

Review

Toll-like receptors and NOD-like receptors in rheumatic diseasesWilliam J McCormack¹, Andrew E Parker¹ and Luke A O'Neill²¹OPSONA Therapeutics Ltd, Institute of Molecular Medicine, Trinity Centre for Health Sciences, St James' Hospital, Dublin 8, Ireland²School of Biochemistry & Immunology, Trinity College Dublin, College Green, Dublin 2, IrelandCorresponding author: Luke A O'Neill, laoneill@tcd.ie

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Arthritis Research & Therapy 2009, **11**:243 (doi:10.1186/ar2729)**Abstract**

The past 10 years have seen the description of families of receptors that drive proinflammatory cytokine production in infection and tissue injury. Two major classes have been examined in the context of inflammatory joint disease – the Toll-like receptors (TLRs) and NOD-like receptors (NLRs). TLRs such as TLR2 and TLR4 are being implicated in the pathology of rheumatoid arthritis, ankylosing spondylitis, Lyme arthritis and osteoarthritis. Nalp3 has been identified as a key NLR for IL-1 β production and has been shown to have a particular role in gout. These findings present new therapeutic opportunities, possibly allowing for the replacement of biologics with small molecule inhibitors.

In the present review we will summarise the current state of knowledge in TLRs and NLRs, and also speculate on their roles in the pathogenesis of autoinflammatory joint diseases.

Toll-like receptors

The past 10 years have seen over 11,000 papers published on TLRs, which is a testament to the importance placed upon them by inflammation biologists and immunologists. Ten TLRs occur in humans, and the roles of nine of them (TLR1 to TLR9) have been determined [1].

Introduction

Proinflammatory cytokines such as TNF, IL-6 and IL-1 have proven to be excellent therapeutic targets for diseases such as rheumatoid arthritis (RA). More recently, however, attention has focused on the mechanisms whereby these cytokines are induced. In this regard there has been remarkable progress in the elucidation of receptors that drive their production as well as other inflammatory mediators. This progress has led to a renaissance of interest in innate immunity among immunologists, since these receptors also sense microbial products to drive host defense.

TLR2 senses lipopeptides from bacteria, with TLR1/2 dimers sensing triacylated lipopeptides and TLR2/6 dimers sensing diacylated lipopeptides. In addition, TLR2 also senses zymosan from fungi. The structure of the TLR1/2 dimer has been solved [2], as has the structure of TLR4 in complex with its ligand lipopolysaccharide from Gram-negative bacteria that are presented to TLR4 by MD2 [3]. TLR4 can also sense F protein from respiratory syncytial virus and glycerophosphatidylinositol anchors from parasites [4,5]. This provides a receptor repertoire to respond to all pathogens that infect humans.

Two particular classes – the Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which are pattern recognition receptors (PRRs) – have been most extensively studied. Certain TLRs (for example, TLR2, TLR4 and TLR9) and certain NLRs (for example, Nalp3) have been implicated in various inflammatory arthropathies. More recently evidence has been presented that these TLRs and NLRs might also be activated by noninfectious endogenous signals, making them even more attractive as important drivers of cytokines in diseases with no obvious infection.

The signaling pathways activated by TLRs have also been worked out in great detail and involve the selective recruitment of adapter proteins (MyD88, Mal, Trif and Tram) [6]. These lead to activation of NF- κ B, which is a major response to TLRs. Certain TLRs (TLR4 and nucleic acid-sensing TLRs) can also engage with a pathway leading to the activation of the transcription factor interferon regulatory factor-3. Both NF- κ B and interferon regulatory factor-3 are required for the induction of a wide range of cytokines.

