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Associations of toll-like receptor (TLR)-4 single nucleotide polymorphisms and rheumatoid arthritis disease progression: An observational cohort study

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ABSTRACT

Objective: To examine the associations of toll-like receptor (TLR)-4 single nucleotide polymorphisms (SNPs) with disease progression in rheumatoid arthritis (RA).
Methods: A total of 1188 RA patients were genotyped for TLR4 SNPs (rs1927911, rs11536878, and rs4986790). Measures of disease activity were examined, including Disease Activity Score-28 (DAS28), Multidimensional Health Assessment Questionnaire (MD-HAQ), Clinical Disease Activity Index (CDAI), and Simplified Disease Activity Index (SDAI). Genetic associations with these longitudinal measures were examined using generalized estimating equations in both univariate and multivariate analyses. Analyses were then stratified by antigen specific anti-citrullinated peptide antibody (ACPA) status including antibody to citrullinated fibrinogen and citrullinated histone H2B.
Results: Disease activity measures progressed less over time in the homozygous minor allele group of rs1927911 including DAS28 ($p < 0.001$), CDAI ($p = 0.008$), and MD-HAQ ($p = 0.015$) in univariate analysis and DAS28, CDAI and SDAI in multivariate analysis. Disease activity progression among those homozygous for the minor allele tended to be lower in the groups with positive ACPA though major differences by autoantibody status were not identified. There were no associations of TLR4 rs11536878 and rs4986790 SNPs with RA disease activity progression.
Conclusions: In this population, TLR4 rs1927911 genotypes are associated with disease activity independent of other covariates.

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1. Introduction

Toll-like receptors (TLRs) are proteins that identify pathogen-associated molecular patterns and initiate signaling pathways that result in both innate and adaptive immune responses. The resulting chronic inflammation and the well-recognized interactions of TLRs with numerous endogenous ligands have implicated this pathway in a number of disease states including rheumatoid arthritis (RA) [1,2]. In particular, TLR4 has been identified as an important avenue of investigation in understanding RA pathogenesis in addition to serving as a possible target in future disease interventions.

It has been demonstrated, for instance, that TLR4 is overexpressed in RA synovium and both citrullinated proteins and immune complexes containing citrullinated proteins are potent stimulators of TLR4 [3–7], important given the speculated role of citrullinated neo-antigen in the etiopathogenesis of RA. More specifically, fibrin stimulates TLR4 on RA synovial fibroblasts resulting in elevated levels of interleukin (IL)-6 and IL-8 and citrullination of fibrin by peptidylarginine deiminases results in even higher cytokine levels than that observed following stimulation with unmodified fibrin [4]. It has also been shown that citrullinated fibrinogen also stimulates TLR4 on macrophages resulting in increased TNF-α and chemokine production; the effect of which can be amplified by fibrinogen immune complex formation [3]. Investigations using animal models of inflammatory arthritis also implicate TLR4 in RA. Mice with non-functional TLR4 or mice deficient of MyD88 (an adaptor protein in the TLR4 signaling pathway) are protected from inflammatory arthritis [8–10]. Furthermore, TLR4

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knockout mice with T cell-mediated arthritis are protected from severe arthritis and demonstrate lower numbers of Th17 cells, and lower levels of IL-17 suggesting that TLR4 is centrally involved in the adaptive immune response characteristic of the disease [11,12].

TLR4 might also serve as a therapeutic target in RA [13]. Arthritis prone mice treated with TLR4 antagonists display lower IL-1β expression in both chondrocytes and synovial tissues in addition to having substantially less severe disease [14]. The converse is also true in that stimulating TLR4 with lipopolysaccharides derived from Gram-negative bacteria cell membranes leads to more severe arthritis [11]. Based on these studies it is reasonable to infer that TLR4 might be involved in the pathophysiology of RA and that genetic variation in TLR4 might also influence the natural course of RA.

