EXTENDED REPORT

No evidence for involvement of the *Toll-like receptor 4* (*TLR4*) A896G and *CD14*-C260T polymorphisms in susceptibility to ankylosing spondylitis

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Objectives: Ankylosing spondylitis (AS) is a multifactorial and polygenic disease. Apart from HLA, other genes very probably play a role in disease susceptibility. Indigenous bacteria of the gastrointestinal flora appear to play a role in the pathogenesis of the disease; therefore, genes controling the innate and acquired immune response are good candidates to study disease susceptibility. CD14 and Toll-like receptor 4 (TLR4) are key receptors for the sensing of bacteria. The *CD14* C-260T and *TLR4* A896G single nucleotide polymorphims are associated with aberrant signal transduction for bacterial agonists.
Methods: The distribution of the *CD14* C-260T and *TLR4* A896G polymorphisms was studied in genomic DNA from 113 unrelated white Dutch AS patients and 170 ethnically matched healthy controls. The diagnosis of AS was made according to the modified New York criteria. The *CD14* C-260T and *TLR4* A896G polymorphisms were genotyped by PCR-RFLP methods.
Results: No significant differences were found between patients and controls in the frequencies of the carriership of the less frequent *CD14*-260T allele (odds ratio 0.65; 95% confidence interval 0.37 to 1.15) or the *TLR4* 896G allele (1.68; 0.67 to 4.19).
Conclusions: There is no evidence for involvement of the *CD14* C-260T or *TLR4* A896G polymorphisms in susceptibility to AS. An important role of bacteria and genetic predisposition of the innate immune system in cases of AS cannot be excluded by these findings. Therefore, studies of the surprisingly highly polymorphic candidate genes in this field should be continued.

Despite the discovery of the association between ankylosing spondylitis (AS) and HLA-B27 over thirty years ago,[1 2] the precise role of the adaptive and innate immune system in the pathogenesis of this common rheumatic disorder has not been clarified.

Apart from genetic factors, bacteria are thought to be crucial in the pathogenesis of AS, the prototype of the spondylarthropathies. Over the past decades it has been shown that bacterial infections can trigger the onset of at least one type of spondylarthropathy, reactive arthritis (ReA). ReA occurs frequently after infectious diarrhoea caused by *Shigella flexneri, Salmonella typhimurium* and *enteritides, Yersinia enterocolitica*, or *Campylobacter jejuni*,[3] or can be caused by urogenital infections with *Chlamydia trachomatis* (for a review, see Zeidler and Schumacher[4]). However, so far no specific pathogen has been consistently linked to the development of AS.[5]

The acquired and the innate immune systems play important roles in the host defence against pathogens. Innate immunity refers to the inborn resistance that is already present the first time a pathogen is encountered; it does not require prior exposure and is not modified significantly by repeated exposures to the pathogen over the life of an individual. Acquired immunity refers to resistance that is weak or absent on first exposure, but that increases dramatically with subsequent exposures to the same specific pathogen.

Cluster of differentiation 14 (CD14) and Toll-like receptor 4 (TLR4) are, together with the MD-2 protein, part of the lipopolysaccharide (LPS) receptor complex of the innate immune system. LPS is a glycolipid specific to gram negative bacterial cell walls. CD14 is the receptor of monocytes/macrophages for complexes of LPS and LPS binding protein (LBP).[6] LPS is transferred from a CD14/LBP complex to a TLR4/MD-2 membrane receptor complex at the cell surface.[7] A transmembrane signal leads to nuclear factor (NF)-κB activation, subsequent induction of gene transcription and release of pro-inflammatory cytokines, such as tumour necrosis factor α, initiating inflammation.[8]

Besides LPS, other bacterial and host agonists are reported to be recognised by TLR4 including *C. trachomatis* and host heat shock protein 60 (HSP60) ligands.[9]

Although, to the best of our knowledge there is no evidence for linkage in the region of the *CD14*-260 single nucleotide polymorphism (SNP) on chromosome 5q31, functional studies imply this gene may be of relevance to AS. The *CD14*-260 promoter SNP enhances transcriptional activity,[10] and is associated with enhanced monocyte CD14 expression.[11 12] Individuals carrying the TT genotype have significantly higher serum levels of soluble CD14 than have those with CC or CT genotypes and they have increased density of CD14 in blood monocytes.[11 12]