NOD-like receptors

NLRs are intracellular sensors of pathogen-associated or endogenous danger-associated molecular patterns. The NLR

AIM2 = absent in melanoma-2; CpG = cytosine phosphate guanine; dsDNA = double-stranded DNA; HMGB1 = high-mobility group box protein 1; IFN = interferon; IL = interleukin; NALP = Nucleotide-binding domain-containing, leucine-rich repeat-containing, and pyrin domain-containing protein; NF = nuclear factor; NLR = nucleotide-binding domain-like and leucine-rich repeat receptors; NOD = nucleotide-binding oligomerisation domains; OA = osteoarthritis; PRR = pattern recognition receptor; RA = rheumatoid arthritis; RAGE = receptor for advanced glycation end products; shRNA = short hairpin RNA; SLE = systemic lupus erythematosus; snRNP = small nuclear ribonucleoproteins; TLR = Toll-like receptor; TNF = tumor necrosis factor.

family consists of 22 cytoplasmic proteins including the NOD and NALP subfamilies, with the 14 NALPs representing the largest subfamily. NLR family members share common structural features, including a nucleotide binding domain (nucleotide binding site or NACHT domain) central to the molecule, flanked by a leucine rich-repeat domain at the C-terminus and a caspase-recruitment domain and a pyrin domain at the N-terminus.

The best characterised NLR is NALP3, which when activated forms a large oligomer able to interact with intermediate proteins ASC and Cardinal, creating a complex able to recruit procaspase-1. Through an autocatalytic process, procaspase-1 is then activated – resulting in a multimeric structure termed the inflammasome, which is able to induce maturation and secretion of proinflammatory cytokines IL-1 β and IL-18 [7].

Gain of function mutations in the NALP3 gene leading to elevated levels of processed IL-1 β cause hereditary periodic fever syndromes in humans, including Muckle-Wells syndrome, chronic infantile cutaneous neurologic articular syndrome and familial cold-induced autoinflammatory syndrome [8]. Fever, joint pain and systemic inflammation are common features of these disorders and provided the first clue that the inflammasome has a potential role in rheumatic diseases [9]. The effectiveness of IL-1 β blockade (Anakinra) in treating inherited periodic fever syndromes has transformed the understanding and management of these disorders and has implications for future therapies in rheumatic diseases.

Important links and synergies are evident between TLRs and NLRs. TLRs are required to induce pro-IL1 β , and the Nalps then activate caspase-1 to process it, so both act in concert for IL-1 production [10]. Another important aspect is the link between these receptors and adaptive immunity. Nalp3 has been shown to be a target for the adjuvant Alum, although whether it is required for antibody production is less clear. TLRs, however, are important for inducing the T-cell costimulatory molecules CD80 and CD86. This is particularly the case with TLR4, which achieves this via induction of IFN β [11]. B cells and T cells have also been shown to express certain TLRs – TLR9 has been shown to induce B-cell proliferation [12], whilst TLR2 has been shown to be present on regulatory T cells and to activate them [13]. These kinds of studies highlight the role of innate immunity in the adaptive response, and the two responses are increasingly seen as inter-linked.

Rheumatoid arthritis

There has been a longstanding hypothesis that infection plays a role in the initiation of RA (Figure 1). Molecules of microbial origin have been found in the joints of patients with RA [14,15], where they can trigger inflammatory reactions through PRRs. These inflammatory reactions damage the host tissue, releasing molecules (danger signals) that can activate the PRRs resulting in vicious cycles of inflammation.

