TRAM) is another adaptor molecule associated with TLR4, necessary for the interaction with TRIF [88]. Generally, activation of the MyD88-dependent pathway leads to activation of signalling intermediaries such as IRAK, TRAF-6 and TANK-binding kinase (TBK)-1 proteins, MAPKs and transcription factor NF- κ B [89]. Activation of NF- κ B results in increased expression of various pro-inflammatory cytokines such as TNF- α , IL-6 and chemokines. Stimulation of the MyD88-independent TRIF-dependent pathway leads to activation of the transcription factor IRF proteins and IFN response. Recruitment of IRF3 to the respective DNA binding site initiates the transcriptional activation of IFN- β and IFN-stimulated genes. ERK: extracellular-signal regulated kinase; JNK: c-jun N-terminal kinase; AP-1: activating protein-1; MyD88: myeloid differentiation primary response protein 88.



patients with non-inflammatory arthritis [117–120]. In addition to RASFs, increased expression and activity of TLR2 and TLR4 has also been detected on macrophages isolated from RA synovium [117]. Stimulation of cultured RASFs with TLR3 and TLR7 ligands resulted in significant

up-regulation of chemokines, cytokines, metalloproteinases and type I IFNs [115, 119, 120]. Similarly, TLR2, TLR3 and TLR4 activation in RASF results in osteoclastogenic activity, MMP release and up-regulation of vascular adhesion molecule-1 [118, 121–123]. Notably, while

TABLE 2 The co-receptors and accessory proteins utilized by DAMPs to bind to respective TLRs

TLR2	
CD14/MD2	HSP60 [102], HSP70 [33], biglycan [73]
CD14	Surfactant protein A [39, 48], surfactant protein D [40]
MD2	Gp96 [34], HMGB1 [36]
P2X ₄ /P2X ₇	Biglycan [101]
TLR6/CD14	Versican [74]
CD44/MD2	HA fragments [103]
CXCR4	PAUF [44]
TLR4	
CD14 / MD2	HSP60 [102], HSP70 [33], oxidized LDL [70], S100A8, S100A9, biglycan [73]
CD14	Lactoferrin [59], surfactant protein A [48], surfactant protein D [40]
MD2	GP96 [34], HMGB1 [36], fibronectin EDA [45], saturated fatty acids [71]
P2X ₄ /P2X ₇	Biglycan [101]
CD44/MD2	HA fragments [103]
TLR6/CD36	Serum amyloid A [104], oxidized LDL [104]
TLR7	
LL37	ssRNA [105]
BCR	RNA-associated autoantigens [106]
TLR9	
LL37	dsRNA [107]
BCR	IgG-chromatin complexes [108]
CD32	DNA-containing ICs [38, 109]
IgM	IgG-chromatin complexes [81]
RAGE/HMGB1	DNA-containing ICs [110]
TLR8	
LL37	ssRNA [105]

RAGE: receptor for advanced glycation endproducts.

stimulation of TLR3 and TLR8 increased TNF- α release in RA membrane cultures, activation of TLR7 or TLR9 did not, suggesting that TLR3 and TLR8 may be of more importance [124]. Inhibition of myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adapter protein (TIRAP), the adaptors for TLR2 and TLR4, reduces cytokine synthesis in RA membranes, indicating that these TLRs also play a key role [122]. A recent study that utilizes double immunostaining of various cell types derived from human RA joints showed that TLRs 1–6, TIRAP and TIR domain-containing adapter-inducing IFN- β (TRIF) are most strongly expressed in dendritic cells, followed by macrophages and the least in fibroblasts [125]. Collectively, several cell types in human RA joints express high levels of functional TLRs.

Evidence from animal experimental models

Many experimental models of arthritis require TLR ligand administration for disease induction; for example, bacterial DNA, streptococcal cell wall, zymosan and the use of complete Freund's adjuvant (CFA) in CIA [126–128]. Therefore disease amelioration in animals lacking specific