Single-nucleotide polymorphisms (SNPs) within the TLR4 gene have been investigated in RA with most studies focusing on prevalence and disease risk [15–24]. A limited number of studies, however, have examined the potential genetic associations with disease progression with results that have been inconsistent across available reports [17,20,21,23]. As part of the present study, we utilized longitudinal clinical data from a well characterized observational cohort of RA patients to determine if TLR4 polymorphisms were associated with RA disease progression. We also explored whether these associations were impacted by anti-citrullinated peptide antibody (ACPA) status, since disease-specific ACPA has been demonstrated to contribute to immune complex formation and TLR4 signaling.

2. Methods

2.1. Study participants

The Veterans Affairs Rheumatoid Arthritis (VARA) registry is a longitudinal observational cohort consisting of U.S. Veterans that initiated enrollment in 2003 [25,26]. VARA participants from 11 enrolling VA sites (Birmingham, AL, Brooklyn, NY, Dallas, TX, Denver, CO, Iowa City, IA, Jackson, MS, Little Rock, AR, Omaha, NE, Portland, OR, Salt Lake City, UT, and Washington, DC) comprised the study cohort for this analysis. All VARA patients satisfied the American College of Rheumatology (ACR) 1987 classification criteria for RA [27]. This study was approved by the institutional review boards at each of the participating centers and the VARA Scientific Ethics and Advisory Committee.

2.2. Genetic data

To limit population stratification, the study was limited to individuals self-reporting Caucasian race/ethnicity. TLR4 genotyping was performed using banked DNA from 1188 available Caucasian patients. Patient serum and whole blood were collected at enrollment. A QiaAmp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) was used for genomic DNA extraction. The complete coding region of the TLR4 gene, the intronic sequence and approximately 6 kb of 5′ genomic and 2 kb of 3′ genomic areas, was resequenced from CEPH Corriel DNA as part of the Innate Immunity Program in Genomic Applications. Using publically available software (Genome Variation Server, University of Washington, Program in Genomic Applications), a haplotype-tagging strategy was initially examined using an algorithm to identify polymorphisms with a minor allele frequency ≥ 10% and a within-bin linkage equilibrium (LD) exceeding 0.7. There were a total of 18 TLR4 SNPs identified with a minor allele frequency exceeding 10%, resulting in 6 bins. Of these, two SNPs from two bins were selected a priori (rs11536878 and rs1927911) and genotyped as part of a strategy to reduce possible false discovery. An additional SNP with a reported minor allele frequency < 5%, rs4986790, was selected for analysis based on preliminary data implicating this polymorphism, along with rs11536878 and rs1927911, in RA or other systemic inflammatory disease states as discussed in Section 4 of this report.

Genotyping was completed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom Inc., San Diego, CA, USA). Multiplex PCR assays and associated extension reactions were designed using SpectroDesigner software (Sequenom Inc.). Primer extension products were loaded onto a 384-element chip with a nanoliter pipetting system (Sequenom) and analyzed by a MassArray mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The resulting mass spectra were analyzed for peak identifications using SpectroTyper RT 4.0 software (Sequenom). For genotyping quality control, all SNPs exhibited call rates exceeding 99% and all three polymorphisms were found to be in Hardy–Weinberg equilibrium.

