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SPONDYLOARTHRITIS (MA KHAN, SECTION EDITOR)

Functional Genomics and Its Bench-to-Bedside Translation Pertaining to the Identified Susceptibility Alleles and Loci in Ankylosing Spondylitis

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Abstract Ankylosing spondylitis (AS) is a highly heritable disease for which there is a great unmet need for improved therapies. Genetics research has identified several major pathways involved in the disease, from which treatments have either now entered clinical practice or are in development. In particular, therapies targeting the IL-23 pathway were repositioned for use in AS following the discovery of multiple genes in the pathway as determinants of AS risk. Discovery of the association of aminopeptidase genes with AS, and subsequently with psoriasis, inflammatory bowel disease and other conditions, has triggered research into therapies targeting this pathway. The AS-genetic associations point to involvement of gut mucosal immunity in driving disease, and metagenomic studies have provided strong support that AS is a disease driven by interaction between the gut microbiome and host immune system, providing a rationale for the exploration of gut-targeted therapies for the disease.

Keywords Functional genomics · Ankylosing spondylitis · Genetics · Single nucleotide polymorphism · IL-23 · Aminopeptidase · Microbiome

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Introduction

There is a great need for improved therapies for the disease ankylosing spondylitis (AS). Current therapies including tumor necrosis factor (TNF) inhibition are quite effective in the majority of cases in controlling AS-related inflammation, but although they likely slow the rate of progression of the joint ankylosis that is the major cause of long-term disability in the disease, ankylosis still progresses despite effective treatment. These medications do not induce true drug-free remission, are associated with significant side effects, are injectable and require a cold chain and are extremely expensive, with biological agents used in treatment of immune-mediated arthritis costing the Australian government more than the combined cost of chemotherapy reagents for oncology. Whilst hypothesis-driven research in AS has provided valuable information about the aetiopathogenesis of the disease, hypothesis-free genetics research over the past decade has massively expanded what we know about the pathways involved in AS. New therapies have already been introduced as a consequence of these discoveries, and more are in the development pipeline. In this article, we seek to highlight some of the key developments that have occurred because of the genetics revolution in AS.

Genetic Studies in AS

The association of HLA-B27 with AS was first reported in the early 1970s and remains one of the strongest genetic associations with any common human diseases. Nonetheless, genetic epidemiology studies and subsequently successful gene mapping studies have demonstrated that many genes are involved in AS, potentially numbering in the thousands (reviewed in [1]). To date, 113 association signals have been discovered in AS, of which 47 achieve definitive genome-wide levels of

statistical association and the remainder have been discovered through studies of overlapping genetic risk with the related diseases psoriasis, inflammatory bowel disease and primary sclerosing cholangitis [2••, 3, 4••, 5, 6]. These findings explain ~30 % of the genetic risk of the disease and indicate that a very large number of genetic associations remain to be identified.

The major histocompatibility complex harbors multiple genes involved with AS, not just *HLA-B27*. This includes protective alleles (*HLA-B*07:02* and *HLA-B*57:01* and other risk alleles including *HLA-B*13:02*, *HLA-B*40:01, HLA-B*40:02*, *HLA-B*47:01* and *HLA-B*51:01*) [7••]. Associations also exist with other HLA genes including *HLA-A, -C, HLA-DRB1* and *HLA-DPB1*. These associations help explain the co-occurrence of AS with other seronegative diseases, such as Behcet's disease (for which *HLA-B51* is the key HLA association) and inflammatory bowel disease (which shares the association with *HLA-DRB1*0103*). These findings, and the differential association of *HLA-B27* subtypes with AS, inform research into mechanisms underpinning the association of HLA alleles with AS, as discussed below.