TLRs and adaptor molecules is perhaps not unanticipated [129–131]. Nevertheless, the use of models not reliant on TLRs for disease induction supports the role of TLRs in persistent inflammation. In a serum-transfer model, disease duration was shortened in TLR4 null mice [132]. Mice lacking IL-1RA develop spontaneous arthritis due to increased production of IL-17 [133, 134]. Significantly reduced disease severity was observed in IL1-RA^{-/-} and TLR4^{-/-} double-knock-out mice and in the presence of a TLR4 antagonist [135]. TLR4 is postulated to be the upstream driving force of IL-17 production in this model. Intriguingly, IL-1RA^{-/-} and TLR2^{-/-} double-knock-out mice showed increased disease severity, casting doubt on the pro-inflammatory role of TLR2 in this context, while TLR9 did not affect disease development [135]. Interestingly, repeated *in vivo* low-dose administration of synthetic TLR7 ligand, which resulted in the tolerance of TLR2, TLR7 and TLR9, suppressed joint inflammation in a serum-transfer arthritis model [136]. Taking into account the results obtained from TLR2 and TLR9 knock-out models, TLR7 may play a key role in this context. Another recent study proposed TLR3 as the central player in the pristane-induced arthritis rat model; receptor expression was significantly up-regulated during early disease stages, disease augmentation occurred upon TLR3 stimulation, and small interfering RNA (siRNA) targeting TLR3 *in vivo* reduced disease severity [137].

Although TLR8 is up-regulated in human RA tissues, little information has been derived from animal studies. Unlike its phylogenetically related TLR7 and human TLR8, murine TLR8 is not activated by its natural ligand viral ssRNA, leading to the general belief that TLR8 is biologically dysfunctional in mice [138]. Similarly, TLR10 is not present in mice [24]. Moreover, the intrinsic details of TLR signalling and activation of target genes is species specific. For example, TLR4 and TLR3 stimulation in murine cells leads to NF- κ B activation and TNF- α and IL-6 synthesis, while in human cells this is only observed with TLR4, but not TLR3 engagement [139, 140]. Interestingly, nickel-induced contact hypersensitivity, mediated via TLR4 activation, is a phenomenon specific to humans, as murine TLR4 is not activated by nickel [141]. These data highlight the fact that cross-species data extrapolation should be undertaken cautiously.

Endogenous TLR activators in the pathogenesis of RA

Infection has long been speculated to be an underlying factor in RA pathogenesis. Early studies reported the presence of peptidoglycan, bacterial and viral DNA in joints of RA patients, implicating PAMP activation of TLRs as a potential driver of disease [142, 143]. However, this theory remains controversial, as pathogenic molecules are also found at comparable levels in the joints of healthy individuals [144]. A large body of evidence has documented the presence of endogenous TLR ligands in RA tissues, predominantly TLR4 and/or TLR2 agonists. These include HSPs, fibrinogen, fibronectin EDA, HMGB1, tenascin-C and serum amyloid A (reviewed in [99]). Moreover, administration of exogenous DAMPs induces

joint inflammation *in vivo*. For example, intra-articular (IA) injection of fibronectin EDA resulted in joint swelling [145]. We have also shown that injection of tenascin-C induced joint inflammation, while mice deficient in tenascin-C exhibited rapid inflammation resolution [47]. Moreover, tenascin-C stimulated the release of pro-inflammatory mediators in explant cultures from RA patients. Interestingly, necrotic cells from SF of RA patients stimulated release of cytokines in RASFs through TLR3 ligation [119]. RA SF and serum also stimulated Chinese hamster ovary (CHO) and human embryonic kidney (HEK)-293 cells overexpressing TLR4 in an LPS-independent manner [120,135]. Notably, IC-containing citrullinated fibrinogen augmented TNF- α release from macrophages via co-stimulation of TLR4 and fragment crystallisable gamma receptor (Fc γ R) *in vitro*, providing insights into how citrullination could potentiate the pro-inflammatory effects of DAMPs [146].