2.3. Patient characteristics and outcome measures

Outcome measures examined included the Disease Activity Score based on a 28 joint count (DAS28), the Multidimensional Health Assessment Questionnaire (MD-HAQ; range 0–3), the Clinical Disease Activity Index (CDAI; range 0–76), and the Simplified Disease Activity Index (SDAI; range 0–86) [28–31]. Outcomes were measured at enrollment and collected longitudinally during routine follow-up visits. The range of follow-up visits was 0 to 60 with a mean of 10 visits. Because time between visits varied based on physician discretion and patient need, disease duration at the time of each visit was selected as the time variable for this analysis. Patient characteristics collected at enrollment included age, gender, self-reported race/ethnicity, body mass index (BMI; kg/m^2), education (high school degree or higher), smoking history (ever vs. never), rheumatoid nodules (yes/no), swollen joint count (range 0–28), tender joint count (range 0–28), high sensitivity C-reactive protein (hsCRP, mg/l), erythrocyte sedimentation rate (ESR; mm/h), methotrexate (MTX; yes/no), and anti-TNF-α therapy (yes/no). Comorbidities including cerebrovascular disease, diabetes mellitus, hypertension, hyperlipidemia, ischemic heart disease, chronic kidney disease, interstitial lung disease, chronic obstructive pulmonary disease (COPD), and depression were collected and summed as a comorbidity count (range 0–9). ACPA was measured using second-generation anti-cyclic citrullinated peptide (anti-CCP) antibody ELISA (Diastat, Axis-Shield Diagnostics, Dundee, Scotland) with levels > 5 U/ml considered positive. Antigen-specific ACPA, including antibody to citrullinated fibrinogen (anti-cFb) and antibody to citrillinated histone H2B (anti-cH2B) was selected for the analysis a priori and measured using a bead-based multiplex antigen array on the BioPlex platform, which measures disease-specific autoantibody reactivity to citrullinated peptides [32]. Positivity for antigen-specific ACPA was set using previously derived values [33]. Prior analyses have suggested that cH2B and cFb are among the primary antigens driving autoimmunity in RA with antibody targeting these autoantigens implicated in immune complex formation that drives TLR4 signaling [3,34]. Rheumatoid factor (RF) was measured with nephelometry (Siemens Healthcare Diagnostics, Munich, Germany), levels ≥ 15 IU/ml were considered positive.

2.4. Statistical analysis

Univariate generalized estimating equations (GEEs) with exchangeable correlation were used to evaluate the association of TLR4 SNPs with disease progression (DAS28, HAQ, CDAI, and SDAI). To analyze disease progression over time we used an interaction product term that included the gene of interest and disease duration. Preliminary univariate analysis demonstrated clinical significance only for TLR4 SNP rs1927911 therefore rs4986790 and rs11536878 were not included in subsequent analyses (data not shown). Baseline clinical characteristics were compared based on rs1927911 genotype using analysis of variance (ANOVA) for continuous variables and chi square test for categorical variables. Genotype frequencies were compared with the expected population distributions, described in the HapMap project, using chi square tests [35]. Multivariate GEE was also done to evaluate the

association of TLR4 SNP rs1927911 with disease progression (DAS28, HAQ, CDAI, and SDAI) accounting for potential confounders. Covariates including age, gender, comorbidity count, anti-CCP positivity, RF positivity, smoking status, MTX, and anti-TNF therapy were forced into the model and additional covariates with a univariate p-value < 0.2 (BMI) were also included in the final multivariate analysis. In addition to limiting genotyping to three SNPs of interest, we used a two-fold strategy to further mitigate possible type I error (incorrectly rejecting the null hypothesis). First we used a conservative Bonferonni correction based on 3 SNPs examined, resulting in a p-value < 0.0167 (0.05/3) required to reach statistical significance. Secondly, bootstrapping was used to reduce selection bias and minimize the false discovery rate as described elsewhere [36]. In brief, bootstrapping is a multiple resampling technique that is an alternative approach to obtaining an independent sample for minimizing estimation bias in locus specific genetic effects [36].

To determine if the association of TLR4 SNPs and disease progression was dependent on ACPA status, univariate and multivariate GEE analyses were stratified by ACPA positivity and by the aforementioned antigen-specific ACPA. All statistical analyses were performed using Stata, version 13.1 (StataCorp., Stata Statistical Software).

3. Results

3.1. Patient characteristics and genotype frequency

There were 1188 Caucasian RA patients included in the analysis. Patients were predominantly male (93%) with a mean age of 64.6 years and mean disease duration of approximately 12 years at enrollment. Patients were largely anti-CCP (78%) and RF (77%) positive. The prevalence of anti-cFb antibody (23%) and anti-cH2B antibody (34%) positivity was lower. Mean BMI was 28 kg/m^2, more than 80% had ever smoked, and 86% had a high school education or more. Patient characteristics by TLR4 rs1927911 genotype are shown in Table 1. There were 677 (57%) patients with TLR4 rs1927911 genotype CC, 434 (36.5%) with TC genotype, and 77 (6.5%) were homozygous for the minor allele (TT genotype).