There is also substantial overlap between the non-MHC genetic factors involved in the various seronegative diseases, particularly between inflammatory bowel disease (IBD) and AS [2••, 6, 8]. Major differences also exist which help inform the mechanisms underpinning the different presentations of these diseases. For example, AS is not associated with the major IBD autophagy gene *ATG16L1*, or the ER stress gene *Xbp1*. Combined dysfunction of these two genes is thought to be a key mechanism underlying IBD [9] and may explain why in AS, the gut inflammatory process is relatively mild, despite involvement of the same key cytokine pathways. The more that is learnt about the specific genes that are involved in these conditions, the more we will understand about the mechanisms leading to their clinical similarities and differences.

The two major pathways discovered to date in AS genetics research involve the aminopeptidase pathway of antigenic peptide handling and the IL-23 cytokine pathway. More recent discoveries include involvement of genes involved in NFKB inactivation, DNA methylation, bacterial sensing in the gut, gut mucosal immunity and TCR signaling [2••]. In this review, we will focus on the aminopeptidase and IL-23 pathways, which are the most advanced in terms of therapeutic translation, and finally review evidence that AS is caused by interaction between the host immune system and the gut microbiome.

Aminopeptidases in AS

The first genetic associations with aminopeptidase genes in AS were well placed to fit elegantly into disease models concerning HLA-B27 peptide presentation to the immune system. Initial predictions pertained to the involvement of a B27-restricted immunogenic peptide, generated through aberrant aminopeptidase processing, in spurring a breakdown in immunological tolerance and misplaced immune autoreactivity. Hypotheses have since expanded to encompass observations of altered cellular activity and cytokine production in AS patients, suggesting alternate roles for aminopeptidase polymorphisms and B27 in pathology. The Wellcome Trust Case Control Consortium (WTCCC) was the first to identify a disease association with two non-synonymous SNPs (rs30187 and rs27044) in the endoplasmic reticulum aminopeptidase 1 *(ERAP1)* gene (originally *ARTS-1*) and nearby markers in the aminopeptidases *ERAP2* and *LNPEP* [10]. Molecular studies have demonstrated that ERAP1 preferentially cleaves N-termini extended peptides, transported into the endoplasmic reticulum via the transporter associated with antigen processing (TAP), to 8-9mers, which are the optimal lengths for binding to MHC class 1 molecules [11]. The enzyme has also been shown to cleave cell surface receptors of key pro-inflammatory cytokines, including interleukin-6-receptor (IL-6R) [12], interleukin-1 receptor type 2 (IL-IR2) [13] and tumor necrosis factor receptor (TNF-R) [14]. Work has asserted that cytokine serum levels do not appear to correlate with *ERAP1* polymorphisms in AS patients [15], nor do the levels of these receptors vary in *ERAAP*-deficient compared with wild-type mice, and therefore this role is no longer considered physiological ([3] no. 67).

The crystal structure of ERAP1 was published in 2011 [16]. Scrutiny of the Lys528Arg amino acid substitution resulting from the AS-protective rs30187(T->C) mutation placed the ancestral lysine residue on the surface of the enzyme where it forms polar interactions with surrounding amino acids when the enzyme is in a closed conformation. The disease-protective arginine substitution at this position had been previously reported to result in a decrease in peptide processing efficiency [17], subsequently suggested to be a consequence of disrupted chemical interactions between this and adjacent residues, hindering the open to closed transition of the molecule during catalysis [16]. Adding weight to the functional significance of this polymorphism, GWAS performed on a dataset of 3023 AS cases and 8779 controls by the WTCCC2 and Australo-Anglo-American Spondylitis Consortium (TASC) revealed a remarkable epistatic interaction between the *ERAP1* SNP rs30187 and *HLA-B27* ($P = 7.3 \times 10^{-6}$) [3]. The association of *ERAP1* with AS was observed only in *HLA-B27*-positive individuals and completely absent in *HLA-B27*-negative AS, consistent with the functional mechanism of the association being effects on HLA class I antigen processing.

Functional studies comparing the HLA-B27:05-bound peptidome from cell lines carrying distinct *ERAP1* variants have demonstrated that the increased enzymatic activity afforded by Lys528 results in an elevated degree of peptide epitope destruction [18•] but also increases production of some peptides relative to the protective variant [19•].

