Intestinal and enthesis innate immunity in early axial spondyloarthritis

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Abstract

Axial SpA (axSpA), encompassing AS, is a multifactorial disease that localizes to sites of high spinal biomechanical stress. Much has been written on T cells and adaptive immunity in axSpA, which is understandable given the very strong HLA-B27 disease association. Extra-axial disease characteristically involves the anterior uveal tract, aortic root, lung apex and terminal ileum. Under recent classification, axSpA is classified as an intermediate between autoimmunity and autoinflammatory disease, with the latter term being synonymous with innate immune dysregulation. The purpose of this review is to evaluate the ‘danger signals’ from both the exogenous intestinal microbiotal adjuvants or pathogen-associated molecular patterns that access the circulation and endogenously derived damaged self-tissue or damage-associated molecular patterns derived from entheses and other sites of high biomechanical stress or damage that may serve as key drivers of axSpA onset, evolution, disease flares and eventual outcomes.

Key words: danger signals, pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), axial spondyloarthritis (axSpA), enthesitis

Introduction

Axial SpA (axSpA) predominantly affects the spine and sacroiliac joints and encompasses AS and non-radiographic axSpA (nr-axSpA), the chronic prototypic seronegative inflammatory rheumatic disease belonging to the family of SpAs [1]. The enthesis is defined as a region of tough fibrous tissue where tendon, ligament or joint capsule inserts into bone [2, 3]. Enthesitis is understood to be the cardinal lesion in axSpA, with synovitis and osteitis being intimately associated [4]. The innate and adaptive immune system are functionally integrated and usually act together. Much has been written about the role of adaptive immunity in axSpA, which is understandable given the HLA-B27 association and many other genetic pointers towards adaptive immunity [5]. Given the proclivity for simultaneous inflammation in extra-axial biomechanically stressed sites, including the aortic root, lung apex and terminal ileum, and the fact that these tissues are antigenically diverse, it is hard to explain disease in terms of an arthritogenic peptide theory, thus the role of local tissue factors and innate immune responses merits greater consideration. The purpose of this review is to summarize the evidence on the role of innate immunity in disease initiation in axSpA. We will split the role of innate immunity into two major categories, innate immunity in the gut in axSpA and its role at physically stressed sites (Figs 1 and 2).
It is increasingly clear that a relationship between intestinal inflammation and axSpA exists. A total of 5–10% of axSpA patients develop clinically diagnosed IBD and up to 60% have evidence of subclinical gut inflammation [6]. A cause-and-effect relationship is best established in reactive arthritis, a member of the SpA family, where enthesitis and synovitis develop after a distant infection, usually genitourinary or gastrointestinal with Campylobacter, Salmonella, Yersinia or Shigella [7]. Severe acute presentations of sacroiliitis have been reported mimicking infection, supporting innate-driven mechanisms [8]. The first line of innate immunity is not the immune cells, but the barrier tissue itself, and there is compelling evidence for barrier dysfunction in animal models and in AS. Increased gut permeability has been demonstrated among patients and first-degree relatives of axSpA [9, 10]. The leakage of epithelial and endothelial membrane forming the gut–epithelial barrier and gut–vascular barrier can result in bacterial translocation with bacterial products such as lipopolysaccharide (LPS), LPS binding protein and fatty acid binding proteins, thus activating resident immune cell populations and inducing inflammation, osteitis and synovitis [11]. In addition to gut-resident myeloid cells and infiltrating neutrophils, Paneth cells, a subset of specialized secretory host-defense epithelial cells located in the small intestines, have been shown to secrete IL-23 and activate key IL-23 responsive cells such as innate lymphoid cells of group 3 (ILC3), γδ T cells and mucosal-associated invariant T (MAIT) cells, which can recirculate from the gut and seek axSpA relevant sites of inflammation [12–16].