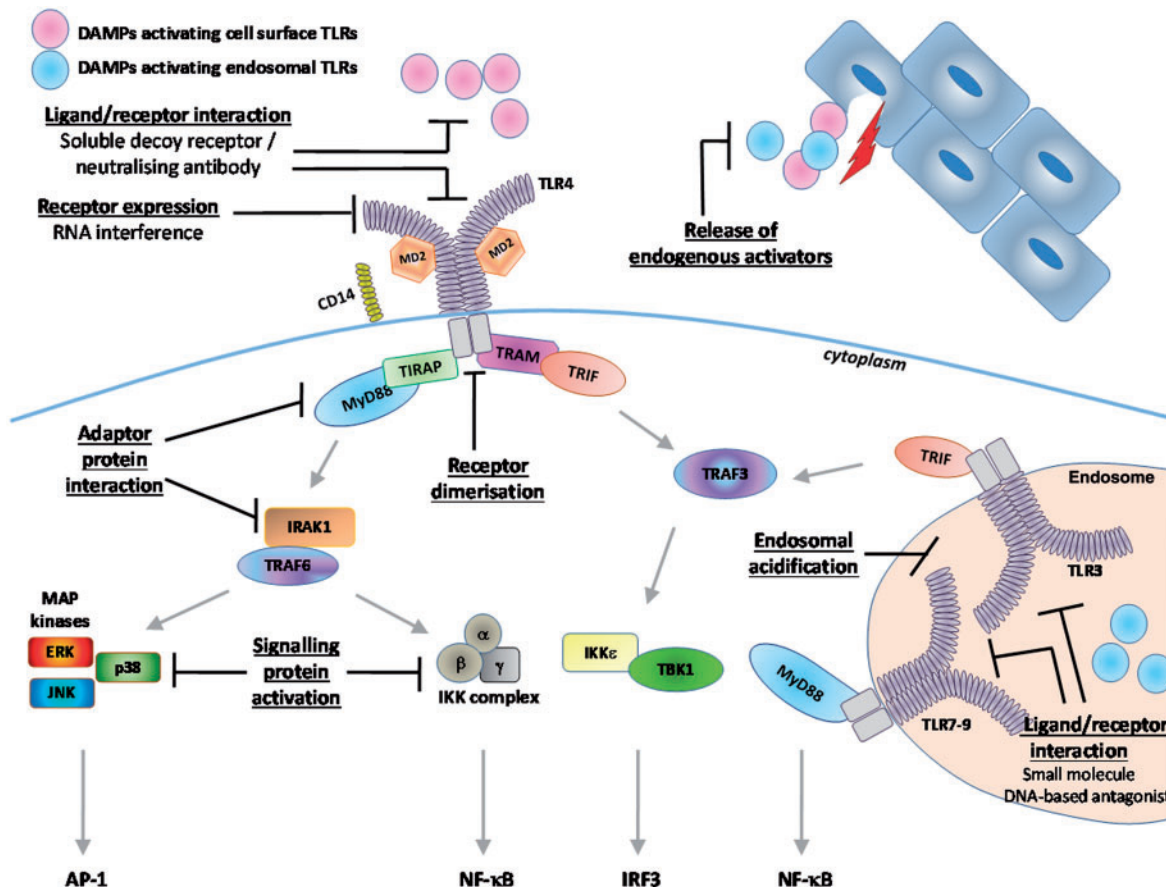
Therapeutic targeting of TLRs

The clinical efficacy of classical RA treatment regimens has significantly improved patient care, but is limited to

certain subsets of patients. Moreover, disease remission remains rare and biologic agents are expensive. Thus there is an urgent need for more effective, cheaper therapeutics [147]. Given the evidence supporting a role for TLRs in RA pathogenesis, these receptors may represent good targets for therapeutic intervention.

Several strategies exist to ablate TLR-triggered inflammatory responses (Fig. 3). Modulation of receptor/ligand interaction can be mediated by soluble decoy receptors and neutralizing antibodies. As DAMPs have been implicated in TLR activation in RA, preventing the release of endogenous ligands is also an option. Additionally, down-regulation of TLR expression may be useful, and the *in vivo* application of siRNA targeting TLR3 has been recently demonstrated [137]. Receptor dimerization is a prerequisite for TLR activation, thus offering another potential approach. Also, molecules serving as intermediary proteins downstream of TLR signalling may be good targets. Therapeutic targeting of TLRs for several pathological conditions, including oncogenesis, infection and autoimmune diseases, has been reviewed elsewhere [148–152]. This review section focuses on the recent advances in inhibiting TLR activation in RA, including

Fig. 3 Site of action for antagonists of TLR signalling pathways and endogenous activators. Potential strategies to block DAMP-induced inflammatory responses both intracellularly and extracellularly are indicated. TLR4 is shown here as an example of cell surface TLRs. The nature of intervention and the type of inhibitor are indicated.



preclinical and clinical studies. A summary of compounds targeting TLRs that are currently being developed by pharmaceutical companies is listed in Table 3.

