

# Contribution of Toll-Like Receptors and the NLRP3 Inflammasome in Rheumatoid Arthritis Pathophysiology

Sarah Unterberger 

Kevin A Davies

Srinivasa Bhargav Rambhatla

Sandra Sacre 

Brighton and Sussex Medical School,  
University of Sussex, Falmer, Brighton,  
BN1 9PS, UK

**Abstract:** Rheumatoid arthritis (RA) is a progressive autoimmune disease that is characterized by inflammation of the synovial joints leading to cartilage and bone damage. The pathogenesis is sustained by the production of pro-inflammatory cytokines including tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6, which can be targeted therapeutically to alleviate disease severity. Several innate immune receptors are suggested to contribute to the chronic inflammation in RA, through the production of pro-inflammatory factors in response to endogenous danger signals. Much research has focused on toll-like receptors and more recently the nucleotide-binding domain and leucine-rich repeat pyrin containing protein-3 (NLRP3) inflammasome, which is required for the processing and release of IL-1 $\beta$ . This review summarizes the current understanding of the potential involvement of these receptors in the initiation and maintenance of inflammation and tissue damage in RA and experimental arthritis models.

**Keywords:** rheumatoid arthritis, IL-1, IL-6, TNF, toll-like receptor, NLRP3 inflammasome

## Rheumatoid Arthritis

Rheumatoid arthritis (RA) affects 0.5–1% of the population. It is a systemic disease, characterized by an erosive symmetrical polyarthritis, where widespread synovial inflammation affects both large and small peripheral joints. In addition to joint destruction, the accompanying systemic inflammation can lead to comorbidities including pulmonary inflammation, vasculitis and an increased risk of cardiovascular disease.<sup>1</sup> RA is regarded as a classic polygenic autoimmune disease, primarily on the basis that 70–80% of patients have autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). However, not all patients express these autoantibodies, and they are neither necessary nor sufficient to cause disease, but are predictive of a more aggressive disease course with greater joint erosion.<sup>2</sup> The heritability of RA is estimated to be ~50% in ACPA positive patients, while seronegative RA is much lower at ~20%.<sup>3</sup> However, the disease concordance in identical twins is around 12–15%, suggesting a role for environmental factors. To date, over 100 genetic loci have been associated with RA, though the exact relationship of many of these loci to the disease remains to be determined.<sup>4</sup>

Within the RA joint, peripheral blood mononuclear cells infiltrate the synovial fluid and the synovial membrane, alongside expansion of tissue resident fibroblast-like

Correspondence: Sandra Sacre  
Tel +441273 872865  
Email s.sacre@bsms.ac.uk

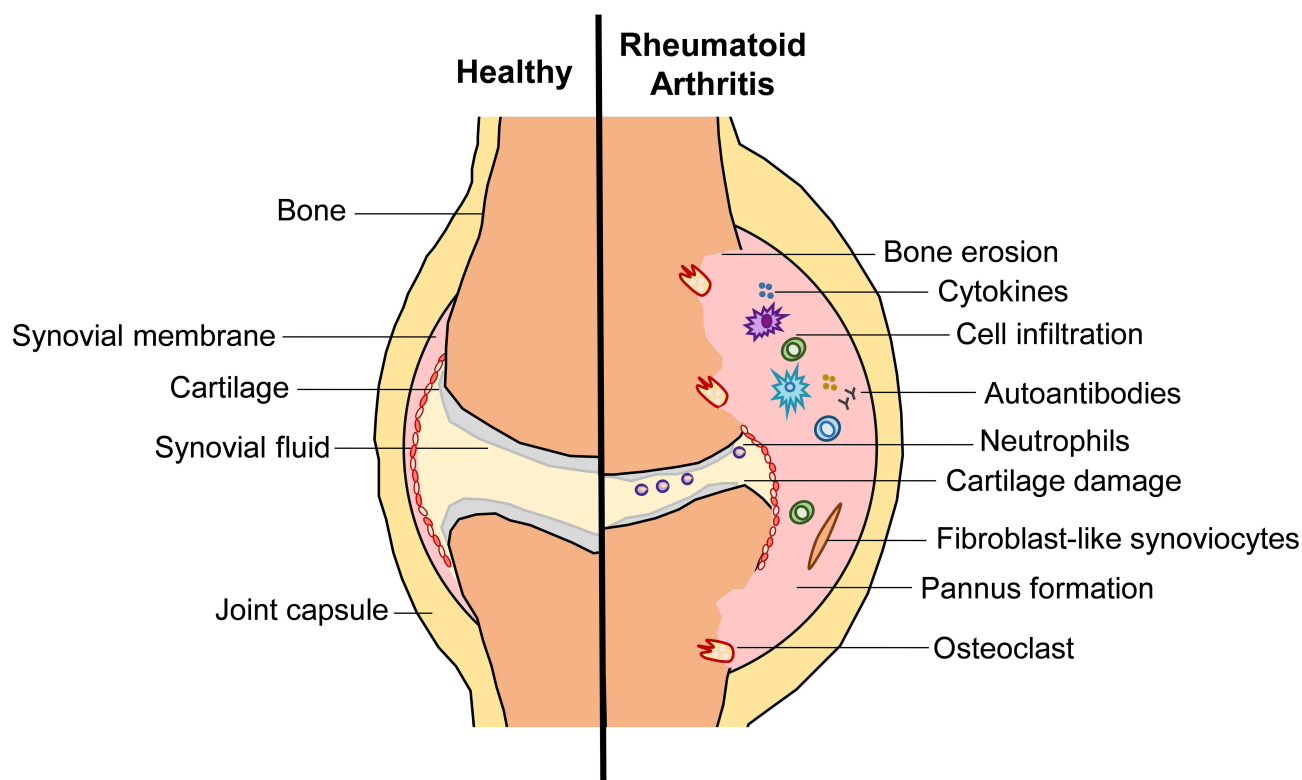
synoviocytes (FLS) leading to the formation of a pannus (Figure 1). These cells are highly activated releasing pro-inflammatory factors, such as Interleukin (IL)-1, IL-6, IL-17, tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF) and matrix metalloproteases (MMPs).<sup>1</sup> Furthermore, neutrophils that accumulate in the synovial fluid undergo NETosis releasing citrullinated proteins that can be recognized by ACPA.<sup>5</sup> This sustained inflammatory environment, leads to the recruitment of further cells into the joint space, whilst FLS invade the cartilage matrix alongside activated osteoclasts, degrading the surrounding cartilage and bone.<sup>1</sup>