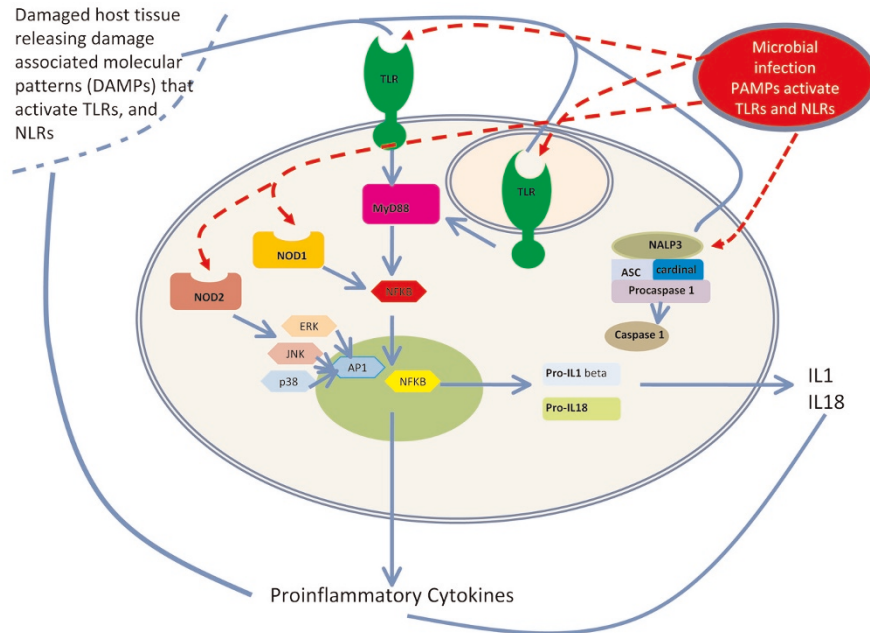
This sterile inflammation induced by endogenous danger signals released from the inflamed host tissue is thought to lead to the pathological joint destruction associated with RA. There is increasing evidence that TLRs, and more recently NLRs, have a role in RA pathology.

Ospelt and colleagues comparatively analysed the expression of TLRs in synovial tissues during the early and late stages of RA, and found that TLR3 and TLR4 were elevated in both early and late RA samples compared with samples from osteoarthritis (OA) synovium [16]. These results concur with studies from Brentano and colleagues, who also detected elevated levels of TLR3 expression in RA synovial fibroblasts over OA synovial fibroblasts [17]. Similarly, elevated levels of TLR7 have also been detected in synovium from RA patients compared with OA patients or healthy volunteers [18]. In addition to synovial fibroblasts, differences in TLR expression/activity have also been detected in macrophages isolated from synovium of RA patients. Huang and colleagues discovered elevated levels of TLR2 and TLR4 activity in macrophages isolated from RA synovium compared with control synovium [19]. Spontaneous production of proinflammatory cytokines and matrix metalloproteinases from RA synovial membrane cultures has been shown to be inhibited by overexpressing dominant negative constructs of Mal and MyD88, essential adaptor molecules for TLR2 and TLR4 signaling [20].

A later study investigating the use of a novel TLR4 antagonist has shown the most convincing evidence for TLR involvement in RA, as shown in Figure 2 [21]. In this study, two mouse models of RA were used to test a TLR4 antagonist for efficacy. An IL1-receptor antagonist knockout model, where the mice develop arthritis spontaneously, was run alongside a collagen-induced arthritis model that requires the use of an adjuvant containing TLR ligands. In both models the TLR4 antagonist showed impressive therapeutic effects. Another study by the same group crossed TLR2, TLR4 and TLR9 knockout mice with the IL1-receptor antagonist knockout mice that spontaneously develop arthritis [22]. Agreeing with the results from their TLR4 antagonist study, Abdollahi-Roodsaz and colleagues found that IL1 $\text{rn}^{-/-}$ TLR4 $^{-/-}$ animals are protected against arthritis whereas IL1 $\text{rn}^{-/-}$ TLR2 $^{-/-}$ animals develop a more severe arthritis – suggesting an anti-inflammatory role for TLR2 in this model. A lack of TLR9 did not affect the progression of arthritis. The anti-inflammatory nature of TLR2 in the IL1-receptor antagonist knockout models is in contrast to results obtained in a streptococcal cell wall induced model of arthritis, where mice deficient for TLR2 were shown to have a reduced severity of arthritis [23]. TLR4 has been shown to be involved in the chronic erosive stage of arthritis in this model of disease [24].

