The potential importance of Toll-like receptors in ankylosing spondylitis

Cells involved in innate immunity scan for pathogens via extracellular and intracellular (endosomal) pattern recognition receptors (PRRs). Engagement of PRRs by a specific ligand results in downstream activation of intracellular inflammatory cascades. There is emerging evidence indicating that one class of PRR, the Toll-like receptor (TLR) plays a potential role in the pathogenesis of spondyloarthropathies. Since certain Gram-negative bacteria are known to act as triggers for reactive arthritis, there has been much interest in studying the role of TLRs in spondyloarthropathies. In this article, we introduce the immunology of TLRs followed by a discussion of their potential role in ankylosing spondylitis.

KEYWORDS: ankylosing spondylitis ▪ pathogen recognition receptors ▪ spondyloarthritis ▪ Toll-like receptors

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Ankylosing spondylitis (AS) belongs to a class of spondyloarthridities (SpA) that includes: psoriatic arthritis (PsA), reactive arthritis (ReA), undifferentiated spondyloarthritis and enteric spondyloarthritis or arthritis associated with inflammatory bowel disease (IBD). Among the SpAs, AS shows a particularly strong genetic association with *HLA-B27*. However, *HLA-B27* itself, only accounts for ~45% of the overall genetic risk for disease [1]. *HLA-B27* subtypes vary between populations, but virtually all subtypes demonstrate association with AS [2]. Other susceptibility genes recently identified include *IL23R*, *ERAP1* and *KIR* polymorphisms [2], and more recently dysregulation of *NR4A2*, *TNFAIP3* and *CD69* have been reported [3].

In ReA, inflammation is triggered after certain bacterial infections of the gut and genitourinary tract in genetically susceptible individuals [4]. Approximately 20% of ReA patients eventually evolve into AS [5]; 80% of patients with ReA express HLA-B27 [6]. A recent study by Ge *et al.* has shown an increased replication and survival of *Salmonella* in HLA-B27-transfected macrophages by upregulation of certain key *Salmonella* genes [7]. This may be an indication towards inability of clearance or persistent low-grade infection, which predisposes the HLA-B27 population to develop ReA and or AS.

Initial response to infection involves activation of the innate immune system. Cells involved in innate immunity such as dendritic cells, macrophages and neutrophils recognize potential pathogens via pattern recognition receptors (PRRs). This large family of receptors includes Toll-like receptors (TLRs), RIG-I-like receptors and Nod-like receptors [8]. TLRs are found both on the cell surface, in other words extracellular as well as on intracellular membranes, whereas RIG-I-like receptors and Nod-like receptors are both exclusively intracellular molecules.

TLRs

TLRs are type I transmembrane proteins that contain a large, leucine-rich repeat in an extracellular region and Toll/IL-1 receptor domain in a cytoplasmic region [9]. Ten members from the family of TLRs have been recognized in humans. TLR1, 2, 4, 5 and 6 are expressed on cell surface membranes while TLR3, 7, 8 and 9 are present on intracellular endosomal membranes. They recognize specific pathogen-associated molecular patterns or endogenous antigens termed danger-associated molecular patterns. TLRs are constitutively expressed in cells that interface with the environment, such as epithelial cells in the skin, ocular surface cells, airway epithelium and GI tract [10,11]. Furthermore, as TLRs are expressed on both adaptive immune cells and those that interface with the environment they bridge both sides of the immune system.

While TLRs protect against infection, inappropriate activation of TLR pathways can also lead to chronic inflammation and autoimmunity. Endosomal TLRs in particular have been implicated in autoimmune diseases such as systemic lupus erythematosus. It has been shown that activation of plasmacytoid

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dendritic cells occurs after internalization of nucleic acid immune complexes via engagement of TLR7 and 9 [12]. The activated plasmacytoid dendritic cells secrete type I IFN and other mediators that promote survival of autoreactive B cells [12].