In clinical practice, the most widely used and effective therapies are designed to dampen down inflammatory processes. Historically, non-steroidal anti-inflammatory drugs and corticosteroids were used. However, for the last 20 years, the mainstay of therapy in RA have been biological therapies targeting pro-inflammatory cytokines or their receptors, eg anti-TNF antibodies or IL-6 receptor antibodies. Although modestly effective, those that target IL-1 are not frequently used due to the superior performance of the other biologicals.<sup>6</sup> Anti-cytokine activity can also be

mediated by a number of oral Janus Kinases (JAK) inhibitors that have recently been approved for the treatment of RA.<sup>7</sup> However, all of these anti-cytokine therapies target inflammation in RA downstream in the inflammatory process, none are effective in all patients, many lose their efficacy with time and all have significant side effects. Thus, there is great interest in exploring upstream inflammatory mechanisms, with a view to the identification of new therapeutic targets. Over the past two decades there has been a considerable focus on understanding the contribution of toll-like receptors (TLRs) and more recently the nucleotide-binding domain and leucine-rich repeat pyrin containing protein-3 (NLRP3) inflammasome in sustaining inflammation and joint destruction in RA.

## Toll-Like Receptors

TLRs are a family of innate pattern recognition receptors that induce pro-inflammatory cytokines in response to both microbial-associated molecular patterns (MAMPs) and endogenous danger signals termed damage-associated molecular patterns (DAMPs). In humans, there are 10 TLRs that are differentially expressed on both immune



**Figure 1** Pathological changes in a rheumatoid arthritis joint. In established RA, the inflamed synovial membrane forms a pannus, due to infiltration of peripheral blood cells and proliferation of fibroblast-like synoviocytes. These cells are highly activated releasing pro-inflammatory mediators and autoantibodies within the joint sustaining the inflammatory process. This is accompanied by cartilage damage and osteoclast-mediated bone erosion leading to invasion of the pannus tissue and irreversible deformation of the joint.

and non-immune cells. TLRs 1, 2, 4, 5 and 6 are predominantly expressed at the plasma membrane, whereas TLRs 3, 7, 8 and 9 are localized to the endosome.<sup>8</sup> TLR10 is the least characterized member of the family and has been suggested to function both at the cell surface and within the endosomal compartment.<sup>9</sup>

TLRs are type 1 integral membrane receptors that share a common structure, consisting of an ectodomain of leucine-rich repeats (LRR) where they engage their ligands and a cytoplasmic toll-interleukin-1 receptor homology (TIR) domain, also shared by the IL-1 receptor, from where they initiate signaling. Upon activation, TLRs form homo or heterodimers bringing their TIR domains into close proximity, permitting the recruitment of the TLR adaptor proteins, myeloid differentiation response protein 88 (MyD88), MyD88 adaptor-like (MAL), TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) and TRIF-related adaptor molecule (TRAM). Generally, MyD88 is engaged by all TLRs except TLR3, MAL by TLR2 and TLR4, TRIF by TLR3 and TLR4 and TRAM by TLR4.<sup>10</sup> However, TLR adaptor proteins have been shown to signal from TLRs outside this general consensus in a cell type dependent manner. For example, in murine bone marrow-derived macrophages (BMDM), TLR7 and TLR9 require MAL with TLR7 also suggested to use TRAM.<sup>11,12</sup> Furthermore, TRAM may also function as an adaptor protein for TLR2 in primary human FLS, human umbilical vein endothelial cells and murine embryonic fibroblasts.<sup>13</sup>

Dependent on the adaptor proteins recruited, various signaling pathways are engaged that culminate in the activation of transcription factors that include nuclear factor- $\kappa$ B (NF- $\kappa$ B), activator protein-1 (AP-1) and interferon regulatory factors (IRFs) to induce pro-inflammatory cytokines such as TNF, IL-1 $\beta$ , IL-6 and type I interferon.<sup>8</sup> Following TLR activation, IL-1 $\beta$  is translated as a biologically inactive 31kDa precursor requiring proteolytic cleavage by caspase-1 to an active mature 17kDa molecule before being released from cells. This process requires the formation of the inflammasome, a cytosolic multi-protein complex to first activate pro-caspase-1.<sup>14,15</sup>