Table 1
Rheumatoid arthritis patient characteristics at enrollment stratified by TLR4 rs1927911 genotype (means ± SD or %).[a, b]

	TLR4 rs1927911 genotype		
	CC n = 677	TC n = 434	TT n = 77
Age, years	65.1 ± 11.1	63.8 ± 10.7	64.5 ± 9.3
Disease duration, years	11.5 ± 11.5	12.0 ± 11.5	12.5 ± 12.8
Gender, % men	92.6	93.3	93.5
Body mass index, kg/m^2	27.7 ± 5.6	28.4 ± 6.0	28.8 ± 4.8
≥ High school education, %	85.0	86.7	84.9
Comorbidity count (0 to 9)	1.9 ± 1.4	2.0 ± 1.4	1.9 ± 1.4
Ever smoker, %	80.7	81.6	75.3
Positive rheumatoid nodules, %	36.6	37.3	41.8
Swollen joint count (0 to 28)	4.2 ± 5.3	4.8 ± 6.1	4.6 ± 6.4
Tender joint count (0 to 28)	5.3 ± 6.9	5.7 ± 7.1	5.3 ± 8.0
Erythrocyte sedimentation rate (mm/h)	24.4 ± 22.0	24.4 ± 23.7	23.8 ± 21.2
High sensitivity C-reactive protein (mg/l)	1.2 ± 2.1	1.2 ± 1.9	1.3 ± 2.0
Disease-modifying treatments, %			
Methotrexate	54.6	53.3	54.2
Anti-tumor necrosis factor (TNF)	24.2	25.1	20.8
Anti-CCP antibody positive, %	77.3	77.8	81.6
Rheumatoid factor positive, %	76.4	76.3	79.2

[a] Chi squared was used for binomial and categorical comparisons and analysis of variance or Kruskal–Wallis test (when normality assumption was not satisfied) was used to compare means for continuous data.

[b] Body mass index reached threshold of statistically significant difference comparing values for all three genotypes (p-value < 0.05); all other differences by genotype were non-significant.

3.2. Baseline disease activity

At baseline the mean (±SD) DAS28 was 3.88 (±1.61); MD-HAQ was 0.90 (±0.61); CDAI was 16.68 (±13.61); and SDAI was 18.06 (±14.33). Clinical severity scores distributed by rs1927911 genotype (CC vs. CT vs. TT) were similar at baseline for DAS28 (p = 0.908), MD-HAQ (p = 0.651), CDAI (p = 0.818), and SDAI (p = 0.898).

3.3. Univariate and multivariate associations of TLR4 SNPs with outcomes

Results from final univariate and multivariate analysis are summarized in Table 2. In unadjusted analysis, the heterozygous genotype group (CT) for TLR4 SNP rs1927911 did not substantially differ from the homozygous dominant allele group (CC) in clinical measures of disease progression including DAS28, MD-HAQ, CDAI, and SDAI. However, the homozygous minor allele group (TT) had significantly less disease activity over time compared to the homozygous dominant allele group (CC) for DAS28, MD-HAQ, and CDAI, and marginally significant less SDAI in unadjusted analyses. In multivariate analysis, the findings persisted and remained highly significant for DAS28 (p < 0.001), CDAI (p = 0.010) and SDAI (p = 0.014), although the association with MD-HAQ (p = 0.018) did not reach statistical significance (α = 0.0167).

3.4. Analyses stratified by ACPA status

In stratified analyses, the homozygous minor allele (TT) remained inversely associated with DAS28 in the anti-CCP antibody positive group but lost significance in the anti-CCP antibody negative group (Table 3). However, the effect size (β) was greater in the anti-CCP antibody negative group and lost significance in this group due at least in part to the smaller sample size of anti-CCP antibody negative patients in our cohort (n = 263). Similar results were identified for CDAI and SDAI, and no differences were observed for MD-HAQ (Table 3). When stratifying by antigen-specific ACPA, the association of the TT genotype (vs. CC) was significant only in the larger anti-cFb antibody negative group for DAS28 (p = 0.001), although the corresponding effect sizes were smaller than that observed in the anti-cFb antibody positive group (Table 4). Effect sizes corresponding to the TT genotype (vs. CC), however, were substantially larger in the anti-cFb antibody positive group for CDAI and SDAI compared to the negative group although these associations did not reach statistical significance. When the analysis was stratified by anti-cH2B antibody status; the homozygous minor allele (TT) remained significantly associated with lower DAS28 values for both autoantibody positive and negative groups, although there were no significant associations with MD-HAQ, CDAI or SDAI values after stratification (Table 5). Effect sizes based on the TT genotype, reflected by the β-coefficients, were numerically higher for DAS28, CDAI, and SDAI in the anti-cH2B antibody positive group compared to the anti-cH2B antibody negative group.