While the exact mechanism linking the gut to axSpA pathogenesis is not completely understood, it is believed that the interplay between the microbiome and the intestinal immune system contributes to intestinal innate immune cell activation [17]. It also remains unclear when gut barrier dysfunction with abnormal permeability occurs. However, it is evident that axial disease evolution might be related to changing spinal biomechanics in the later teenage years [18].
It is not understood exactly how intestinal inflammation leads to axial inflammation, but possibilities include innate and adaptive cell circulation and skeletal homing, translocation of adjuvants to the gut or shared dysregulated immune mechanisms [17]. It has been postulated that while IL-23 acts directly on tissue-resident enthesis immune cells, IL-23 might activate circulating immune populations that then migrate and seek enthesis, inducing inflammation [4]. Translocation of bacterial products into the gut submucosa and circulating blood provides evidence of the concept of so-called leaky gut, linking bacteria to activation of immune cells in circulating blood and/or enthesis resident immune cells [11]. Similarly, observations highlight the role of the gut as the primary site for the differentiation, expansion and priming of innate cells prior to recirculation to axSpA target sites [19]. Reported de novo severe SpA in ameliorated IBD following α4β7 integrin blockade is further clinical evidence of the intimate connection intertwined at the gut–joint axis and innate-driven mechanisms [20]. Perhaps upregulation of the mucosal vascular cell adhesion molecule 1 (MADCAM-1), the homing integrin (α4β7) ligand, in the gut and inflamed bone marrow of patients with axSpA provides additional evidence of the importance of recirculation of gut-primed innate cells [20]. Finally, microbial products, also known as pathogen-associated molecular patterns, act as adjuvants, defined as substances enhancing antigen-specific immune response [21]. It is possible that bacterial adjuvants act synergistically with mechanical factors in activating innate immune responses and providing necessary co-stimulatory signals [21].

The role of HLA-B27 in shaping the gut microbiome was investigated in an HLA-B27 transgenic rat (murine SpA model) using biome representational in situ karyotyping and 16S rRNA gene sequencing, revealing significant differences in caecal microbiota [22]. A larger proportion of radiographic axSpA patients express HLA-B27 as compared with non-radiographic axSpA [23]. The robust association of HLA-B27 with axSpA provides a strong indication that adaptive immunity may be able to sculpt intestinal innate immunity and hence might provide insights into the molecular influence on phenotype (i.e. radiographic vs non-radiographic axSpA), but adaptive immune mechanisms are not discussed further in this article. Moreover, intestinal dysbiosis is
accompanied by alterations of tight junctions between epithelial cells forming the lining of the gut, which is a hot area of research [11].

Pattern recognition receptors in innate immunity in the gut and the enthesis

Pathogen-associated molecular patterns in the gut

The innate immune system possesses a repertoire of germ-line encoded receptors that recognise conserved molecular structures found in pathogens [so-called pathogen-associated molecular patterns (PAMPs)]. Somewhat surprisingly, it was discovered that the same family of receptors become activated on engaging the products released from stressed and damaged cells [termed damage-associated molecular patterns (DAMPs)] [24]. These pattern recognition receptors (PRRs) are subdivided into four major families: Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid–inducible gene 1-like receptor (RLR) and C-type lectin receptor (CLR). Signalling through PRRs triggers a downstream cascade of pro-inflammatory programs within innate immune cells that can initiate antigen-specific adaptive immune responses [24].

TLRs are a group of transmembrane-spanning receptors each having several distinct ligand and cellular localizations. TLRs signal through myeloid differentiation factor 88 (MyD88), except TLR3, which utilizes Toll/IL-1 receptor domain containing adapter-inducing IFN-β (TRIF) for signalling [25]. Subsequent activation of downstream proteins results in upregulation of genes involved in the inflammatory process, antigen presentation, activation of adaptive immunity or induction of cell death [25]. Given the essential role of microbiota and bacteria in axSpA, TLR4, which recognizes LPS from Gram-negative bacteria, was investigated for its association with axSpA, with gene expression profiling showing TLR4 and TLR5 upregulation in axSpA patients [26]. Polymorphisms in TLR4 were associated with axSpA, further adding to the evidence supporting their role in increasing susceptibility to disease [27, 28].