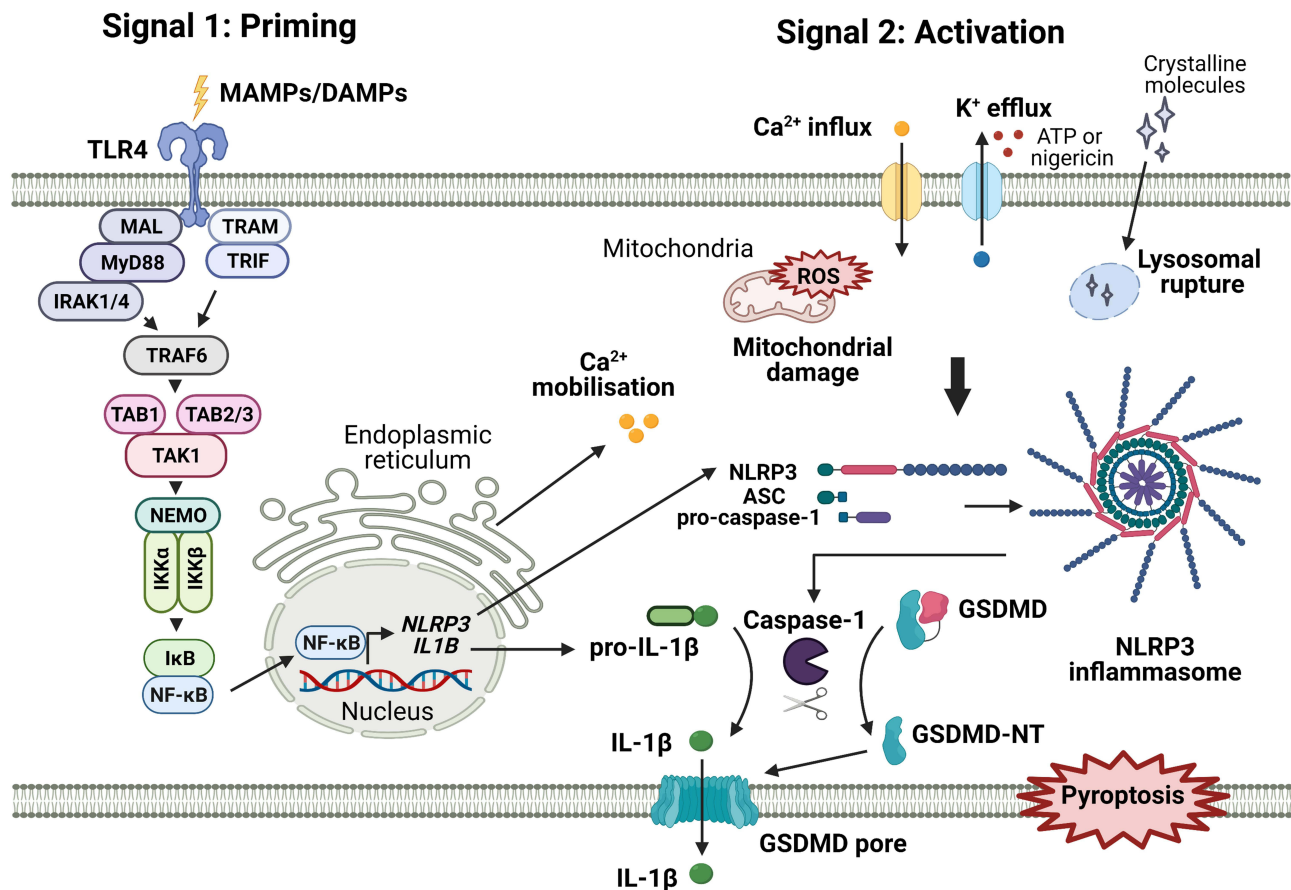
## The NLRP3 Inflammasome

Several types of inflammasome have been identified, of which the nucleotide-binding domain and leucine-rich repeat pyrin containing protein-3 (NLRP3) inflammasome is the most extensively studied. It consists of a LRR domain at the C-terminus considered to be the sensing

domain, a central nucleotide-binding domain (NBD or NACHT domain) and a pyrin domain (PYD) at the N-terminus.<sup>16</sup> Upon activation, NLRP3 oligomerizes with the adaptor protein apoptosis-associated speck-like protein containing CARD (a caspase activation and recruitment domain) (ASC), which then recruits and activates pro-caspase-1 to form the inflammasome (Figure 2).<sup>17</sup> Once activated, caspase-1 cleaves pro-IL-1 $\beta$  and gasdermin D (GSDMD) into two fragments. The GSDMD-N terminus fragments then oligomerize forming pores in the cell membrane which facilitate IL-1 $\beta$  release. This also initiates pyroptosis, an inflammatory form of cell death characterized by cell swelling and rupture, leading to the release of the cytoplasmic contents.<sup>18,19</sup>

To produce mature IL-1 $\beta$ , most cells require two distinct signals. A priming step is required to activate NF- $\kappa$ B to initiate the transcription of pro-IL-1 $\beta$  and NLRP3, which is expressed at low levels under resting conditions.<sup>20</sup> A second signal is then needed to stimulate the assembly of the NLRP3 inflammasome to enable the processing and release of IL-1 $\beta$  (Figure 2). Priming has been demonstrated following activation of several different receptors. Ligands that activate TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 all induce IL-1 $\beta$  release.<sup>21,22</sup> Upregulation of NLRP3 expression has been observed in murine macrophages following activation of TLR2, 3 and 4.<sup>23</sup> In addition, activation of TLR2, TLR3, TLR4, TLR7, NOD2 or stimulation with TNF, IL-1 $\alpha$  or IL-1 $\beta$  results in the cleavage of caspase-1 in the presence of ATP, where ATP alone is not sufficient; further demonstrating the requirement for priming.<sup>20,24</sup> NLRP3 is also activated by a diverse range of stimuli, suggesting a role as a sensor of cellular stress. These stimuli include reactive oxygen species, mitochondrial dysfunction, ion fluxes due to K<sup>+</sup> or Cl<sup>-</sup> efflux, Na<sup>+</sup> influx and Ca<sup>2+</sup> mobilization and lysosomal damage due to uptake of crystalline molecules, such as monosodium urate and cholesterol crystals.<sup>25</sup> In contrast, primary human monocytes engage an alternative pathway; TLR4 can induce IL-1 $\beta$  release in the absence of a separate NLRP3 activation signal or the induction of pyroptosis.<sup>26</sup>