Alongside the fundamental contribution of HLA class 1 peptide presentation in moderating the tolerogenic and autoimmune potential of the adaptive immune cell repertoire, correct folding of class 1 molecule is dependent on the formation stability of heterotrimers complexed with high-affinity peptide. It is possible that suboptimal peptides may have profound effects on the structural integrity of HLA-B27.

Previous studies on the CD8-positive T-cell repertoire in *HLA-B27*-positive AS patients have demonstrated shifts in adaptive T-cell populations hinting at immune cell engagement with and expansion in response to a stimulating peptide. Early studies detected CD8+, and to a lesser degree CD4+, T-cell oligoclonal expansion in AS patients [20–22]. Mass sequencing of the TCRβ CDR3 has detected expanded populations exhibiting a pro-inflammatory phenotype and CDR3 sequence similarity to clones identified in HLA-B27-positive reactive arthritis patients, and T-cells responsive to the HLA-B27 presented self-peptide pVIPR (RRKWRRWHL) [23, 24]. A true immunogenic peptide, if it exists, is predicted to be a microbial mimic of a self-peptide that leads to loss of tolerance and autoimmunity (the 'arthritogenic peptide' theory).

As discussed in the sections, HLA-B27 may operate in AS by interacting with the gut microbiome in a manner that leads to increased invasion of the gut wall, driving IL-23 production. In this hypothesis, *ERAP* variants may operate to cause disease either by risk variants leading to destruction of antigenic epitopes (and hence relative mucosal immunodeficiency) or their creation (either leading to immune activation as in the arthritogenic peptide theory, or potentially immunity against protective gut bacterial species). Consistent with immunodeficiency theories, there is moderate evidence that HLA-B27 is a chronicity factor for reactive arthritis [25] and is associated with less bacterial killing in some cellular studies [26].

ERAP1 polymorphisms have recently been tied to surface expression of HLA-B27-free heavy chain homodimers ($B27_2$) [27••], and aberrant B27 structures are believed to be the consequence of slow complex folding in the absence of engagement with a suitable peptide. $B27_2$ structures are recognized by the killer immunoglobulin-like receptor KIR3DL1 expressed in certain natural killer cells and T-cells, and populations expanded in the blood and synovial fluid of spondyloarthritis patients [28, 29]. Engagement of KIR3DL2, up-regulated upon activation of CD4+ T-cells, with $B27_2$ promotes increased cell survival, differentiation into a Th17 phenotype and secretion of the potent inflammatory cytokine IL-17 [30••].

The discovery of a gene-gene interaction between *ERAP1* variants and *HLA-B40* in HLA-B27-negative AS, HLA-B51 and Behcet's disease, and HLA-Cw6 in psoriasis, suggests that these HLA class I alleles likely all operate by similar mechanisms to induce disease. Further research is required into how these other HLA class I alleles are involved in these clinically related diseases. These genetic findings are clearly consistent with mechanisms involving the canonical functions of HLA class I alleles in adaptive immunity, but evidence of KIR mechanisms similar to that proposed for HLA-B27 for the other HLA alleles is at this point lacking.

Additional to the primary functional consequence of rs30187 on ERAP1 enzymatic activity, early *ERAP1*-targeted analyses revealed disease associations with a large number of additional polymorphisms previously identified as those influencing *ERAP1* expression [31]. There are at least two *ERAP1* variants associated with AS, one being the nsSNP rs30187 and the other tagged by the SNP rs10050860 [3]. Co-inheritance of disease-protective alleles at rs30187 and rs10050850 decreases disease risk by three to four times [3], emphasizing the pathogenicity afforded by overexpression of hyperactive ERAP1 variants. In the largest AS GWAS study to date, two additional aminopeptidase genes, *ERAP2* and *NPEPPS*, were found to be associated with disease [4••], at which the *ERAP2*-protective polymorphism rs2248374(G) is known to result in nonsense-mediated decay of an alternative transcript and result in complete absence of ERAP2 expression [32]. Collectively, these findings speak to the potential therapeutic benefit of inhibiting aminopeptidase function in suppressing underlying molecular causes of immune-mediated pathology in AS.