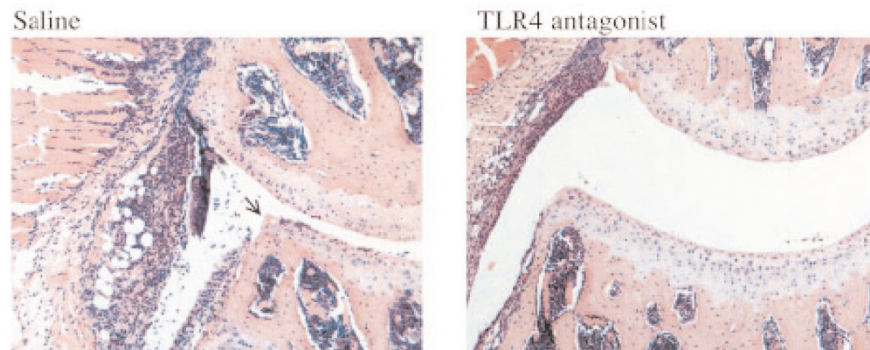
As already mentioned, the role of TLRs in RA is believed to be driven by inflammation in response to danger signals (endogenous host cell molecules released from stressed

Figure 1



Signaling through pathogen-associated and damage-associated molecular patterns drives chronic inflammation in diseases like rheumatoid arthritis. Bacterial DNA, peptidoglycans, muramyl dipeptide and viral molecules have been found in arthritic joints. These microbial pathogen-associated molecular patterns (PAMPs) can drive inflammation through the membrane-bound (Toll-like receptor (TLR)) and cytosolic (NOD-like receptor (NLR)) pattern recognition receptors (PRRs). The resulting release in inflammatory cytokines can drive the damage of host tissue releasing damage-associated molecular patterns (DAMPs), such as high-mobility group box protein 1, GP96, heat shock proteins and ATP, which also activate both types of PRR resulting in a vicious cycle of inflammation.

Figure 2



Treating spontaneous arthritis with a TLR4 antagonist suppresses the clinical and histological characteristics of arthritis. Abdollahi-Roodsaz and colleagues have recently shown that treating collagen-induced arthritis (left-hand side) with a TLR4 antagonist suppresses the clinical and histological characteristics of arthritis (right-hand side). Histological images of knee joints are shown, stained with hematoxylin and eosin. Arrow indicates inflammatory cell influx and chondrocyte cell death. Image taken from [21]. Reproduced with permission of John Wiley and Sons.

cells) as well as TLR ligands of microbial origin. Similar to the microbial TLR ligands, endogenous TLR ligands have been found in the joints or serum of RA patients and their levels have been correlated with disease activity scores [25]. These ligands – including heat shock proteins, fibronectin, high-mobility group box chromosomal protein-1 (HMGB1) and

breakdown products of heparan sulfate and hyaluronic acid – activate TLR2, TLR4, or both. The most recent addition to the growing list of endogenous TLR ligands is GP96 [26]. GP96 is a heat shock glycoprotein detected at high levels in RA synovial tissues that is capable of activating TLRs. Like HMGB1, this endogenous ligand has been shown to drive

inflammation by signaling through both TLR2 and TLR4. Considering the extensive evidence linking TLR signaling and RA pathology, it is surprising that no TLR polymorphisms have been identified involved in the susceptibility and severity of RA [16,27,28].

While TLRs appear to be the principal PRRs implicated in RA pathology, evidence is emerging that NLRs may also have a role in RA. NOD1 and NOD2 have been shown to be expressed in RA synovial tissue samples, and the microbial ligand for NOD2, muramyl dipeptide, has been detected in RA synovium [29,30]. Using NOD1 and NOD2 knockout mice, Joosten and colleagues have shown a proinflammatory role for NOD2 and an anti-inflammatory role for NOD1 in a streptococcal cell wall induced model of arthritis [30].