As a potent inducer of inflammation and cell death, NLRP3 activity needs to be tightly regulated. In addition to a low expression in resting cells, further regulation can be achieved through post-translational modifications including phosphorylation, ubiquitination, nitrosylation and sumoylation.<sup>27-30</sup> Furthermore, several proteins are suggested to interact with NLRP3 to regulate



**Figure 2** Two signal model for classical NLRP3 inflammasome activation by TLR4. During the priming stage, activation of TLR4 by MAMPs or DAMPs upregulates NLRP3 and pro-IL-1 $\beta$  expression through NF- $\kappa$ B activation. This is closely followed by activation and assembly of the NLRP3 inflammasome, which can be induced by various stimuli including K<sup>+</sup> efflux, Ca<sup>2+</sup> signaling, mitochondrial dysfunction, and lysosomal rupture. Upon activation, caspase-1 cleaves pro-IL-1 $\beta$  and GSDMD resulting in pyroptosis and IL-1 $\beta$  release. Created with BioRender.com.

**Abbreviations:** TLR, toll-like receptor; MAMPs, microbe associated molecular patterns; DAMPs, damage-associated molecular patterns; MAL, MyD88 adaptor-like; MyD88, myeloid differentiation primary response 88; IRAK, IL-1R-associated kinase; TRIF, TIR-domain-containing adaptor protein-inducing IFN- $\beta$ ; TRAM, TRIF-related adaptor molecule; TRAF, TNF receptor-associated factor; TAK, TGF- $\beta$ -activated kinase; NEMO, NF- $\kappa$ B essential modulator; IKK, I $\kappa$ B kinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NLRP3, nucleotide-binding domain and leucine-rich repeat pyrin containing protein-3; IL, interleukin; ASC, apoptosis-associated speck/like protein containing a CARD; GSDMD, gasderminD; ROS, reactive oxygen species.

inflammasome assembly.<sup>25</sup> Most recently, a member of the TIR domain protein family, sterile alpha and TIR motif containing 1 (SARM1) was demonstrated to regulate NLRP3 through its TIR domain, inhibiting ASC oligomerization and caspase-1 activation.<sup>31</sup>

Together, the ability of TLRs and NLRP3 to respond to DAMPs and stimuli induced by cellular stress makes them key candidates for sustaining inflammation in sterile inflammatory diseases such as RA. Moreover, induction of pyroptosis following NLRP3 activation would potentially release further DAMPs with the potential to activate TLRs sustaining a chronic cycle of inflammation.

## TLRs in RA

In early studies of TLRs in RA, their potential involvement in RA pathophysiology became evident from studies

of arthritis models using TLR deficient mice. In addition, we also demonstrated a role for the TLR adaptor proteins MyD88 and MAL in spontaneous production of cytokines and MMPs from human RA synovial membrane cultures.<sup>32</sup> Since then, a wealth of research studies has demonstrated the upregulation of potential endogenous TLR ligands within the serum and synovial joints of RA patients (Table 1), with all members of the TLR family having been associated with RA in some way (Table 2). However, for several TLRs, it is yet to be determined whether changes in expression or function are a cause or a consequence of inflammation in RA.

## TLR2

To signal, TLR2 forms a heterodimer with TLR1 or TLR6 and possibly TLR10, which will be discussed later. TLR2

**Table 1** Endogenous Toll-Like Receptor Ligands Associated with Rheumatoid Arthritis

TLRs	Endogenous Ligands
TLR2	High-Mobility-Group-Protein B1 <sup>37</sup>
	Heat shock protein 96 <sup>39</sup>
	Serum amyloidA <sup>127</sup>
	SNAP-associated protein <sup>128</sup>
	Heat shock protein 60 <sup>34</sup>
TLR3	RNA released by necrotic synovial fluid cells <sup>54</sup>
TLR4	High-Mobility-Group-Protein B1 <sup>37</sup>
	Citrullinated fibrinogen-containing immune complexes <sup>69</sup>
	Tenascin-C <sup>129</sup>
	S100 Calcium Binding Protein A8 <sup>130</sup>
	Soluble biglycan <sup>131</sup>
	Heat shock protein B8 <sup>132</sup>
	Heat shock protein 96 <sup>39</sup>
	Heat shock protein 60 <sup>34</sup>
	Alpha-enolase <sup>133</sup>
TLR5	High-Mobility-Group-Protein B1 <sup>76</sup>
TLR7	Single-stranded RNA <sup>81</sup>
	miR-let-7b <sup>88</sup>
	Small extracellular vesicles -derived-miR-574-5p <sup>87</sup>
TLR8	Small extracellular vesicles -derived-miR-574-5p <sup>87</sup>
TLR9	DNA fragments <sup>94,134</sup>
TLR10	

**Abbreviation:** TLR, toll-like receptor.

is highly expressed in RA blood and synovial fluid monocytes and synovial lining macrophages.<sup>33,34</sup> Compared with osteoarthritis (OA), TLR2 is also elevated at the mRNA level in RA synovial tissue, with the highest expression associated with patients that do not respond to anti-TNF treatment.<sup>35</sup> Correspondingly, TLR1, 2 and 6 mRNA levels in whole blood are reduced in patients that respond to anti-TNF therapy.<sup>35</sup> However, within the synovial tissue TLR6 mRNA is not upregulated and TLR1 expression is mainly increased in seropositive RA.<sup>35,36</sup> In addition to increased expression of TLR2, several endogenous TLR2 ligands are also present within RA serum

and synovial tissue indicating the potential for TLR2 activation in RA pathogenesis (Table 1). Indeed, High-Mobility-Group-Protein B1 (HMGB-1) can stimulate TLR2 on RA monocytes to induce IL-23, IL-6 and IL-17 promoting the differentiation of Th17 cells.<sup>37,38</sup> Also, extracellular heat shock protein 96 within the RA synovium correlates with inflammation and synovial lining thickness.<sup>39</sup>