Recently, allele *CD14*-260T has been found to be significantly positively associated with ulcerative colitis (UC) in Japan.[13] However, in Germany, the allele *CD14*-260T and the genotype TT were increased in Crohn's disease (CD) but not in UC.[14] More recently, the same group of investigators found allele *CD14*-260T and TT genotype frequencies of the *CD14* SNP to be significantly increased only in patients with CD carrying at least one of the susceptibility variants Arg702Trp,

Abbreviations: AS, ankylosing spondylitis; CD, Crohn's disease; HSP, heat shock protein; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; NF, nuclear factor; SNP, single nucleotide polymorphism; TLR, Toll-like receptor; UC, ulcerative colitis

Gly908Arg, or Leu1007fsinsC in the *CARD15* gene.[15] Both UC and CD can have disease manifestations that belong to the spondylarthropathies.

So far, there have been no studies addressing the possible association of the *CD14*-C260T SNP with AS, although carriage of the *CD14*-260T allele might increase the susceptibility of female patients to the development of chronic spondylarthropathy.[16]

The *TLR4* gene is located on chromosome 9q32-q33 at 115.8 Mb. Genome scanning in AS identified linkage to a region on chromosome 9q. Marker D9S1826 on chromosome 9q34 at 133.9 Mb achieved a lod score of 2.8 ($p<0.0005$), and marker D9S1682 at 120.4 Mb achieved a lod score of 2.3 ($p<0.005$), both suggestive of linkage.[17] Given the distance between the *TLR4* gene and the D9S1826 and D9S1682 markers, it is unlikely that *TLR4* can warrant the linkage to the region on chromosome 9. Nevertheless, similar to the situation in *CD14*, functional studies imply that *TLR4* might be of relevance to AS.

The A896G SNP is located in the ectoplasmic receptor domain of *TLR4*, resulting in an aspartic acid to glycine substitution at position 299. Recently, the A896G SNP was shown to be associated with ''an endotoxin hyporesponsive phenotype'' in concert with other genetic changes or acquired factors that influence the complex physiological response to LPS.[18] However, monocytes from individuals heterozygous for allele *TLR4* 896G do not exhibit a deficit in recognition of LPS from several bacterial strains.[19] Moreover, the heterozygous *TLR4* A896G polymorphism does not influence LPS induced human whole blood cytokine release[20]. Other agonists for TLR4 have not yet been tested functionally.

The present study was designed to study the frequency of the *CD14* C-260T and *TLR4* A896G SNPs in AS to find out whether these polymorphisms contribute to disease susceptibility and clinical manifestations.

PATIENTS AND METHODS

Subjects

After informed consent, 113 AS patients were recruited from the outpatient Department of Rheumatology of the Jan van Breemen Institute. All AS patients fulfilled the diagnosis of AS, according to the modified New York criteria.[21] Controls were 170 randomly selected healthy blood donors from the Amsterdam region. All subjects were unrelated Dutch whites.

Methods

Genotyping the *CD14* C-260T SNP (NCBI SNP cluster ID: rs2569190) was performed with forward primer 5′-TCACC TCCCCACCTCTCTT-3′ and 5′-CCTGCAGAAT_CCTTCCTGTT-3′. Digestion overnight with *Hae*III (New England Biolabs, UK) of the 107 bp amplicons was followed by separation by electrophoresis on 4% agarose gels, staining with ethidium bromide, and visualisation under ultraviolet light, and resulted in two fragments of 83 and 24 bp (C allele) or 107 bp (T allele).

Genotyping of the *TLR4* A896G SNP (NCBI SNP cluster ID: rs4986790) was performed as described by Morré *et al*.[22]

Table 1 Demographic and clinical characteristics of patients with ankylosing spondylitis (n = 113)

Characteristics	
Age, years*	52.6 (12.5; 19 to 79)
Age at first complaints, years†	23.0 (19.0 to 31.5)
Age at actual diagnosis, years†	34.0 (25.0 to 42.5)
Years between first complaints and diagnosis†	7.0 (2.0 to 12.5)
Women	12.5%
HLA-B27 positive	98%
Iridocyclitis	40%
Peripheral arthritis	37%
First degree relatives with AS	28%

*Mean (SD; range), †median (interquartile range).