TLRs recognize various ligands. For instance, TLR4 recognizes lipopolysachharide (LPS) of Gram-negative bacteria via MD2, a ligand binding TLR4 coreceptor. The interaction of LPS with TLR4/MD2 is facilitated by CD14, a glycosylphosphatidylinositol-anchored protein that is expressed on the cell surface. TLR4 also recognizes and binds certain endogenous ligands (e.g., fibrin, heat shock proteins, heparin sulfate and hyaluronic acid), some of which are breakdown products from injured cells [13]. MD2 is critical for TLR4 oligomerization and resultant downstream signaling through either MyD88-dependent or TRIF-dependent pathways [14]. The ligand of TLR5 is flagellin found in Gram-negative bacteria. TLR5 signals mainly via MyD88 pathways. Heterodimers of TLR1 and TLR2 recognize triacylate lipoproteins, while heterodimers of TLR1 and 6 recognize diacylated lipoproteins. This allows for recognition of components from Gram-negative bacteria, Gram-positive bacteria and mycoplasma as well as other pathogens [9,13,15].

In the case of AS, a direct link between an infectious pathogen and AS has not been definitively proven. Moreover, it not clear if the putative infectious triggers(s) are exogenous pathogens or commensal organisms. Pöllänen *et al.* have hypothesized that microbial pathogen-associated molecular patterns from urogenital and or GI tract (chronic prostatitis, pelvic inflammatory disease or asymptomatic bacterial biofilms) may stimulate TLR and cause chronic inflammation which ensues in the form of enthesitis or arthritis in AS and ReA [16]. These hypotheses have yet to be experimentally verified.

To the extent that TLRs play a primary role in pathogenesis of AS is unclear. This has however sparked studies focusing on single nucleotide polymorphisms (SNPs) especially of the TLR4 receptor as a possible genetic marker. Cantaert *et al.* hypothesized that a genetic variant of TLR2/4 adaptor protein TIRAP (SNP C539T), which encodes for myelin and lymphocyte protein, may be under-represented and may have an exaggerated innate response to pathogens in SpA [17]. This polymorphism has been implicated in providing some pathogenic protection from severe infections in certain African and UK populations and the allele results in an attenuated response to bacterial pathogens [17]. The underlying correlation between susceptibility to infection and axial SpA was therefore explored. They studied 204 patients with AS however, they did not find a functional association between this SNP and axial SpA [17].

Two SNPs in the *TLR4* gene (Asp299GIy and Thr399IIe) can alter the extracellular domain of this receptor. These mutations have been implicated with an exaggerated response to infection and in IBD. Snelgrove *et al.* have shown a modest association between these variants and AS [18]. However, these findings were not replicated in other studies and no association was found between these alleles and in AS [19–21]. Similarly, altered TLR2 receptor signaling and effector mechanisms have been postulated to be involved in developing ReA [4]. *TLR2* variants especially 753Q variant (A allele), located in the Toll/IL-1 receptor binding domain was shown by Tsui *et al.* to be associated with acute ReA after *Salmonella* outbreak [4]. They also reported the 631H variant to be associated with articular symptoms in infected males [4]. They proposed that this gender preference association may be implicated in higher prevalence of ReA in males than females, however, this needs to be validated with further studies.

Ultimately, although an attractive candidate gene from the mechanistic perspective, to what extent these SNPs in TLR4 and TLR2 are genetic risk factors in AS still remains to be proven. In fact, genetic association studies have not revealed any TLR SNPs to be associated with AS at the genome-wide significance level [22].

Potential mechanistic roles of TLRs in SpA

More than two-thirds of SpA patients display microscopic signs of gut inflammation and 6–13% of these eventually develop IBD [5]. Thus, it has been hypothesized that alterations in gut flora may lead to sensitization of the immune system, which then results in development of arthritis and gut inflammation.

HLA-B27 transgenic rats housed in germ-free state conditions do not develop gut and peripheral joint inflammation [23]. Taurog *et al.* showed that these *B27* transgenic rats are disease free if they are raised in a germ-free environment (genital and skin lesions are

unaffected by the germ-free state), however, introduction of gut flora caused development of gut and joint inflammation supporting a role of commensal flora in sensitizing the immune system and subsequent development of inflammation [23]. In an IBD cohort studied by Turkcapar *et al.*, a group of 162 patients with IBD were analyzed. They found that SpA and AS were present in 45.7 and 9.9% of patients, respectively. Among the SpA subjects, 63% were HLA-B27 and in the AS subjects, 100% were HLA-B27 [24]. This data suggest that there may be some overlap in the pathogenesis of IBD and SpAs [25,26].