IL-23 Pathway—Immunogenetics and Implications for Drug Development

In the last 10 years, our understanding of the role played by IL-23 signaling in the pathogenesis of immune-mediated diseases (IMDs) such as AS, IBD and psoriasis has expanded greatly. Indeed, the convergence of advances in understanding IL-23 basic biology with drugs targeting IL-23 signaling [33••, 34•] and their use in IMD is a leading example of translational science in the past decade.

IL-23 is a heterodimeric member of the IL-12 cytokine family composed of a p40 subunit that is shared with IL-12 and an IL-23-specific p19 subunit. IL-23 is produced by activated myeloid cells, endothelial and epithelial cells. Whilst the source(s) of IL-23 in AS has not been fully characterized, IL-23 secretion from epithelial cells is markedly increased in the terminal ileum of AS patients [35]. Under homeostatic conditions, IL-23 is an important regulator of mucosal immunity, mediating antimicrobial responses and defense against extracellular bacteria at the gut epithelium. The trigger for the enhanced IL-23 secretion observed in the gut of AS patients is unknown, but one leading theory is that the AS epithelium mounts a vigorous response to subtle alterations in the local microbiome (discussed below). IL-23 signaling is then potentiated by disease-associated variants in IL-23 signaling pathway genes.

The IL-23 receptor heterodimer is made up of an IL-23 receptor subunit complexed with IL-12Rβ1. Il-23 signaling causes intracellular recruitment of the tyrosine kinases Jak2 and Tyk2 to intracellular domains on IL-23R and IL-12Rβ1, respectively, which allow for recruitment and phosphorylation of Stat3, homodimerization of phospho-Stat3 (pStat3) and ultimately translocation of pStat3 homodimers to the nucleus to effect transcription of downstream targets, IL-17 and IL-22.

The first suggestion that IL-23 signaling played an important role in AS pathogenesis came from GWAS that described variants in the gene encoding the IL-23 receptor (*IL23R)* [10]. Since then, variants in many IL-23 pathways genes have also been shown to confer increased susceptibility to developing AS including *Tyk2* [4••], *STAT3* [6], *IL12B* [6], *IL6R* [36], *IL27* [4••], *CARD9* [3] and *JAK2* [2••]. Variants in IL-23 signaling pathway genes are common across a number of IMD including psoriasis and IBD [2••, 8]. Precisely how these variants contribute to disease remains unclear. The role of even the best studied of the IL-23 pathways genes, *IL23R*, remains enigmatic. One of the most extensively studied SNPs in *IL23R*, rs11209026 results in the amino acid switch R381Q. The protective variant conferred approximately twofold protection against AS [10]. Several studies have demonstrated that carriage of the R381Q-protective variant resulted in reduced IL-23-induced STAT3 phosphorylation [37, 38]. Di Meglio and colleagues have shown that individuals homozygous for the protective R381Q variant display almost complete loss of IL-23 signaling and have impaired Th17 responses [39••]. Whether other IL-23 pathway risk variants also lead to gain of IL-23 function is not clear, but this evidence does indicate that the key *IL23R* risk variant operates to cause disease by increasing IL-23 signaling.

Understanding the complexity and nuances of IL-23 signaling in AS has important implications for the clinical use of biologics targeting IL-23. CD4 T-cells were considered for many years to be the dominant source of IL-17, but studies in IL-23R reporter mice showed that CD4 T-cells make up a very small proportion of the total pool of IL-23-responsive IL-17-secreting cells [40]. Many cell types have been shown to have enhanced IL-17 secretion in AS (reviewed in [41]). Recent literature suggests that IL-22 expression is also increased in AS [42, 43]. However, we have no understanding of hierarchy or redundancy among all the cell types responding to IL-23. Moreover, we know little about where in the body IL-23 is driving disease pathogenesis.