Lyme arthritis and TLR2

Lyme arthritis is caused by infection with the tick-borne spirochete *Borrelia burgdorferi*. A subacute inflammatory arthritis develops in 60% of individuals not treated at the time of the tick bite, and is associated with invasion of the joint tissue by spirochetes. Immune responses of the host toward *B. burgdorferi* are predominantly mediated by the recognition of proteins modified with tripalmitoyl-S-glyceryl-cysteine by TLR2 [31]. TLR2 knockout mice have been shown to be hyporesponsive to vaccination with lipopeptides, and hyporesponsiveness in humans is linked with low levels of TLR1 expression [32]. In contrast to the studies in the TLR2 knockout mice, a polymorphism resulting in a nonfunctional TLR2 receptor (Arg753Gln) *in vitro* has been shown to be protective from the clinical symptoms of late-stage infection with *B. burgdorferi* [33].

Systemic lupus erythematosus, Toll-like receptors and the AIM2 inflammasome

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease, the cause of which has not yet been fully elucidated. Immune complexes of autoantibodies to chromatin and RNA protein particles (snRNP) are characteristic of SLE and play an important role in the pathogenesis of the disease. Increased levels of serum IFN α have been found in many patients with SLE, and these levels correlate with disease severity and disease markers such as the DNA autoantibodies. Evidence for the crucial role of type 1 interferon in the pathology of lupus comes indirectly from findings that patients with nonautoimmune disorders treated with recombinant IFN α produce autoantibodies to DNA and develop clinical syndromes that resemble SLE [34,35].

There is good evidence that TLRs are involved in SLE. TLR9-expressing B cells are expanded in SLE patients with active disease, and this is correlated with levels of autoantibodies against DNA [36]. Activation of endosomal TLRs is believed to drive the elevated levels of IFN α that promote and maintain SLE disease progression. Nephritis is a condition associated with SLE, and in a murine model of the disease (MRL^{lpr/lpr})

immunisation with unmethylated CpG, an exogenous TLR9 ligand, aggravates the condition [37]. This is consistent with observed association of lupus flares with viral infection. Using TLR7 and TLR9 oligonucleotide-based inhibitors, mammalian DNA and RNA in the form of immune complexes from SLE patient serum have been shown to act as endogenous ligands for TLR7 and TLR9, respectively [38]. In lupus-prone (NZB x NZW)F1 mice that spontaneously develop symptoms similar to human lupus, administration of a TLR7/TLR9 dual oligonucleotide inhibitor showed efficacy at suppressing the production of autoantibodies, reducing kidney damage and increasing survival of treated mice [39]. In the MRL^{lpr/lpr} lupus model, mice deficient for MyD88 failed to produce DNA autoantibodies [40]. In the same lupus animal model, TLR7 deficiency has shown reduced autoimmune disease as expected, while TLR9 deficiency resulted in exacerbated autoimmune disease [41].

The pathogenic rather than protective effect observed in the TLR9 knockout in the MRL^{lpr/lpr} lupus mouse model does not correlate with the earlier *in vitro* studies linking TLR9 activation to disease progression. It has been suggested that human–mouse differences in the expression, distribution and functional response of TLR7 and TLR9, as well as drawbacks in the animal model used, may explain the pathogenic effect observed in the TLR9 knockout MRL^{lpr/lpr} mouse model [42]. Three studies have failed to correlate a certain set of polymorphisms in TLR9 with SLE [43-45]; however, a Japanese group recently identified two alleles that downregulated TLR9 expression in a reporter assay but are associated with increased SLE susceptibility [46]. This linkage would indicate that the TLR9 knockout data from the MRL^{lpr/lpr} mice may be correct and that TLR9 has an anti-inflammatory function in SLE.