Statistical analysis

Allele and genotype frequencies were tested for Hardy-Weinberg equilibrium by χ^2 test. To compare frequencies, χ^2 or Fisher's exact test was used. The possible influence of interaction of the two studied polymorphisms in patients and controls was analysed by logistic regression. A two sided p value <0.05 was considered significant. The magnitude of association was expressed as odds ratio with a 95% confidence interval (CI). Statistical analysis was performed by SPSS 10.0 for Windows.

RESULTS

Characteristics of the 113 AS patients are summarised in table 1. Genotype and allele frequencies in AS patients and controls for the *CD14* C-260T and the *TLR4* A896G SNP in table 2. The genotype frequencies in the control group did not deviate from HWE equilibrium. No significant differences were observed between AS patients and controls in the frequencies of carriership of the *CD14*-260T and *TLR4* 896G alleles (table 2). Logistic regression analysis showed absence of modification of the odds ratios (OR). Thus, no interaction between the *CD14* C-260T and *TLR4* A896G polymorphisms was found (data not shown).

No significant associations were found between carriage of each of the alleles *CD14*-260T and *TLR4* 896G with sex, existence in past or present of peripheral arthritis or acute anterior uveitis, age at first complaints, years between first complaints and actual diagnosis of AS, or the number of patients with at least one first degree family member with AS (table 3).

DISCUSSION

The studied SNPs reported to affect gene function in two genes that play a role in innate immunity, *CD14* C-260T and *TLR4* A896G, are not significantly associated with the susceptibility to or the clinical manifestations of AS.

Table 2 Allele and genotype frequencies of the *CD14* C-260T and the *TLR4* A896G polymorphisms in ankylosing spondylitis patients and controls

	AS patients (n = 113)	Controls (n = 170)		p
CD14 C-260T				
Allele				
C	111 (49.1%)	178 (52.4%)	OR = 0.88	0.45
T	115 (50.9%)	162 (47.6%)	95% CI = 0.63 to 1.23	
Genotype				
CC	23 (20.4%)	48 (28.2%)	$\chi^2 = 2.87$	0.24
CT	65 (57.5%)	82 (48.2%)		
TT	25 (22.1%)	40 (23.5%)		
TLR4 A896G				
Allele				
A	219 (96.9%)	322 (94.7%)	OR = 1.75	0.21
G	7 (3.1%)	18 (5.3%)	95% CI = 0.72 to 4.26	
Genotype				
AA	106 (93.8%)	153 (90.0%)	$\chi^2 = 1.64$	0.44
AG	7 (6.2%)	16 (9.4%)		
GG	0 (0%)	1 (0.6%)		

No significant difference was found between patients and controls in the frequencies of the carriership of the *CD14*-260T (OR 0.65; 95% CI 0.37 to 1.15) or *TLR4* 896G (OR 1.68; 95% CI 0.67 to 4.19) allele.

Table 3 Clinical characteristics of patients with ankylosing spondylitis (n = 113) in relation to carriage of the *CD14*-260T or the *TLR4* 896G allele

	TLR4 896G				*CD14* 260T			
Characteristics	**Carrier**	**Non-carrier**	**OR (95% CI)**	**p**	**Carrier**	**Non-carrier**	**OR (95% CI)**	**p**
Female/male	1/6	12/85	1.18 (0.13 to 10.67)	1.0	11/72	2/19	1.45 (0.30 to 7.11)	1.0
PA (+/−)	3/4	35/60	0.78 (0.16 to 3.68)	1.0	33/48	5/16	0.46 (0.15 to 1.36)	0.15
AAU(+/−)	5/2	34/57	0.24 (0.04 to 1.30)	0.11	31/48	8/11	1.13 (0.41 to 3.11)	0.82
Familial(+/−)	2/4	23/61	0.75 (0.13 to 4.40)	0.67	21/51	4/14	0.69 (0.20 to 2.36)	0.56
Age at first complaints, mean (SD) (range)	23.83 (6.18) (18 to 32)	25.64 (9.78) (9 to 55)		0.57*	25.17 (9.39) (9 to 51)	25.89 (10.37) (16 to 55)		0.63*
Years to actual diagnosis, mean (SD) (range)	9.08 (11.43) (1 to 32)	9.27 (8.83) (1 to 40)		0.77*	8.99 (8.79) (1 to 37)	10.33 (9.74) (1 to 40)		0.43*

AAU, acute anterior uveitis; PA, peripheral arthritis; familial, patients with at least one first degree family member with AS; +/−, present/not present; years to actual diagnosis, number of years between the first complaints and the actual diagnosis of AS. *Mann-Whitney test.