In IBD, there are data suggesting that TLR dysregulation may be involved. Heuschen *et al.* examined patients with ulcerative colitis and showed an increase of TLR5 expression in mucosa of patients with pouchitis whereas TLR3 downregulation in normal pouch mucosa. They hypothesized that this was secondary to increase in anaerobic bacteria after pouch formation resulting in TLR activation by flagellin whereas TLR3 is downregulated to suppress inflammation [27]. In the case of IBD, mucosal barrier disruption definitely plays a role. However, interestingly from both genetic and experimental studies a protective role of inflammasome Nod-like receptor (NLrp3, in particular) against colitis has been shown [28].Whether extrapolation of this disrupted role of the inflammasome in chronic inflammation in axial SpA occurs or not is yet unknown.

De Rycke *et al.* [29] and Yang *et al.* [30] have shown that there is constitutive increase in TLR4 expression in peripheral blood mononuclear cells (PBMCs) from patients with AS, compared with healthy controls. Increased TLR2 and 4 expression was also found in SpA (including PsA, AS and undifferentiated spondyloarthritis) synovium when compared with osteoarthritis or rheumatoid arthritis (RA) synovium [29]. Treatment with infliximab or etanercept reduced the expression of TLR2 and 4 in the SpA synovium and PBMCs [29]. More recently, Assassi *et al.* also demonstrated using whole blood gene-expression profiling that TLR4 and 5 expression is significantly increased in AS but not healthy controls or systemic lupus erythematosus patients [31]. Similarly, in earlier reports both TLR4 and 5 levels declined significantly during and after treatment with anti-TNF agents [30].

Increased protein levels of TLR1, 2 and 9 have also been reported in AS monocytes from AS patients who were not on anti-TNF drugs [32]. These observational studies suggest association between increased TLR expression and AS, however, by no means demonstrate causality. Other possibilities are that TLR expression is a common pathway further downstream to a more proximal event and itself is a nonspecific indicator of activation of a more general inflammatory response in SpAs.

The expression of TLR2 and 4 has been investigated in PsA. In a small study, Candia *et al.* [33] examined PBMC $CD14^+$ monocytes in PsA patients and found transiently increased expression of TLR2 (but not TLR4) in immature dendritic cells *in vitro*. The significance of this finding is presently unclear. However, a recent report by Myles and Aggarwal found that elevated levels of TLR2 and 4 in peripheral blood and synovial fluid monocytes, in enthesitis-related juvenile chronic arthritis, produce more IL-6 and MMP-3 compared with controls upon LPS stimulation [34]. This implies that dysregulated TLR expression may drive exaggerated inflammatory responses.

Consequences of TLR activation

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While the causal role of TLRs in AS remains to be established; it is clear that engagement of TLRs result in activation of the NF-κB pathway, which promotes secretion of proinflammatory cytokines such as IL-6, IL-12p40, TNF and type I IFNs and these may drive inflammation in AS.

Moreover, certain cytokines such as TNF have been shown to increase the expression of TLRs [35]. Indeed, serum and synovial fluid levels of p40 IL-12/IL-23 have been shown to be increased in SpA compared with osteoarthritis patients [36]. Smith *et al.* have reported that PBMC-derived macrophages obtained from AS patients (half of whom were on anti-TNF therapy) had a 'reverse' IFN-γ signature that suggests impaired activation of genes that may affect the clearance of pathogens and contribute to the activation of the IL-23/IL-17 pathway [37]. It has also been shown that TLR2 and 4 stimulation differentially skews the balance of T cells in mouse models of RA [38]. ILR1-R and TLR2 knockout mice revealed severe arthritis and had reduced populations of Th2 and Treg cells. However, TLR4-deficient mice were protected against arthritis, which is likely mediated via Th-17 cells through IL-1 and IL-23. The IL-17 pathway may likely be playing a crucial role in inflammation of AS. More recently, it has been shown by Appel *et al.* that cells involved

in innate immunity (neutrophils and myeloid precursor cells) were predominant in the facet joints of advanced AS patients and these secrete IL-17 [39]. Innate immune cells producing IL-17 may have a greater relevance in inflammation in AS than the adaptive Th-17 pathway producing IL-17 and consequently driving inflammation. All these pathways are likely intertwined however, and more studies are needed to elucidate these mechanisms.