Several reports have identified a nominal association between polymorphisms in caspase recruitment domain-containing protein-9 (CARD-9) and axSpA, placing it as a strong functional candidate [29]. CARD-9 is an adapter protein expressed exclusively on myeloid cells that mediates downstream signalling of Dectin-1, the NK receptor C-type lectin-like PRR recognizing β-glucan (fungal cell wall polysaccharide) in response to fungal infection, resulting in secretion of pro-inflammatory cytokines including TNF, IL-6 and IL-23, among other cytokines [30]. The strongest evidence for the importance of gut barrier cells in SpA comes from the striking NOD2 association with distal ileal inflammation in Crohn’s disease [31] and it is noted that 50% of AS cases have subclinical ileocolonoscopy or histologic changes reminiscent of Crohn’s disease [32]. NOD2 is an intracellular cytosolic PRR belonging to the NLR family, which mediates the activation of mitogen-activated protein kinase signalling components such as p38 and the c-Jun N-terminal kinase pathway through the amino-terminal CARD domain. NOD2 has two CARD domains and is readily activated by muramyl dipeptide (MDP), a ubiquitously present peptidoglycan motif found among both Gram-positive and negative bacteria (Fig. 3) [33]. CARD-9 is an essential component of the NOD2 signalling pathway and induces the maturation of dendritic cells (DCs) to antigen presenting cells and priming naïve cells to Th-1 or Th-17 phenotypes [31].

DAMPs at the enthesis or circulating blood

Parallel to its role in identifying non-self and defence against pathogens, the innate immune system has evolved to serve a housekeeping role, including repair and removal of damaged tissue from structures releasing signals, also known as DAMPs [34]. Several molecules that behave as DAMPs have a primary endogenous role, including transport or chaperoning. However, upon release from damaged cells and arriving within the incorrect extracellular compartment they bind to PRRs and activate an innate immune cascade, a response that is identical to that activated by PAMPs. An example of such molecules includes molecules derived from the intracellular compartment, including high-mobility group box 1 (HMGB1) proteins, S100 protein, uric acid, adenosine triphosphate, heat shock proteins (HSPs), DNA and RNA, among others [34]. Following tissue injury, DAMPs can be secreted from extracellular matrix due to degradation; such products include heparan sulphate, low molecular weight hyaluronan, fibrinogen, fibronecctin and biglycan, among others, which are capable of directly activating TLRs and even some NLR receptors (Fig. 4) [35].

HSP70 plays a central role by acting as a chaperone and folding catalyst, including the folding of synthesized proteins, refolding of aggregated proteins and translocation of secretory proteins and control of activity of regulatory proteins. Extracellular HSPs activate the innate immune system through activation of TLR2, TLR4 and CD91 [36]. The association between HSP70 gene polymorphisms in axSpA was investigated and showed a significant difference in HSP70-1 and HSP70-2 genotypes between axSpA patients and healthy controls [37]. Also, it is possible that impairment of HSP function could result in the accumulation of unfolded protein responses that have been incriminated in SpA pathogenesis [38]. Another important intracellular DAMP, HMGB1, is located in the nucleus and plays an important role in gene expression. When HMGB1 is secreted it binds to TLR2, TLR4, TLR9 and RAGE and results in nuclear factor (NF)-xB activation and inflammation induction [36]. Serum levels of HMGB1 in axSpA patients were reportedly higher than those of controls [39]. Another report demonstrated an association between serum levels, disease activity and inflammatory markers [40]. While these factors may all be linked to axSpA,
Cytokines as danger signals in both the gut and enthesis

Tissue microdamage, including bone microcracks, and soft tissue microscopic inflammatory and reparative changes have been reported in normal healthy entheses. Injury to tissue is capable of innate immune system activation by the liberation of so-called DAMP receptors, resulting in cytokine release, including IL-23, IL-1β and TNF [41, 42]. Independent of DAMPs, liberation of pro-inflammatory cytokines that can also act as danger signals and drive inflammation. The best example of a cytokine danger signal in the experimental setting is the TNF model, where physical stress on the enthesis drives stromal liberation of TNF, resulting in enthesitis, synovitis and also colitis that localizes to the terminal ileum.

Resident immune cells including γδ T cells and ILC3, known targets of IL-23, then respond by producing IL-17 and TNF, among other cytokines [42]. This response drives homeostatic repair at enthesal sites. However, in SpA, repair circuits are dysregulated and failure to resolve inflammation results in disease initiation.