4. Discussion

TLR4 is a fundamental constituent of the innate immune system in recognizing lipopolysaccharide endotoxin of Gram-negative bacteria [37]. More recently, TLRs have also been implicated as a key component in the recognition of endogenous damage-associated molecular patterns (DAMPs) released from locally damaged cells and other immune cells as danger signals [2,38]. Once activated, a number of intracellular signaling events occur which can result in augmented cytokine expression. Specifically in RA, TLR4 activation has been shown to result in elevated levels of IL-1β, TNF-α, IL-6, IL-10, and IL-17 [6,7,39,40]. Furthermore, blocking TLR4 or its adaptor molecules appears to mitigate this response and has therefore been proposed as a possible therapeutic target in RA [14,41,42]. It therefore leads to suggest that TLR4 SNPs might also alter disease severity in RA by modifying TLR4 function,

Table 2
Associations of TLR4 rs1927911 with rheumatoid arthritis disease progression over time.[a, b, c]

Genotype	Univariate			Multivariate		
	β-Coef	95% CI	p-Value	β-Coef	95% CI	p-Value
DAS28						
CC	Ref.	–	–	Ref.	–	–
CT	−0.007	−0.022, 0.008	0.351	−0.007	−0.022, 0.008	0.374
TT	−0.042	−0.063, −0.021	<**0.001**	−0.045	−0.068, −0.022	<**0.001**
MD-HAQ						
CC	Ref.	–	–	Ref.	–	–
CT	0.004	−0.005, 0.012	0.385	0.003	−0.004, 0.011	0.397
TT	−0.014	−0.026, −0.003	**0.015**	−0.014	−0.026, −0.002	0.018
CDAI						
CC	Ref.	–	–	Ref.	–	–
CT	−0.080	−0.228, 0.068	0.292	−0.075	−0.226, 0.077	0.334
TT	−0.293	−0.509, −0.077	**0.008**	−0.359	−0.633, −0.085	**0.010**
SDAI						
CC	Ref.	–	–	Ref.	–	–
CT	−0.088	−0.236, 0.061	0.247	−0.066	−0.232, 0.101	0.438
TT	−0.276	−0.507, −0.046	0.019	−0.342	−0.618, −0.067	**0.015**

[a] DAS = Disease Activity Score, MD-HAQ = Multidimensional Health Assessment Questionnaire, CDAI = Clinical Disease Activity Index, SDAI = Simplified Disease Activity Index.
[b] Multivariate model adjusted for age, gender, comorbidity count, body mass index, smoking, rheumatoid factor positivity, anti-cyclic citrullinated peptide antibody positivity, methotrexate and anti-tumor necrosis factor use.
[c] Bolded p-values were considered to reach significance after Bonferroni correction ($p < 0.0167$).

its gene expression, or possibly through close linkage with other culprit gene mutations.

Utilizing data collected from U.S. Veterans, we examined the association of TLR4 SNPs with RA disease activity over time. We found that individuals with the homozygous minor allele of TLR4 rs1927911 had lower levels of disease activity during the course of their disease as measured by DAS28 and other composite measures, an association that was independent of other covariates examined for DAS28 in addition to both CDAI and SDAI.