Additionally, RA patient monocytes produce higher levels of cytokines compared to healthy donors upon activation of TLR1/2 and TLR2/6.<sup>33,40,41</sup> However, despite increased TLR1 in seropositive RA, we found no association of RF or ACPA status with the level of TLR1/2 cytokine production; although TLR1/2 induced IL-6 did correlate with DAS28.<sup>36,40</sup> TLR2 activation of RA FLS also strongly induces Receptor activator of nuclear factor kappa-B ligand (RANKL) promoting osteoclastogenesis and TLR2 activated M2 macrophages derived from RA patient monocytes exhibit an impaired anti-inflammatory activity.<sup>42,43</sup> In addition to cytokine production, TLR2 has also been demonstrated to promote cell invasion and migration in RA synovial explants.<sup>44</sup> Indeed, inhibition of TLR2 in RA synovial explants with the anti-TLR2 antibody OPN301 led to a decrease in spontaneous cytokines, MMPs and FLS migration in response to explant conditioned media.<sup>45</sup>

Similar to RA, experimental arthritis models also report increased TLR2 expression, which is decreased in studies where anti-inflammatory agents are used to ameliorate disease.<sup>35,46–48</sup> However, variable results have been described for TLR2 in the pathogenesis. In the IL-1Ra<sup>-/-</sup> spontaneous arthritis model, mice develop a more severe disease in the absence of TLR2 due to a modulation of T cell balance from T helper (Th)2 and regulatory T cells (Tregs) towards Th1 cells, suggesting a protective role for TLR2.<sup>49</sup> However, TLR2 has been shown to be important in the development of arthritis induced by intra-articular injection of streptococcal cell wall fragments, with TLR2<sup>-/-</sup> mice having a reduced disease severity.<sup>50</sup> Furthermore, in the murine collagen induced arthritis (CIA) model, TLR2 becomes elevated in blood samples during the pre-onset stage and then falls during early arthritis, suggesting a possible role in disease induction.<sup>35</sup>

## TLR3

TLR3 is also highly expressed in the RA synovium in both early and established disease, where it is potentially

**Table 2** Summary of Some of the Main Associations of TLRs with RA Pathogenesis in Human and Animal Disease Models

	Rheumatoid Arthritis	Experimental Animal Models
<b>Altered TLR expression</b>	↑TLR1, <sup>35,36</sup> ↑TLR2, <sup>33–35</sup> ↑TLR3, <sup>51,52</sup> ↑TLR4, <sup>35,51,63–66</sup> ↑TLR5, <sup>77</sup> ↑TLR8, <sup>35,36,81</sup> ↑TLR9, <sup>33,56,95</sup> ↑TLR10 in B cells <sup>104</sup> and NK cells <sup>103</sup> and ↓TLR10 in PBMCs from active RA vs inactive. <sup>101</sup>	↑TLR2 <sup>46–48</sup> (pre-onset of disease in CIA <sup>35</sup> ) ↑TLR3 (CIA model, Rat PIA model) <sup>59,61</sup>
<b>Association with disease activity</b>	DAS28 correlates with TLR1/2 induced IL-6 from RA monocytes <sup>40</sup> and expression of monocyte TLR5 <sup>77</sup> and TLR7. <sup>81</sup> Synovial TLR4 expression correlates with synovitis. <sup>36</sup> Patients expressing the less inflammatory TLR8 MIV variant exhibit reduced disease severity. <sup>82</sup> TLR10 missense mutation associated with increased disease severity. <sup>102</sup> ↑TLR10 expression in B cells <sup>104</sup> and ↓TLR10 in PBMCs <sup>101</sup> associated with active disease and increased severity.	Murine CIA model: TLR4 and TLR7 deficient mice and therapeutic inhibition of TLR4 or the endosomal TLRs reduces disease severity. <sup>71,73,85,89,90</sup> hTLR8tg mice are more susceptible to CIA. <sup>93</sup> TLR9 activation by apoptotic cells is protective. <sup>96</sup> Rat CIA model: Intraarticular knockdown of TLR7 reduced disease. <sup>91</sup> Rat PIA model: MiRNA suppression of TLR3 reduces disease. <sup>60</sup> Prophylactic TLR9 inhibition reduces disease onset. <sup>99</sup> IL-1Ra-/- spontaneous arthritis model: TLR2-/- more severe disease. <sup>49</sup> Inhibition of TLR4 suppresses disease. <sup>73</sup> SCW model: TLR2-/- <sup>50</sup> and TLR9-/- <sup>99</sup> show reduced severity. K/BxN serum transfer model: TLR9-/- <sup>99</sup> no effect, TLR4-/- mice are protected. <sup>72</sup> Intraarticular injection of bacterial DNA induces arthritis in C57/BL6 mice. <sup>97</sup> AIA rat model: TLR4 antagonist reduces joint inflammation and bone damage. <sup>74</sup>
<b>Pro-inflammatory cytokine production</b>	Activation of TLR1/2 and TLR2/6. <sup>33,40,41</sup> TLR4 activated RA SF macrophages <sup>65</sup> and TLR5 activated monocytes induce elevated cytokines. <sup>40</sup> Inhibition of TLR2 <sup>45</sup> and TLR8 <sup>83–85</sup> reduces spontaneous cytokine release from RA synovial tissue.	TLR8 expression correlates with pro-inflammatory joint cytokines in hTLR8tg CIA. <sup>93</sup> Inhibition of TLR4 suppresses disease and reduces cytokine release from FLS in the AIA rat model. <sup>74</sup>
<b>Osteoclastogenesis</b>	TLR2, <sup>42</sup> TLR3, <sup>58</sup> TLR5, <sup>78</sup> TLR7 and TLR8 <sup>87</sup>	TLR4 (CIA model), <sup>70</sup> TLR5 (CIA model) <sup>78</sup> and TLR9 (rat PIA model) <sup>99</sup>
<b>Cell invasion/migration</b>	TLR2 <sup>44,45</sup> TLR5 <sup>78</sup>	