Currently >100 gene polymorphisms have been identified to be associated with axSpA, several of which are associated with innate immune signalling, including interferon regulatory factor 5 (IRF5), TNF, NOS and IL-12B, or are potentially involved in innate immune sensing, such as IL-1R, IL-6R, IL-23R and prostaglandin E2 receptor 4 [43, 44].

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Several DAMPs have been identified and are released from both extracellular and intracellular compartments following stress, tissue injury or cell death, all of which might occur at sites of high physical stressing, including the entheses in early SpA. Upon release, DAMPs bind to their respective pattern recognition receptors and induce inflammatory responses that are essential in driving normal repair and homeostasis. Uncontrolled, DAMPs result in hyperactivation of innate immune signals and responses driving and exacerbating inflammatory diseases.

Evidence from genetic studies, animal models and therapeutic studies firmly implicates the IL-23–IL-17 axis in the pathogenesis of SpA [53]. Enthesal immune cells, which were only recently discovered, can produce TNF-α transcripts and upregulate IL-17, IL-22 and IL-23 in vitro [54–56]. IL-23, a heterodimeric cytokine of the IL-12 family, is mainly produced by innate cells including macrophages, DCs and monocytes. IL-23 induces IL-17 production from a wide range of cells, including Th-17, CD8+ T cells and γδ T cells, which results in stromal cells and immune cell activation and promotion of inflammation [2]. It is becoming increasingly evident that IL-17 can be independent of IL-23, probably explaining the ineffectiveness of IL-23 targeting in axSpA [57, 58]. Moreover, IL-23 deficiency has been shown to play an important role in the initiation but not the persistence of experimental SpA in the HLA-B27 transgenic murine model [59].

In a recent report, CD14+ cells with the ability to secrete IL-23 were confirmed in the human enthesis [55]. IL-23 overexpression through minicircle DNA injection induced experimental SpA and is essential for enthesitis and entheseal new bone formation by acting on γδ T cells [4]. It is hypothesized that misfolding of HLA-B27 triggers a stress response in the endoplasmic reticulum (ER), known as unfolded protein response, resulting in IL-23 production; however, conflicting reports exist regarding ER stress response [60]. Furthermore,
activation of prostaglandin E2 receptor 4 (EP4), the protein product of prostaglandin EP4 receptor (PTGER4) promotes development of Th17 cells by increasing the IL-23 expression level. PTGER4 polymorphisms have been shown to be associated with axSpA [61]. In addition to the increase in IL-23 secretion, it has been shown that prostaglandin E2 (PGE2) levels were significantly elevated in mice patellar and Achilles tendons in response to rigorous treadmill training [62]. Moreover, treatment of tendon stem cells with PGE2 decreased their proliferation ability but induced both adipogenesis and osteogenesis [62]. It is clear that PGE2 plays a central role as a mediator of inflammation, as evidenced by the clinical efficacy of NSAIDs (PGE2 inhibitor) in diminishing the inflammation and pain in patients with axSpA [63].

**Innate immune cells involved in axSpA**

Macrophages are a heterogeneous population and are broadly subdivided into M1 (classical/pro-inflammatory) and M2 (alternative, anti-inflammatory). Histological assessment of early and active sacroiliitis showed abnormal enthesal architecture with increased vascularity and cellular infiltration with a predominant macrophage cell infiltration in the fibrocartilage [64]. Recently, human enthesis was shown to harbour a population of CD14+ myeloid cells capable of IL-23 and TNF production [55]. Stimulation of CD14+ cells with adjuvants induced chemokine ligand 20 (CCL20) from the enthesis, which functions as a chemoattractant to IL-17-producing cells expressing its ligand chemokine receptor 6 (CCR6) [55]. DCs link innate and adaptive immunity and impaired DC function has been linked to a predisposition to experimental SpA [65], impaired T cell interactions with impaired co-stimulation and altered cytoskeletal dynamics in early disease [66].