Targeting TLR4

Natural TLR antagonists and soluble TLRs (sTLRs) can suppress TLR-mediated inflammation [160–166]. For example, LPS from *Bartonella quintana* and ST2 protein expressed by mast cells and Th cell type 2 (Th2) inhibit TLR4-mediated signalling in experimental models of arthritis, resulting in disease amelioration [160,161]. Other naturally occurring molecules include single-immunoglobulin IL-1 receptor related (SIGIRR) and HSP10/chaperonin 10 [162,167]. SIGIRR, a negative regulator of TLR4 signalling, suppresses cytokine production in cells derived from RA synovium [162]. The recombinant analogue of chaperonin 10, XToll, is now being tested in a Phase II clinical trial for RA treatment given by subcutaneous injection. The adipocytokine C1q/TNF-related protein-3 (CTRP-3) has been shown to inhibit the binding of LPS to TLR4 [166]. Interestingly, a plant derivative, RSCL-0520, when administered *in vivo*, reduced paw swelling in rats [168]. A small carbohydrate molecule developed by the same group specifically inhibits TLR2 and TLR4 [169]. sTLR4 has been reported by several groups to inhibit LPS signalling, but the effects of these soluble receptors have yet to be demonstrated *in vivo* [163–165].

The crystallographic structure of LPS/MD2/CD14 interacting with TLR4 has facilitated the synthesis of inhibitory molecules interrupting this binding, such as MD2 mimetic peptides and a cationic amphiphile compound, the effects of which remain to be determined *in vivo* [170, 171]. Sulforaphane and OSL07, an oxazolidinone derivative, prevent dimerization of TLR4 and suppress downstream signalling activation; the former exhibiting anti-inflammatory properties *in vivo* [172, 173]. mAb NI-0101/ α , developed by NovImmune, has also been found to inhibit TLR4 activation [154].

Targeting TLR2

Soluble forms of TLR2 have been found in human plasma, breast milk and amniotic fluid [174, 175]. Significantly, sTLR2 is a competent modulator of TLR2-mediated responses in an experimental model of peritoneal inflammation without affecting the host's ability to fight bacterial infection [176]. Opsona Therapeutics is currently developing antibodies targeting TLR2: OPN-305, which abrogates

cytokine release [155], and OPN-301, which inhibits spontaneous cytokine release in human RA explant cultures as efficaciously as anti-TNF- α antibody [177].

Targeting TLRs 3/7/8/9

Several drugs possess off-target effects on endosomal TLRs 3/7/8/9 in addition to their classical mechanisms of action. The anti-malarial drug chloroquine and related derivatives have long been used as anti-inflammatory drugs in various disorders, including SLE, psoriasis and RA (reviewed in [178]). The underlying mechanism is attributed to the ability of these drugs to suppress the activation of endosomal TLRs, presumably by suppressing endosomal acidification, a fundamental prerequisite for the activation of endosomal receptors. New evidence has also suggested that these drugs might act by binding directly to nucleic acid ligands, thereby masking the receptor-binding domain [179]. Small molecules such as mianserin and imiquimod inhibit the production of TNF- α from human RA synovial membranes; the former inhibits TLRs 3, 7, 8 and 9 while the latter targets TLR8 [124]. VTX-763 is a small molecule inhibitor targeting TLR8 that is being developed by VentiRx Pharmaceuticals; it is currently in the preclinical phase [158]. Interestingly, it has also been recently reported that anti-depressant drugs fluoxetine and citalopram inhibit the endosomal TLRs in addition to their ability to interfere with the serotonin re-uptake process [180]. These psychotropic drugs improved disease outcome in a CIA model and reduced cytokine release from human RA synovial membranes. However, it will be a challenging task to incorporate these drugs into monotherapy applications for autoimmune diseases due to the high doses required and associated adverse effects.

Short DNA oligodeoxyribonucleotides (ODNs) called immunoregulatory sequences (IRSs) also inhibit endosomal TLRs [181–183]. Prophylactic administration of ODNs in CIA and CpG-induced arthritis abrogated disease progression [184, 185]. Idera is now developing a novel DNA-based TLR7/9 antagonist, IMO-3100, that has shown promising results *in vivo* for several autoimmune disease models. Phase I clinical trials involving healthy subjects are under way and so far IMO-3100 is reported to be well tolerated with no major adverse effects, in addition to reducing the release of cytokines such as TNF- α and IL-1 β in these subjects. Idera is also exploring the possibility of IMO-3100 for the treatment of RA, SLE and

TABLE 3 Clinical and preclinical candidates available in inhibiting TLRs for the treatment of RA