**Abbreviations:** AIA, adjuvant induced-arthritis; CIA, collagen-induced arthritis; DAS28, disease activity score 28; FLS, fibroblast-like synoviocytes; hTLR8tg, human TLR8 transgenic; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; MIV, methionine l valine; miRNA, microRNA; NK cells, natural killer cells; PBMCs, peripheral blood mononuclear cells; PIA, pristane-induced arthritis; RA, rheumatoid arthritis; SCW, streptococcal cell wall; SF, synovial fluid; TLR, toll-like receptor; ↑, upregulated; ↓, downregulated.

activated by dsRNA released from cells within the joint.<sup>51,52</sup> In culture, RA FLS release RNA under hypoxic conditions, as would be present in the pannus and correspondingly extracellular RNA has been detected within the RA synovial lining layer of patient samples.<sup>53</sup> Additionally, RNA released from necrotic synovial fluid cells has been shown to stimulate TLR3 on FLS in culture and significantly increased levels of dsRNA are present in the synovial fluid of RA patient with an erosive disease course.<sup>54,55</sup> Upon activation of TLR3, FLS induce IL-6, MMPs, B cell activating factor (BAFF) and VEGF that

support inflammation, cartilage damage, angiogenesis, B cell activation and can enhance Th1 and Th17 cell expansion.<sup>56,57</sup> In addition, TLR3 activation of monocytes induces osteoclast differentiation, which is further enhanced by TLR3 induced RANKL released from FLS.<sup>58</sup>

Elevated TLR3 expression has also been observed in the CIA model and in rat pristane induced arthritis (PIA), where treatment with methotrexate to suppress disease also prevented TLR3 induction.<sup>59</sup> Likewise, suppression of TLR3 with the microRNA mimic miRNA-26a ameliorates disease in the PIA model.<sup>60</sup> This increase in TLR3

expression may also be connected with T cell activation, as co-culture of pristane primed T cells or their conditioned media upregulated TLR3 on FLS.<sup>61</sup> This effect may in part be associated with IL-17, a pathogenic cytokine released by Th17 cells, which increases TLR3 expression in FLS in culture.<sup>62</sup>

## TLR4

Increased TLR4 is evident in synovial fluid cells of patients with early and longstanding RA, as well as in RA peripheral blood monocytes and CD8<sup>+</sup> T cells.<sup>35,51,63–66</sup> Furthermore, RA synovial fluid macrophages have an increased cytokine response upon TLR4 stimulation.<sup>65</sup> In particular, seropositive RA patients are reported to have higher levels of synovial TLR4, which positively correlates with synovitis.<sup>36</sup> Within the synovium, TLR4 may be upregulated in RA FLS due to overexpressed histone methyltransferase mixed-lineage leukemia 1, which in turn upregulates TLR4 expression.<sup>67</sup> However, miRNA regulation may also be important, for example, RA FLS have reduced expression of miR-506 which is suggested to limit TLR4 expression.<sup>68</sup>

Elevated TLR4 alongside the presence of a considerable number of TLR4 DAMPs such as HMGB-1 and ACPA immune complexes containing citrullinated fibrinogen within the serum and synovial fluid of RA patients, suggests TLR4 may play an active role in RA (Table 1).<sup>37,69</sup> Indeed, in murine CIA, disease development leads to the upregulation of multiple endogenous TLR4 ligands, which are associated with CIA pathogenesis and promote osteoclast differentiation.<sup>70</sup> Furthermore, in CIA and the K/BxN serum transfer model, TLR4 deficient mice are protected from joint destruction with reduced cell infiltration.<sup>71,72</sup> Additionally, the naturally occurring LPS from *Bartonella Quintana* that antagonizes TLR4, can also therapeutically suppress disease severity in both CIA and the spontaneous IL-1Ra<sup>-/-</sup> model.<sup>73</sup> Similarly, the TLR4 antagonist TAK-242 can suppress the expression of inflammatory cytokines from FLS and reduce local joint inflammation and bone damage in a complete Freund's adjuvant (CFA)-induced arthritis (AIA) rat model.<sup>74</sup> However, despite these encouraging results from experimental models, inhibition of TLR4 with a monoclonal antibody NI-0101 in RA patients produced no benefit in a recent clinical trial.<sup>75</sup>

## TLR5

Similar to TLR2 and TLR4, TLR5 recognizes HMGB-1 as its endogenous ligand (Table 1).<sup>76</sup> TLR5 was initially

associated with RA pathogenesis due to increased expression in RA synovial tissue lining and sublining macrophages and endothelial cells.<sup>77</sup> The expression of TLR5 on peripheral blood monocytes has since been correlated with DAS28, where this elevated expression is reduced in patients receiving anti-TNF treatment, suggesting a possible regulatory effect of TNF.<sup>77,78</sup> Further influence on expression may also come from miRNAs, as miR-3926 which limits TLR5 expression is down-regulated in FLS where TLR5 is accordingly upregulated.<sup>79</sup>

Functionally, a connection between TLR5 and RA pathogenesis may arise through its ability to promote angiogenesis and osteoclastogenesis. RA synovial fluid can induce endothelial cell migration and tube formation and also monocyte chemotaxis in a TLR5 dependent manner.<sup>78,80</sup> Activation of TLR5 on RA peripheral blood mononuclear cells (PBMCs) can also synergise with TNF to facilitate osteoclast precursor cell differentiation.<sup>78</sup> Furthermore, the activation of TLR5 on RA monocytes with flagellin can also induce higher levels of IL-6 and IL-10 than healthy donor monocytes irrespective of RF or ACPA status; however, this did not correlate with DAS28.<sup>40</sup>