The cellular function of NK cells, a major component of the innate immune system, is defined by the balance of activating and inhibitory signals that recognize the MHC class through the highly polymorphic killer cell

**Fig. 5** Innate cell response during cellular stress

(A) MICA is a gene located on chromosome 6 close to the HLA-B locus and is upregulated during stress response to heat shock and DNA damaging conditions and is influenced by some infectious agents, including *Escherichia coli*, among others. (B) NKG2D is an activating receptor of the C-type lectin-like family and is expressed on NK cells, CD8+ T cells, γδ T cells and NK T cells. Upon activation, it has been associated with a pro-inflammatory response. (C) MICA serves as a ligand to NKG2D and upon engagement during a stress response, the complex results in the activation of effector cytolytic function of NKG2D-expressing cells against MICA-expressing cells.
immunoglobulin-like receptor (KIR) [67]. The role of HLA-B27 in disease induction is not well understood. One plausible hypothesis implicates the observed ability of HLA-B27 to form heavy chain homodimers and cell surface expression of heterodimers that bind to innate immune receptors on NK cells, including KIR3DL1, KIR3DL2 and LILIRB2 [68, 69]. This interaction results in a downstream pro-inflammatory response in both NK cells and KIR3DL2-expressing T cells, including Th17 and γδ T cells [70, 71]. NKGD2, an activating receptor of CLR, is expressed on NK cells, CD8+ T cells, γδ T cells and NK T cells and has been associated with pro-inflammatory response when activated. MHC class I chain A related (MICA) is a gene located on chromosome 6 close to the HLA-B locus and codes for stress inducible glycoprotein that results in the activation of NKGD2 (Fig. 5). Polymorphisms in MICA-129 have been associated with spondylitis, and homozygosity to MICA A5.1 conferred increased risk for axSpA after adjustment for HLA-B27 status [72].

Invariant NK T (iNKT) cells represent a small subset of T cells expressing a restricted T cell receptor repertoire, and unlike T cells, iNKT cells recognize glycoproteins through the non-polymorphic MHC class 1 molecule CD1d. iNKT cells secrete IL-17 upon stimulation of either T cell receptor or the constitutively expressed IL-23R, pointing towards an independent and convergent pathway [72]. Interestingly, iNKT cells have been shown to be natural regulators of murine SpA. iNKT cell depletion aggravated gut and joint inflammation in a TNF adenyate–uridylate-rich regulatory element mouse model, which was linked to a greater number of inflammatory DCs [73].

γδ T cells are another distinct subset of the unconventional T cell subpopulation expressing the γδ form of the T cell receptor and capable of recognizing a broad range of antigens through pattern recognition receptors, including TLRs and Dectin-1, supporting their role in the early response to microbes [74]. Several γδ T cell subsets have been identified and have been shown to

| Immune cell population | Role in axSpA | Reference |
|------------------------|--------------|-----------|
| Macrophages            | Active sacroiliitis shows predominant cellular infiltration with macrophages | 64          |
|                        | CD163+ macrophages and CD68+ macrophages from fibrous tissue of axSpA facet joints secrete IL-23 | 85          |
|                        | Normal enthesis harbours a population of CD14+ cells capable of IL-23 and TNF production | 55          |
| Dendritic cells        | Impaired formation of conjugates between dendritic cells and T cells due to impaired accessory molecule function | 65          |
|                        | Defects in co-stimulation, decreased expression of MHC II and altered cytoskeletal dynamics in axSpA patients | 66, 86, 87  |
| NK cells               | HLA-B27 heavy chain homodimers and heterodimers bind and activate NK cells through killer cell immunoglobulin like receptor | 68, 69      |
|                        | High NK cell cytotoxicity in SpA patients compared with controls | 88          |
|                        | Lower expression of A20, responsible for NF-κB inhibition on CD56bright cells in patients with axSpA | 89          |
|                        | MICA serves as a ligand to NKGD2 and the complex results in the activation of effector cytolytic function of NKGD2-expressing cells against MICA-expressing cells | 71          |
| Invariant NK T cells   | iNKT depletion worsened joint inflammation in TNF AU-rich regulatory element mouse model | 73          |
| γδ T cells             | iNKT cells secrete IL-17 upon stimulation of the TCR or IL-23R | 72          |
|                        | γδ T cells population has been shown to be the predominant IL-17A producers at the enthesis in the IL-23-dependent mouse model | 4           |
|                        | γδ T cells are resident in the enthesis and express transcripts associated with the IL-23–IL-17 pathway, including RORC, CCR6 and IL-23R | 58          |
|                        | γδ T cells has been shown to drive IL-17 secretion independent of IL-23 stimulation | 58          |
|                        | γδ T were enriched within inflamed joints of SpA and acts as a major IL-17 secretors | 90          |
| Innate lymphoid cells  | ILC3 groups are relevant to SpA due to secretion of IL-17 and IL-22 in response to activation by IL-23 | 77          |
|                        | Gut-derived ILC3 were expanded in the synovial fluid and bone marrow of patients with axSpA | 77          |
|                        | Normal enthesis harbours a population of resident ILC3 | 79          |
|                        | Psoriatic arthritis is characterized by a skewed ILC homeostasis, with elevated levels of ILC3s, which are potent source of IL-17/IL-22 | 91          |
| MAIT                   | Enriched population in the synovial fluid of patients with axSpA | 16          |
|                        | Secretion of IL-17 independent of IL-23. Role in IL-22 secretion and regulation of bone formation | 82          |
|                        | The number of IL-22+ and IFN-γ+ /IL-17A+ MAIT cells was higher in axSpA as compared with healthy controls | 82          |
be potent producers of IL-17, promoting wound healing and osteogenesis in mice [74, 75]. Recently γδ T cells were described in normal human enthesis, with evidence supporting their entheseal residency and expression of transcripts associated with IL-23/IL-17 signaling, including retinoic acid receptor-related orphan receptor C (RORC), IL-23R and CCR6 [58]. Furthermore, IL-17 secretion from γδ T cells was shown to be driven in the absence of IL-23, providing a potential explanation for anti-IL-23 inefficacy in axSpA [58].