In addition, we utilized antigen specific ACPA to determine if the aforementioned associations with the TT genotype were affected by autoantibody status. We did not observe any major genotypic differences between antigen-specific ACPA positive and negative groups, however the data suggested that disease activity scores may be lower among those with the TT genotype positive for anti-cFb or anti-cH2B antibodies. However, these exploratory findings should be interpreted with caution given the inconsistent results observed with analyses stratified by anti-CCP antibody status compared to those stratifying by antigen-specific ACPA in addition to the substantial overlap in 95% CIs for these genotype effects based on autoantibody status. Although we consider these analyses to be exploratory and hypothesis generating, these results suggest that studies in larger cohorts with precise measurement of antigen-specific ACPA and possibly ACPA-containing immune complexes might prove to be informative, particularly since ACPA-containing immune complexes are activators of TLR4 and appear to induce inflammatory responses in RA [3]. The absence of a strong difference across multiple outcome measures in the antigen-specific ACPA groups suggests that TLR4 activation in RA might not be solely driven by immune complex formation. This parallels other findings suggesting that fibrin and even more so citrullinated fibrin are potent activators of TLR4 even in the absence of ACPA-containing immune complexes [4].

Table 3
Associations of TLR4 rs1927911 with rheumatoid arthritis disease progression stratified by anti-cyclic citrullinated protein (anti-CCP) antibody status.[a, b, c]

Genotype	Anti-CCP Negative (n = 263)			Anti-CCP Positive (n = 919)		
	β-Coef	95% CI	p-Value	β-Coef	95% CI	p-Value
DAS28						
CC	Ref.	–	–	Ref.	–	–
CT	−0.016	−0.063, 0.030	0.485	−0.004	−0.020, 0.012	0.633
TT	−0.081	−0.150, −0.012	0.022	−0.044	−0.070, −0.019	**0.001**
MD-HAQ						
CC	Ref.	–	–	Ref.	–	–
CT	0.001	−0.017, 0.020	0.879	0.004	−0.005, 0.012	0.400
TT	−0.024	−0.050, 0.003	0.080	−0.013	−0.027, 0.002	0.080
CDAI						
CC	Ref.	–	–	Ref.	–	–
CT	0.080	−0.460, 0.619	0.772	−0.083	−0.247, 0.080	0.319
TT	−0.833	−2.364, 0.697	0.286	−0.352	−0.562, −0.141	**0.001**
SDAI						
CC	Ref.	–	–	Ref.	–	–
CT	0.099	−0.392, 0.590	0.692	−0.077	−0.248, 0.093	0.374
TT	−0.891	−2.830, 1.048	0.368	−0.336	−0.565, −0.107	**0.004**

[a] DAS = Disease Activity Score, MD-HAQ = Multidimensional Health Assessment Questionnaire, CDAI = Clinical Disease Activity Index, SDAI = Simplified Disease Activity Index.
[b] Multivariate model adjusted for age, gender, comorbidity count, body mass index, smoking, rheumatoid factor positivity, anti-cyclic citrullinated peptide antibody positivity, methotrexate and anti-tumor necrosis factor use.
[c] Bolded p-values were considered to reach significance after Bonferroni correction ($p < 0.0167$).

Table 4
Associations of TLR4 rs1927911 with rheumatoid arthritis disease progression stratified by anti-citrullinated fibrinogen (anti-cFb) antibody positivity.[a, b, c]

Genotype	Anti-cFb Negative (n = 913)			Anti-cFb Positive (n = 275)		
	β-Coef	95% CI	p value	β-Coef	95% CI	p value
DAS28						
CC	Ref.	–	–	Ref.	–	–
CT	−0.002	−0.018, 0.014	0.785	−0.033	−0.071, 0.006	0.098
TT	−0.042	−0.067, −0.017	**0.001**	−0.079	−0.186, 0.028	0.149
MD-HAQ						
CC	Ref.	–	–	Ref.	–	–
CT	0.004	−0.005, 0.012	0.408	−0.001	−0.022, 0.019	0.889
TT	−0.015	−0.027, −0.002	0.018	−0.015	−0.102, 0.073	0.741
CDAI						
CC	Ref.	–	–	Ref.	–	–
CT	−0.016	−0.196, 0.164	0.864	−0.372	−0.691, −0.053	0.022
TT	−0.284	−0.580, 0.013	0.061	−0.882	−3.307, 1.544	0.476
SDAI						
CC	Ref.	–	–	Ref.	–	–
CT	−0.014	−0.193, 0.166	0.882	−0.443	−0.843, −0.043	0.030
TT	−0.284	−0.583, 0.015	0.063	−0.804	−5.621, 4.014	0.744

[a] DAS = Disease Activity Score, MD-HAQ = Multidimensional Health Assessment Questionnaire, CDAI = Clinical Disease Activity Index, SDAI = Simplified Disease Activity Index.
[b] Multivariate model adjusted for age, gender, comorbidity count, body mass index, smoking, rheumatoid factor positivity, anti-cyclic citrullinated peptide antibody positivity, methotrexate and anti-tumor necrosis factor use.
[c] Bolded p-values were considered to reach significance after Bonferroni correction ($p < 0.0167$).