## TLR7 and TLR8

TLR7 and TLR8 both recognise ssRNA and are expressed at higher levels in RA synovial tissue lining and sublining macrophages, synovial fluid macrophages and peripheral blood monocytes.<sup>36,81</sup> In particular, TLR8 expression is notably raised within the synovial tissue of seropositive RA patients.<sup>36</sup> However, when comparing mRNA levels within RA synovial tissue with OA samples, a strong trend towards increased TLR8 was observed but no difference was detected for TLR7.<sup>35</sup> Interestingly, RA patients carrying the M1V variant of TLR8 that induces lower cytokine levels upon TLR8 stimulation of monocytes exhibit a reduced disease severity.<sup>82</sup> In agreement with this, we have demonstrated that inhibition of endosomal TLRs and in particular inhibitors that target TLR8 can suppress spontaneous cytokine production from human RA synovial membrane cultures.<sup>83–85</sup> However, it is expression of TLR7 but not TLR8 in RA monocytes that is reported to be associated with DAS28 and TNF. Additionally, this study demonstrated that RNA present in RA synovial fluid could stimulate RA monocytes to produce TNF.<sup>81</sup> Although RNA released from cells is quite unstable, LL-37 which is upregulated within the RA synovium can protect it from degradation to enable activation of TLR7

and TLR8.<sup>86</sup> Furthermore, FLS from ACPA+ patients release extracellular vesicles containing miR-574-5p, which activates TLR7 and TLR8 to induce osteoclastogenesis.<sup>87</sup> Similarly, miR-let-7b can activate TLR7 on monocytes to induce TNF and IL-6 and promote differentiation to M1 macrophages when released in extracellular vesicles by synovial fluid macrophages.<sup>88</sup>

Experimental arthritis models have also indicated a pathogenic role for TLR7 and TLR8. We have demonstrated that inhibitors of the endosomal TLRs therapeutically suppress disease in the murine CIA model.<sup>85,89,90</sup> Moreover, mice deficient of TLR7, exhibit reduced disease severity in the CIA model following disease onset. This was associated with decreased IL-17 and elevated levels of Tregs, suggesting a role for TLR7 in regulating T cell responses.<sup>89</sup> In agreement with this data, intra-articular knockdown of TLR7 also improves disease activity in the rat CIA model.<sup>91</sup> In contrast, the investigation of TLR8 has proven more complicated, as unlike in human cells, murine TLR8 does not respond to stimulation with ssRNA.<sup>92</sup> However, transgenic mice expressing human TLR8 have been generated and found to be more susceptible to CIA with TLR8 expression correlated with pro-inflammatory cytokines within the joints.<sup>93</sup>

## TLR9

As a receptor for unmethylated CpG motifs within DNA, TLR9 also has the potential to be activated in RA, where patients have elevated levels of circulating immune complexes containing cell-free DNA compared to healthy controls.<sup>94</sup> In addition, TLR9 is upregulated in FLS, B-cells, monocytes and neutrophils of RA patients.<sup>33,56,95</sup> However, there are few studies of TLR9 in RA pathology, instead, most data have been generated in experimental arthritis models. Although an anti-inflammatory effect has been reported in the CIA model, where addition of apoptotic cells reduced the arthritis score in a DNA and TLR9 dependent manner, most studies indicate a pro-inflammatory role for TLR9.<sup>96</sup> Indeed, intra-articular injection of bacterial DNA containing CpG motifs in C57BL/6 mice induces arthritis.<sup>97</sup> Furthermore, co-activation of TLR9 and the B cell receptor with DNA containing immune complexes can stimulate RF autoreactive B cells.<sup>98</sup> In more recent studies, TLR9 was suggested to participate in the T cell-dependent phase of inflammatory arthritis models. In the rat PIA model, inhibition of TLR9 before the onset of disease reduced the severity of disease, serum IL-6, osteoclast formation and cartilage

degradation, whereas therapeutic inhibition had no effect. In addition, TLR9<sup>-/-</sup> mice demonstrated a reduction in the T cell-dependent phase of streptococcal cell wall-induced arthritis. Whereas TLR9 deficiency had no effect on the T cell-independent K/BxN serum transfer model.<sup>99</sup>

## TLR10

Currently, TLR10 is the least understood of the human TLRs. It has been suggested to form homodimers or heterodimers with TLR1, 2 or 6, permitting engagement with a diverse range of ligands including dsRNA and the TLR1/2 ligand Pam3Cys. Furthermore, depending on the type of dimer formed, TLR10 is suggested to be able to produce a pro-inflammatory or an inhibitory effect.<sup>100</sup> Similarly, mixed results have emerged for the role of TLR10 in RA. In line with TLR10 having an anti-inflammatory role, TLR10 mRNA is expressed at lower levels in PBMCs of RA patients with active disease, whilst a missense mutation (I473T) has been associated with increased disease severity and a lower response to the anti-TNF biological infliximab.<sup>101,102</sup> However, TLR10 is conversely upregulated in RA natural killer cells compared to healthy controls and in B cell subsets where a correlation with disease activity was observed.<sup>103,104</sup> Thus, the function of TLR10 in RA may be complex and cell type dependent.