Compound	Target	Drug class	Clinical status	Company	Reference
Chaperonin 10/XToll	TLR4	Protein	Phase II	Cbio Ltd	[153]
NI-0101/ α	TLR4	Antibody	Preclinical	NovImmune	[154]
OPN-305	TLR2	Antibody	Preclinical	Opsona	[155]
IMO-3100	TLR7/9	DNA-based	Phase I	Idera Pharmaceuticals	[156]
DV-1179	TLR7/9	DNA-based	Preclinical	Dynavax	[157]
VTX-763	TLR8	Small molecule	Preclinical	VentiRx Pharmaceuticals	[158]
SB-681323	p38MAPK	Small molecule	Phase I	GlaxoSmithKline	[159]

psoriasis. DV-1179 is another DNA-based compound, developed by Dynavax, for TLR7/9 inhibition [157].

Targeting downstream signalling mediators

Another approach undertaken to inhibit TLR-mediated responses is to target downstream signalling mediators. Several attempts have been made to inhibit MAPKs in inflammatory diseases, and data obtained from animal studies showed promising results. However, undesirable toxicity revealed during clinical trials rendered these inhibitors impractical as therapeutic agents (reviewed in [186]). Papamimod, a selective p38 MAPK inhibitor, halts disease progression in arthritis animal models, and showed tolerable adverse effects compared with inhibitors of the same kind, but was less efficacious than MTX and without added benefit for patients when given as part of a combination therapy [187, 188]. SB-681323, a p38 MAPK inhibitor developed by GlaxoSmithKline, is currently undergoing trials for the treatment of RA [159]. Newer p38 inhibitors such as PF-3644022 and DBM1285 have recently been reported to be efficacious in several arthritis models, although their clinical application remains to be determined [189, 190].

As part of the NF- κ B pathway, intense efforts have also concentrated on targeting I- κ B kinase β (IKK β). MLN-0415 is one such compound developed by Millenium Pharmaceuticals Inc. that entered Phase I clinical trial, but was discontinued due to adverse side effects. Several other IKK β inhibitors that have been reported to date include ML-120B, SPC-839, DHMEQ and fasudil, all of which have been shown to inhibit the NF- κ B pathway and ameliorate disease progression *in vivo* [191–194].

The adaptor proteins that lie upstream of TLR-induced signalling cascades may be better candidates. MyD88 and TRIF lie in closer proximity to the receptor; this might minimize undesirable off-target effects. Although it has yet to be demonstrated in RA, such an approach holds some promise in reducing inflammation. RDP-58, a decapeptide that disrupts TNF- α receptor-associated factor 6 (TRAF6)/MyD88/IL-1R-associated kinase (IRAK) complex formation, exhibits anti-inflammatory properties in several autoimmune disease models [195–197] and has entered Phase II clinical trials for Crohn's disease and ulcerative colitis [198]. Similarly, ST2825, which prevents homodimerization of MyD88, resulting in reduced NF- κ B activation and cytokine release in mice [199], suppresses antibody secretion in B cells from SLE patients [200].

Targeting endogenous activators

Given the aberrant expression of DAMPs in human RA tissues, one potential therapeutic approach may be to directly modulate their activity. Neutralizing antibodies to HMGB1 reduced inflammation in experimental CIA [201], as did a truncated HMGB-1 containing only the A-box region. A low molecular weight compound, SNX-4414, which was developed as an HSP90 inhibitor, completely suppressed collagen-induced rat paw swelling *in vivo* [202]. Another approach may be to limit expression levels of these endogenous ligands. This requires a

good understanding of the molecular mechanism underlying the induction and release of DAMPs. For example, there exist two modes of HMGB1 release from host cells: it may be liberated passively from necrotic cells, or actively secreted by various stimulated cells including monocytes, macrophages, dendritic cells and synovial fibroblasts (reviewed in [203, 204]). A wide range of compounds such as the neurotransmitter acetylcholine [205], plant-derived flavonoid quercetin [206] and phospholipid stearyl lysophosphatidylcholine [207] suppress the release of HMGB1 in experimental sepsis. The exact inhibitory mechanisms of these molecules are still unclear, and whether they are effective in arthritis models remains to be elucidated.