It remains to be seen whether endosomal TLR agonist or antagonists will be beneficial for the treatment of SLE; however, endosomal TLR signaling certainly appears to be involved in SLE pathology. Interestingly a polymorphism in Mal, the signaling adaptor used by TLR2 and TLR4, has been shown to be protective against SLE [47]. This polymorphism attenuates Mal signal transduction, which would diminish signaling through TLR2 and TLR4 [48]. Interestingly, HMGB1-containing DNA immune complexes that have been shown to bind RAGE on plasmacytoid dendritic cells and B cells [49] have recently been shown to induce proinflammatory cytokine production in macrophages in a TLR2-dependent manner [50]. These results indicate that there may be a more complex interplay between cell surface TLRs, their adaptors and endosomal TLRs in the pathology of SLE.

A cytoplasmic DNA-sensing inflammasome has been described more recently that is NALP3 independent. Absent in melanoma-2 (AIM2) is an interferon-inducible HIN200 family member that binds DNA through the HIN domain and has a pyrin domain that interacts with ASC to activate NF- κ B

and caspase-1. Knockdown of AIM2 using shRNA blocks recognition of cytoplasmic dsDNA in human macrophages [51-53]. SLE is characterised by elevated levels of interferon and by the presence of DNA:antibody complexes. In addition, genetic mapping studies have identified a susceptibility locus for SLE that contains the AIM2 gene, raising the possibility that AIM2 has a role to play in the pathology of SLE. Further studies are required to fully elucidate any link between AIM2 and SLE. The identification of AIM2 may in addition help to explain the results observed by Kawane and colleagues, who observed a TLR-independent polyarthritic phenotype in mice deficient for DNase1 and IFN1 as a consequence of the inability of macrophages to efficiently degrade cytosolic DNA [54].

Ankylosing spondylitis, TLR2 and TLR4

Ankylosing spondylitis is a multifactorial and polygenic inflammatory rheumatic disease with a poorly understood pathophysiology. Apart from HLA, other genes are likely to play a role in disease susceptibility and indigenous bacteria also appear to be involved in the pathology. This suggests that both adaptive and innate immune responses are required for disease progression. Expression studies looking at the CD4⁺CD28^{null} T-cell populations from ankylosing spondylitis patients have shown that TLR2 and TLR4 levels are increased and that this effect can be reduced by therapeutic blockade of TNF α [55]. Polymorphisms in TLR4 have been described and there are several studies that have looked at the association between these polymorphisms and susceptibility to ankylosing spondylitis. There is good evidence for a link between both Asp299Gly and Thr399Ile polymorphisms and ankylosing spondylitis [56], but no link with the Asp896Gly polymorphism [57]. The functional consequences of these polymorphisms and the mechanistic link to ankylosing spondylitis remain to be established. The S180L polymorphism in TIRAP/Mal that has been shown to be protective against SLE [47] has no association with axial spondyloarthritis [58].

Psoriatic arthritis

Psoriatic arthritis is an inflammatory arthritis associated with psoriasis in which the CD8⁺ T cell plays a pivotal role. The data on TLRs in psoriatic arthritis are restricted to a few studies of expression levels of TLR2 and TLR4. Candia and colleagues have shown that TLR2 expression was increased in immature dendritic cells from patients with psoriatic arthritis, although mature dendritic cells did not show statistically significant differences [59]. No effect was seen on TLR4 expression. Conversely, Raffener and colleagues looked at CD4⁺CD28^{null} T cells and showed an increase in surface levels of TLR4 but no effects on TLR2 [55]. Further detailed analysis of TLRs in psoriatic arthritis is required to better understand whether there is a role in the pathogenesis of the disease.

Gout, pseudogout, TLR2 and Nalp3

Gout and pseudogout are crystal-induced arthropathies, gout being the most common autoinflammatory arthritis with

increasing incidence over the past decade [60]. Gout is characterised by elevated serum urate and recurrent attacks of intra-articular crystal deposition of monosodium urate, whereas pseudogout is associated with calcium pyrophosphate dihydrate crystals and has a poorly understood pathophysiology.