## The NLRP3-Mediated Immune Response in Rheumatoid Arthritis

In addition to the induction of proinflammatory cytokines by TLRs in RA, the NLRP3 inflammasome is likely to have a key role in the processing and release of IL-1 $\beta$ . This was first demonstrated in the CIA model, where NLRP3 expression is increased within synovial tissue and correlates with disease severity.<sup>105</sup> Furthermore, when treated with the NLRP3 inhibitor MCC950, CIA mice exhibit a reduction in disease severity, synovial inflammation and cartilage erosion.<sup>106</sup> NLRP3 is also elevated in RA synovial tissue, as well as whole blood and CD4 T cells from RA patients with active disease.<sup>106-108</sup> In addition, active caspase-1 in CD4 T cells correlates with DAS28 and IL-17A in patient sera.<sup>108</sup>

The activation of NLRP3 in RA could be triggered by several different pathways. As discussed previously, TLR activation by DAMPs can induce NLRP3 and pro-IL-1 $\beta$  expression. However, several DAMPs are suggested to additionally activate NLRP3 assembly. Extracellular heat shock



protein 96 which is elevated in RA has a dual action activating TLR2 and NLRP3 in murine macrophages where 2 signals are required for IL-1 $\beta$  release.<sup>39,109</sup> Also, ACPA that can activate TLR4 when complexed with citrullinated fibrinogen can also indirectly stimulate NLRP3 induced IL-1 $\beta$  release in macrophages, due to activation of pannexin channels releasing ATP which then activates P2X7 receptors resulting in K<sup>+</sup> efflux.<sup>69,110</sup> Accordingly, higher levels of IL-1 $\beta$  are detected in the synovial tissue of ACPA+ compared to ACPA- patients and OA patients.<sup>110</sup> In addition, the uptake of colloidal calciprotein particles by RA monocytes at sites of bone erosion has been suggested to activate the NLRP3 inflammasome.<sup>111</sup>

Further enhancement of NLRP3 activity may arise in RA due to dysregulation of molecular regulators of inflammatory signaling. Mice deficient in the RA susceptibility gene A20, also known as tumour necrosis factor- $\alpha$  inducible protein 3 (TNFAIP3), develop a spontaneous erosive arthritis associated with enhanced NLRP3 expression and IL-1 $\beta$  secretion, similar to that observed in RA patients. Moreover, A20 deficient murine BMDM demonstrates hyperactivation of NLRP3 inflammasome, IL-1 $\beta$  release and pyroptosis, suggesting a negative regulatory role for A20 on NLRP3.<sup>112</sup> In addition, the PTPN22 R620W gain-of-function variant associated with RA susceptibility, has also been shown to regulate NLRP3 dephosphorylation and subsequent activation.<sup>113</sup>

Interestingly, the vitamin D receptor has also been suggested to negatively regulate NLRP3 inflammasome assembly through suppressing BRCC3-mediated deubiquitination of NLRP3, which corresponds with the finding that RA patients frequently have low Vitamin D levels that correlate with disease activity.<sup>114,115</sup> More recently, we demonstrated in RA monocytes that a reduced expression of SARM, which negatively regulates NLRP3, was associated with elevated TLR1/2-induced IL-1 $\beta$  and DAS28. Furthermore, RA patients responsive to anti-TNF therapy then displayed a transient increase in the expression of SARM in their monocytes, which was not observed in non-responders.<sup>116</sup>

Further compounding effects on NLRP3 activation have been suggested in the presence of key RA cytokines. TNF can prime cells such as FLS to upregulate NLRP3 and pro-IL-1 $\beta$ . However, FLS require an additional signal to induce inflammasome activation but this can be achieved by extracellular calreticulin which is elevated in RA joint and serum where it correlates with disease activity leading to an increase in IL-1 $\beta$  release.<sup>117–119</sup> In

addition, IL-6 can enhance monocyte NLRP3 overactivation and pyroptosis, induced by pentraxin-3 (PTX3) and C1q which are elevated in RA serum.<sup>120</sup> Furthermore, inhibition of IL-6 in the CIA model reduces NLRP3 activation and IL-1 $\beta$  release.<sup>121</sup>

## Conclusion

Although there is a wealth of information supporting a contribution from both TLRs and the NLRP3 inflammasome in RA pathophysiology, there are still significant gaps in our understanding. Despite two decades of research, therapeutic interventions targeting these pathways have yet to be successfully translated into the clinic. Mechanistic insights have been forthcoming from experimental arthritis models; however, these do not always translate to the human disease, as can be seen with the recent clinical trial of NI-0101 to inhibit TLR4.<sup>75</sup> In recent years, numerous inhibitors targeting TLR activation have entered clinical trials for other inflammatory conditions or Phase I safety trials in healthy volunteers, but other than NI-0101, none have yet entered clinical trials for RA.<sup>122</sup> However, several trials have commenced with inhibitors that target IRAK4 or Bruton's tyrosine kinase (Btk) which lie downstream of many TLRs. A Phase II trial with Fenebrutinib (GDC-0853) a Btk inhibitor showed a moderate improvement in disease activity compared to placebo and a phase IIb trial of PF-06650833 an IRAK4 inhibitor, produced a significant clinical improvement in moderate and severe RA patients compared to placebo control.<sup>123,124</sup> For the TLRs, it will now be important to determine which receptors are pivotal in the disease process rather than simply dysregulated as a downstream consequence of the inflammatory environment. With so many TLRs potentially contributing to RA, it will also be important to gain a better understanding of how their expression and function is affected by LncRNA, miRNAs and shared downstream signaling regulators. This may provide insights into novel ways to limit inflammation. Several small molecular weight drugs have already been developed to inhibit the NLRP3 inflammasome. A phase II clinical trial for RA using CP-456,773 (later renamed MCC950) was discontinued due to liver toxicity; however, several new inhibitors that target NLRP3 activation are in development either at the preclinical stage, in early clinical trials in healthy volunteers or trials for other inflammatory conditions.<sup>125,126</sup> Although inhibition of IL-1 is not as effective as suppressing other cytokines such as TNF and

IL-6 in RA, NLRP3 inhibitors may still have a place alongside these biological therapies. Indeed, with the potential for many different pathways driving inflammation in parallel within the joint, blocking a single pathway may not be sufficient.

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