Work by Sherlock and Cua showed that overexpression of IL-23 alone was sufficient to induce an AS-like inflammatory disease in mice, including evidence of enthesitis [44]. Earlier work also highlighted the importance of IL-23 biology in localized but not systemic immunity [45]. The SKG mouse model of spondyloarthritis has revealed even more subtlety in IL-23 signaling in IMD. In this model, peripheral arthritis, enthesitis and ileitis were all found to be IL-23-dependent. IL-23-mediated enthesitis was driven by IL-17 and IL-22 secretion, but in the ileum IL-17 was pathogenic whilst IL-22 was protective [46•]. The discordancy between local IL-17 and IL-22 responses is intriguing and has important clinical implications for the use in AS, and other IMD, of new biologics targeting components of the IL-23 signaling pathway.

Results from clinical trials show that targeting IL-23/IL-17-driven inflammation is an effective therapeutic strategy in AS [33••, 34•]. The FDA has subsequently approved use of secukinumab, a fully human monoclonal antibody that binds and neutralizes IL-17A, in treatment of AS. Ustekinumab, a monoclonal antibody that binds the IL-12/IL-23-shared p40 subunit, is approved for use in psoriasis and psoriatic arthritis but not yet in AS. A number of other IL-23/IL-17-targeting drugs are in trials at the moment. BI655066, a fully human monoclonal antibody against the IL-23-specific p19 subunit, is undergoing a phase III clinical trial (Clinicaltrials.gov ID NCT02047110). Ixekizumab, a humanized anti-IL-17A monoclonal similar to secukinumab, is also in phase III trials in AS (Clinicaltrials.gov ID NCT02696798) and has demonstrated its efficacy in psoriasis [47]. To date, agents targeting IL-22 have not been trialed in AS. Further studies are needed to clarify the effects of IL-23, IL-17 and IL-22 blockade on extra-articular manifestations of AS.

Improved knowledge of how the gut microbiome contributes to disease is important so that we can understand how disruption of cytokine pathways crucial to gut homeostasis may impact patients. Furthermore, the impact of these new classes of biologics on bone formation remains to be determined. It will also be important to determine the relationship between IL-23-mediated and TNF-mediated inflammation in AS and to assess efficacy of bi-specifics that may target both cytokine pathways.

The Microbiome and Ankylosing Spondylitis

Involvement of the intestinal microbiome in the aetiopathogenesis in AS has been long been suggested, although a definitive link is yet to be established [48–54]. However, there is growing evidence for a role of the intestinal microbiome, with a recent study describing a discrete microbial signature in the terminal ileum (TI) of patients with AS [55••]. The microbial signature that clearly discriminated AS patients from healthy controls (HCs) is composed of seven families of bacteria, with higher abundances of *Lachnospiraceae*, *Ruminococcaceae*, *Rikenellaceae*, *Porphyromonadaceae* and *Bacteroidaceae* and decreases in *Prevotellaceae* and *Veillonellaceae*. Further analysis showed that interactions between these indicator species within the microbial community further shaped the AS microbial community signature [55••]. Interestingly, increases in abundance of *Prevotellaceae* and decreases of *Rikenellaceae* have also been reported in the intestinal microbiome in the HLA-B27

transgenic rat model of SPA [56]. These findings do not yet fully distinguish between effects of AS on the gut microbiome from the converse. Given that in reactive arthritis bacterial infections of the gut or urinary tract cause an AS-related spondyloarthropathy, reactive arthritis, there is strong support that AS itself is driven by interaction between the gut microbiome and the host immune system, leading to interest in targeting the microbiome as a therapy for AS.