Uric acid crystals stimulate dendritic cell maturation, enhance antigen-specific immune responses and directly activate T cells leading to elevated levels of CD70 [61]. The role of the innate immune system in gout has now been firmly established with the realisation that the uptake of monosodium urate crystals by monocytes involves interactions with TLR2 and CD14 [62] and that intracellularly monosodium urate crystal-induced inflammation is mediated by the NALP3 inflammasome [63]. The role of the NALP3 inflammasome was confirmed in a monosodium-urate-induced peritonitis mouse model that mimics an acute gout attack. Intra-peritoneal injection of monosodium urate induces recruitment of neutrophils, and this effect was abrogated when either Anakinra or an anti-IL-1R antibody was co-administered with monosodium urate [63]. This monosodium-urate-induced mouse gout model clearly establishes the role of IL-1 in gout and led to an open-label study of Anakinra in 10 patients with gout that could not tolerate or had failed standard anti-inflammatory therapies. All patients received Anakinra daily for 3 days and all showed rapid positive responses with no adverse effects observed [64]. In addition, there is one report of Anakinra delivering a positive effect in a steroid-resistant pseudogout patient [65].

Osteoarthritis and Toll-like receptors

Synovial inflammation is increasingly recognised as an important pathophysiological process in OA, and endogenous ligands released as a consequence of synovial and cartilage catabolism (for example, fibronectin and hyaluronan fragments) are likely to be recognised by PRRs [66].

Histology and expression studies using isolated chondrocytes and cartilage have shown that human articular chondrocytes predominantly express TLR1, TLR2, TLR3, TLR4 and TLR5 [67-69]. Expression of TLR2 and TLR4 is elevated in OA particularly at sites of lesions in cartilage [67,69]. Treatment of isolated cells with inflammatory cytokines and fibronectin proteolytic fragments results in increased expression of TLR2, and culture in the presence of TLR1/2 or TLR2/6 ligands but not TLR3 ligands results in elevated levels of matrix metalloproteinases and significantly increased collagenolysis and aggrecanolysis [67,69].

OA is also associated with crystal deposition in synovial fluid – in particular, calcium pyrophosphate dihydrate and basic calcium phosphate [70], as well as hydroxyapatite [71] and silicon dioxide [72]. The physiological relevance of crystals to disease pathology is keenly debated but it seems probable that recognition of these crystals by the inflammasome will contribute to local inflammation in the joint [73].



The Scientific Basis of Rheumatology: A Decade of Progress

This article is part of a special collection of reviews, *The Scientific Basis of Rheumatology: A Decade of Progress*, published to mark *Arthritis Research & Therapy's* 10th anniversary.

Other articles in this series can be found at: <http://arthritis-research.com/sbr>

Conclusions and future therapeutics opportunities

The roles of TLRs and Nalp3 in arthropathies are becoming clearer and they remain exciting therapeutic options. One interesting example is in aseptic loosening that occurs in 10% of joint replacements, resulting in revision surgery. Evidence is emerging to suggest that aseptic loosening of total joint replacements is driven through implant debris activation of the inflammasome leading to locally elevated levels of inflammatory cytokines [74]. More obviously Nalp3, TLR2 and TLR4 are attractive targets for RA and OA, whilst TLR7 and/or TLR9 and AIM2 represent therapeutic potentials for joint inflammation in SLE.

There has been considerable focus on identification of small molecule agonists and antagonists of TLRs over the past 5 years, with several successful examples now undergoing clinical evaluation. If the preclinical observations described in the present review [21,39] translate to the clinic, then inhibition of TLRs and NLRs using small molecules may provide viable replacements for current biologic agents. In any event, the hope is that these new insights into innate immunity will ultimately translate into better therapies for inflammatory arthropathies that continue to represent a major burden on humanity.

Competing interests

WJM and AEP are employees of Opsona Therapeutics, a drug discovery and development company focused on the role of TLRs and inflammasome signaling in human immunology. LAO'N is a founder of Opsona therapeutics and is a member of its scientific advisory board.

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