Nevertheless, this study cannot exclude that specific phenotypic features of AS are related to *TLR4* and/or *CD14* genotypes. Therefore, it would be of interest to assess whether the *CD14* C-260T and *TLR4* A896G SNPs are associated with disease severity (C-reactive protein, ESR, Bath Ankylosing Spondylitis Disease Activity Index, Bath Ankylosing Spondylitis Functional Index), anatomical evolution (Bath Ankylosing Spondylitis Metrology Index, sacroiliitis, degree of ankylosis), or involvement of the gut. Moreover, although this study could not find a significant association between *CD14* C-260T and *TLR4* A896G and the clinical manifestations of AS, the number of patients in this analysis was too low to warrant a definitive conclusion.

Bacteria are thought to play a crucial role in the pathogenesis of AS. In animal models such as B27 transgenic rats, in ~50% of the cases, the presence of the bacterial flora is obligatory for development of inflammatory gut lesions and peripheral and axial inflammatory joint lesions, similar to AS. When raised in a germfree environment, inflammatory intestinal or joint disease does not develop until the normal bacterial flora is restored.[23]

AS occurs frequently in patients with newly diagnosed inflammatory bowel disease—that is, UC or CD.[24] On the other hand, patients with spondylarthropathies often have subclinical gut involvement.[25] This gut inflammation is clinically and histologically closely related to CD.[26 27]

It is likely that an altered host response of the innate immune system may contribute to the development of AS. This hypothesis is supported by the findings on genetic susceptibility of CD, which have shifted the focus of research in this disease towards innate immunity. The relationship with acquired immunity provides a good insight into the mechanisms that control inflammation in the gut.[28] Recently, IgA levels of anti-*Saccharomyces cerevisiae* mannan antibodies, a marker of CD, have been found to be significantly higher in patients with spondylarthropathies, and more specifically in AS, than in healthy controls and patients with rheumatoid arthritis.[29]

Besides the association reported with *CD14*,[15] an established genetic association of the innate immune system with CD is the Leu1007fsinsC insertion mutation at position 3020 in the *CARD15* gene, located on chromosome 16p12. This mutation was described as an important susceptibility factor for CD.[30–33] However, studies from our group in the Netherlands,[34] and from Spain[35] and the UK[36] have disproved that the commonest *CARD15* mutations found in these populations contribute to the disease susceptibility in primary AS.

The identification and functional characterisation of mutants, such as those in *CD14*, *TLR*, and *NOD2/CARD15* receptors, provide a new insight into the relationships between bacteria and the host, and between bacteria and the development of disease.[37 38] A previous study in Germany in a population of similar ethnic background to the one we have studied has revealed interactions of the *CARD15* and *CD14* genes that increase the risk for developing CD.[15] Interestingly, so far the associations found in the three genes *CD14*, *CARD15*, and *TLR4* have not been reproduced in all population studies demonstrating the complexity of the interaction between genes and environment. As recently described by Vercelli,[39] ''Genetic variation in innate immunity genes at the interface with the environment may skew the human immune response in critical ways by modulating the impact of pathogen exposure''. Therefore, although we found no significant associations between the *CD14*-C260T and *TLR4* A896G SNPs with AS in general or with clinical characteristics, the significance of functional polymorphisms in genes that are involved in innate immunity deserve to be studied in this disease, in which bacteria and HLA appear to be important in the pathogenesis.

These are early days in the studies of innate immunity in AS and in many other chronic inflammatory diseases. It is possible that polymorphisms in other receptors sensing other bacteria than the ones recognised by *CD14*, *TLR4*, and *CARD15* may prove to be important in understanding the synergy between clinical manifestations, epidemiology, molecular biology, and pathophysiology of inflammation.

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