ILCs are a family of heterogeneous resident effector cells at the epithelial barrier surfaces of the gut and are involved in tissue remodelling and maintenance of organ homeostasis and inflammation [76], with ILC3 being most relevant in SpA due to IL-17 and IL-22 secretion in response to activation by IL-23 [77]. It has been demonstrated that gut-derived IL-17-producing ILC3 is increased in the peripheral blood, synovial fluid and bone marrow of patients with axSpA and expressed the homing integrin α4β7 [78]. Recently it was shown that peri-entheseal bone and entheseal soft tissue harboured a population of ILC3 that was entheseal resident [79]. The frequency of entheseal ILC3 was lower than that reported in the gut, possibly due to the lack of exposure of enthesis to the external environment [79].

Another innate immune source of IL-17 are MAIT cells, a population of unconventional innate T cells that recognise non-peptide antigens presented by monomorphic MHC class I molecules through a semi-invariant T cell receptor [80]. As the name suggests, MAIT cells are abundant at barrier surfaces, such as mucosal surfaces, and act as part of the first line of defence against bacteria and yeasts by producing inflammatory cytokines, including IFN-γ, TNF, IL-17A and IL-22, among other cytokines [80]. Evidence from a collagen-induced arthritis murine model highlighted the effector role of MAIT cells in augmenting joint inflammation during the effector phase of arthritis, suggesting arthritogenic potency [81]. MAIT cells have also been shown to be enriched in the synovial fluid of patients with axSpA and expression of IL-17 was dependent on priming with IL-7 but not IL-23 or antigen stimulation [16]. IL-22 secretion by MAIT cells has been documented and associated with the induction of genes implicated in the regulation bone formation and osteogenesis [82].

A strong translational perspective in innate immunity in SpA comes from clinical and experimental studies where anti-IL-17A is effective in axSpA but ineffective for gut inflammation. This was shown to be due to γδ T cell intestinal production of IL-17A independent of IL-23 expression, with IL-17A mediating normal barrier function [83, 84].

**Conclusions**

Innate immune system dysregulation plays a critical role in the pathogenesis of axSpA (Table 1). Evidence from murine models highlight the role of innate immunity in the induction of axSpA. IL-23/IL-17 pathways provide clues to how HLA-B27 contributes to disease pathogenesis through innate immune activation. Several innate immune cells have been identified and their role in the production of disease-relevant cytokines and disease propagation is evident. However, certain areas remain undefined, including the exact role of gut inflammation in axSpA, and further studies are needed to better elucidate the role of innate immunity in the initiation and propagation of inflammation in axSpA.

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