Yang *et al.* also found that the constitutively increased TLR4 expression in AS monocytes strongly correlated with erythrocyte sedimentation rate, C-reactive protein, IL-12, TNF and sTRAIL levels in HLA-B27$^+$ AS subjects [30]. TNF-related apoptosis-inducing ligand or TRAIL (*TNFSF10*) is a glycoprotein that includes a membrane-bound TRAIL and soluble TRAIL.

TRAIL can induce apoptosis in a variety of cells and has been shown to be important in the generation of CD4$^+$ primed, CD8$^+$ memory T cells [40]. Compared with healthy controls, patients with inflammatory arthritis including RA and SpAs possess an expanded population of circulating CD4$^+$CD28$^-$ T cells [41]. Raffeiner *et al.* showed that this subpopulation of T cells from SpA patients significantly expressed more functionally active TLR4 and 2 compared with controls. These levels decreased significantly with anti-TNF treatment [35].

One of the links between innate immunity and adaptive immunity in the case of AS inflammation may be CD4$^+$CD28$^-$ T cells. Cytotoxic CD8$^+$ T cells and natural killer cells produce cytolytic enzymes such as perforin. Normally CD4$^+$ T helper cells lack this phenomenon.

However, a subset of T cells known as CD4$^+$CD28$^-$ T cells express NKG2D, a natural killer cell-related receptor. These T cells also fail to express the CD40 ligand molecule and therefore are unable to costimulate antibody production by B cells [42]. Their signature cytokines include IFN-γ (normally Th-1), IL-4 (normally produced by Th-2) [42]. In AS and other autoimmune diseases, these cells may play an important role [35].

Finally, osteoblasts and osteoclasts also express TLR2 and 4. Pöllänen *et al.* proposed that activation of TLR4 signaling (which increases RANKL and osteoclastogenesis in both osteoblasts and osteoclasts) may contribute to endochondral bone formation, eyndesmophyte, enthesopathy and ankylosis in AS [16]. However, this hypothesis needs further verification by experimental studies.

Future perspective

There are no consistent data demonstrating that TLRs are a genetic risk factor for AS, yet by virtue of the fact that TLRs sit at the interface between the immune system and the environment, there is still considerable interest in the role of TLRs in driving inflammation in AS.

It may be that bacterial components initiate the stimulation of TLRs either by molecular mimickery or triggers a sensitization to an endogenous antigen, which then consistently and persistently activates the adaptive arm of immunity causing a sterile but chronic inflammatory status. The trigger may not be a pathogenic bacteria, it may be a component of commensal resident flora that occurs in a genetically susceptible individual (e.g., flagellin in Crohn's disease). It may be a combination of several factors that have to take place in a genetically susceptible individual to finally manifest disease.

There are still many unexplored avenues and further research is needed to delineate these pathways. Until recently, targeted therapeutics towards inflammatory cytokines have brought us a long way in treatment of autoimmune diseases. Delving deeper in pathogenesis and molecular mechanisms of TLRs may allow for selective inhibition of TLR signaling or their respective coreceptors and adaptor proteins. This may provide us with a new chapter in therapeutic options in the future in AS, SpA and other autoimmune diseases.

Executive summary

- Toll-like receptors (TLRs) are pattern recognition receptors, which are found extracellulary and intracellularly.
- While TLRs protect against infection, inappropriate activation of TLR pathways can also lead to chronic inflammation and autoimmunity.
- In the case of ankylosing spondylitis (AS), genetic association studies have not revealed any TLR single nucleotide polymorphisms to be associated at the genome-wide significance level.
- Observational studies suggest a possible association between TLR dysregulation and AS, however, definitive causality has not been proven.
- At which point and to what extent dysregulation of TLR plays a role in driving inflammation in AS is as of yet unclear and further research is needed to elucidate this information.

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