The potential therapies include prebiotics, probiotics and faecal microbiota transplants (FMTs). These microbiome therapies are increasingly being used as last-line treatments for patients suffering with debilitating *Clostridium difficile* infections where previous conventional treatments have failed. A small study that compared patients on antibiotic treatment only to those who underwent FMT for *C. difficile* showed that FMT was more effective than antibiotics alone at resolving patient symptoms [57] with a clinical remission rate of ~90 % [57–59]. Over the last few years, the number and frequency of FMT being performed in clinics and hospitals have rapidly increased [60]. This new avenue of medicine brings new challenges including FMT sample preparation and screening. The standardized screening required for blood and blood products is not extended to donor faeces for FMT [61, 62]. The human intestinal tract can, at any point in time, contain numerous different combinations of bacteria, viruses and parasites [63]. Whilst they may not be harmful to the donor, potential pathogens as well as the healthy flora may be transplanted and lead to unexpected outcomes such as weight gain [64]. Currently, there is limited long-term safety data on FMT recipients [65]. Research is underway in laboratories such as OpenBiome (http://www.openbiome.org/) in the USA, on patient screening procedures and overall FMT regulation, similar to the model used by the Red Cross [59, 61]. There is currently no US Federal Drug Administration (FDA) regulation with respect to FMT procedures; however, there are draft guidelines [66].

One alternate option to FMT is the use of synthetic microbes, grown in the laboratory and tailored to each individual patient [65, 67]. Whilst FMT is becoming a routine treatment option for *C. difficile* infections, several studies of FMT in patients with Crohn's disease and ulcerative colitis showed variable efficacy [68, 69]. This suggests that whilst the results of FMT in an infection setting have been outstanding, when applied to a genetically complex, multifactorial disease setting, it is not that straightforward.

The role host genetics plays in shaping the intestinal microbiome is poorly understood. A recent study by Blekhman et al highlighted the effect of host genetic variation, particularly in immune-related pathways, in shaping the intestinal microbiome composition [70]. This work followed on from a study in twins that suggested that some members of the intestinal microbiome may in fact be inherited rather than acquired [71••]. Recent animal studies have shown that host gene polymorphisms and deletions result in shifts in microbiota composition and link certain genetic polymorphisms with microbial abundances [56, 72, 73]. This highlights the importance of underlying host genetics in community composition and raises concerns with respect to the long-term viability of FMT in complex genetic disease. The expectation is that since the microbiome interacts with the immune system, then transplanting the gut with 'healthy' intestinal flora will lead to interactions with the immune system which leads to a less inflammatory environment, reduces intestinal disease and provides overall improvement of symptoms and possibly disease. The caveat here is that many genes associated with AS are involved in mucosal immunology and microbial processing, so underlying host genetics may eventually override the transplant. It is therefore likely that in AS, microbiome treatment will require additionally some treatment to protect the healthy microbiome from effects of the host immune system.

To explore the hypothesis that genes predisposing to AS do so by influencing the gut microbiome, either directly or indirectly via the immune system, will require large cohorts of patients and controls with matched genetic, clinical and microbiome data. Larger studies will define the AS microbiome profile and interactions, raising the possibility of the use of the microbiome both as a biomarker and for novel therapeutic interventions.

Conclusions

Whilst less than a third of the genetic risk in AS has been defined, this information has informed successful development of novel therapies, and many others are in development. This highlights the strength of hypothesis-free genetics approaches to provide solid foundations for hypothesis-driven mechanistic research. There is now considerable optimism in the AS research community that this disease will be solvable, and treatments which induce true remission and prevent the long-term consequences of the disease will be developed given sufficient resources for research into this common condition.

Compliance with Ethical Standards

Conflicts of Interest TJK, AH and MEC declare that they have no conflicts of interest. MAB reports grants from Wellcome Trust, grants from NHMRC (Australia), grants from NIAMS (USA), grants from Arthritis Research UK and grants from Arthritis Australia, during the conduct of the study.

Human and Animal Rights and Informed Consent All reported studies/experiments with human or animal subjects performed by the authors have been previously published and were in compliance with all applicable ethical standards (including the Helsinki Declaration and its amendments, institutional/national research committee standards and international/national/institutional guidelines).

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