Fragments of ECM components also constitute DAMPs. Inhibiting the mobilization of these endogenous molecules by targeting the proteolytic enzymes that create them may be therapeutically useful. Heparan sulphate proteoglycan is liberated from intact matrices by neutrophil elastase (NE); and *in vivo* administration of NE-induced septic shock in mice via TLR4, resembling the effect of direct heparan sulphate injection [208]. Moreover, an NE inhibitor, ONO-5046, improves disease outcome in CIA animal models [209] and alpha-1 anti-trypsin, which also inhibits NE, greatly suppressed disease onset and progression in mice [210].

Targeting regulation by microRNAs

MicroRNAs are endogenous RNAs that post-transcriptionally modulate gene expression (reviewed in [211]). MicroRNAs are important immune regulators; each is able to modulate up to several hundreds of inflammatory mediator targets [212, 213]. Not surprisingly, regulation of gene expression by microRNAs has also been extended to the TLR signalling paradigm (reviewed in [214]). MicroRNAs impose several levels of regulation on the TLR signalling axis, for example, miR-155, miR-21 and miR-147 regulate the expression of TLRs 2–4, downstream signalling mediators such as MyD88 and TRIF, as well as transcription factors NF- κ B and IFN response factor 3 (IRF3) (reviewed in [214]). Conversely, miR-21 is a key regulator of inflammatory gene synthesis downstream of LPS activation of TLR4 [215]. Recent studies have reported that miR-155, miR-146a and miR-203 are up-regulated in RASFs, resulting in altered cytokine and MMP synthesis [216–218]. These insights may create a novel approach to limiting excessive TLR activation during inflammation.

Conclusions and future perspectives

Evidence from animal models and human explants highlights a role for TLR2, TLR3, TLR4 and TLR8 in the pathogenesis of RA. Effective targeting of these TLRs will be facilitated by greater knowledge of the biochemical properties and precise molecular interactions between receptor and agonist. This may be helped by computational homology modelling in the absence of many crystal structures [219]. The identity of key pathogenic DAMPs also remains unclear and it is not known how each or all contribute to RA. It will be essential to elucidate if targeting

one specific DAMP will suffice for disease amelioration or if it is more appropriate to target downstream signalling pathways shared by many DAMPs. We also require in-depth knowledge of the molecular interaction of DAMPs with their respective TLRs, in addition to the adaptor proteins and the signalling cascades involved.

It is also tempting to speculate to what extent TLRs might interact or work cooperatively with each other during RA. Some TLRs cooperate with each other in a context-specific manner, resulting in enhanced production of selective downstream target genes [120, 220, 221]. For example, co-stimulation of TLR2 and TLR4 in murine dendritic cells (DCs) leads to enhanced production of IL10, but not TNF- α [220]. Up-regulation of TNF- α and IL-6 expression was observed during combined stimulation of TLR2 and TLR3, while stimulation of TLR2 suppresses the induction of type I IFN-stimulated genes by TLR3 [221]. Co-stimulation of DCs from RA patients with TLR4 and TLR3-7/8 agonists results in synergistic cytokine release [120]. TLRs may also be involved in heterologous receptor activation. For instance, TLR4 interacts with a G protein-coupled receptor, proteinase-activated receptor (PAR)-2 [222]. PAR-2 has been implicated in several diseases, including joint inflammation (reviewed in [223]). Stimulation of PAR-2 results in NF- κ B activation, and in the presence of TLR4 agonist the response is enhanced, leading to synergistic cytokine expression [222, 224]. Further studies showed that such cross-regulation might extend to TLR3 and downstream IRF and signal transducer and activator of transcription (STAT) signalling [225]. Strikingly, in TLR4^{-/-} and MyD88^{-/-} mice, PAR-2 agonist-induced footpad oedema was significantly reduced compared with controls [225]. This provides an *in vivo* correlation of TLR4 and PAR-2 signalling in the context of joint inflammation. Thus TLRs may be involved in distinct novel signalling paradigms, which still remain largely elusive.

Great advances in the past decade have increased our understanding of the role of TLRs in RA pathogenesis. Although there are still substantial gaps in our knowledge, targeting TLRs or their activation by endogenous danger signals represents an attractive therapeutic option. RA is a heterogeneous disorder associated with an unpredictable pattern of therapy response. With the aid of robust biomarkers and molecular signatures, pharmacogenetic identification leading to personalized medicine and effective drug-targeting disease remission might be possible.

Rheumatology key messages

- TLR activation drives persistent inflammation in RA.
- Endogenous molecules generated upon tissue damage may drive TLR activation in RA.
- Targeting TLR activation could provide novel strategies to treat RA and improve patient care.

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