The rs1927911 SNP is located within the intron-coding region of the TLR4 gene on chromosome 9 and, to our knowledge, no other studies have investigated rs1927911 in relation to RA disease activity or severity. The minor allele has, however, been associated with other conditions including a lower risk of myocardial infarction [43] and gastric cancer [44]; and an increased risk of non-healing diabetic foot ulcers [45], normal tension glaucoma [46], bronchiolitis obliterans following lung transplantation [47], chemotherapy induced neutropenia [48], pulmonary tuberculosis [49], and susceptibility to air pollution in childhood asthma [50]. Most of the aforementioned studies suggest a state of reduced inflammation associated with the rs1927911 minor allele that is consistent with our findings. To our knowledge, however, the functional consequences of this mutation (as well as other polymorphisms 'tagged' by this SNP) on TLR4 function and/or expression have yet to be elucidated. To fully understand the mechanisms underpinning the association of this polymorphism with RA disease activity, future studies will be needed to clarify whether this genetic variant exerts any meaningful biological effects.

Several studies have examined the associations with TLR4 Asp299Gly (rs4986790) and RA disease course [15,17,19–21,23,24]. In contrast to rs1927911, the rs4986790 SNP is a functional allele located in the exon gene region of TLR4 and is known to cause an aspartic acid to glycine replacement at codon position 299 altering its extracellular domain and potentially modifying its binding affinity. One might therefore expect the polymorphism to consequently have immunomodulatory properties; however, most studies, now including ours, observed no associations with this SNP with RA disease course [15,17,19,21]. This is in contrast to biologic studies, which have demonstrated lower levels of inflammatory cytokines associated with rs4986790 [18]. This discrepancy might relate at least in part to the up-regulation of TLR4 during inflammation, which might compensate for any decreased binding affinity associated with the functional allele. Since rs1927911 is part

Table 5
Associations of TLR4 rs1927911 with rheumatoid arthritis disease progression stratified by anti-citrullinated histone 2B (anti-cH2B) antibody positivity.[a, b, c]

Genotype	Anti-cH2B Negative (n = 783)			Anti-cH2B Positive (n = 405)		
	β-Coef	95% CI	p value	β-Coef	95% CI	p value
DAS28						
CC	Ref.	–	–	Ref.	–	–
CT	0.001	−0.017, 0.019	0.910	−0.023	−0.048, 0.003	0.079
TT	−0.042	−0.070, −0.014	**0.003**	−0.058	−0.092, −0.024	**0.001**
MD-HAQ						
CC	Ref.	–	–	Ref.	–	–
CT	0.002	−0.007, 0.010	0.651	0.006	−0.015, 0.026	0.576
TT	−0.016	−0.029, −0.002	0.028	−0.009	−0.034, 0.016	0.492
CDAI						
CC	Ref.	–	–	Ref.	–	–
CT	0.030	−0.182, 0.241	0.785	−0.279	−0.632, 0.074	0.122
TT	−0.330	−0.681, 0.020	0.065	−0.443	−0.943 0.056	0.082
SDAI						
CC	Ref.	–	–	Ref.	–	–
CT	0.033	−0.177, 0.243	0.756	−0.289	−0.630, 0.052	0.097
TT	−0.320	−0.678, 0.039	0.081	−0.454	−1.172, 0.264	0.215

[a] DAS = Disease Activity Score, MD-HAQ = Multidimensional Health Assessment Questionnaire, CDAI = Clinical Disease Activity Index, SDAI = Simplified Disease Activity Index.
[b] Multivariate model adjusted for age, gender, comorbidity count, body mass index, smoking, rheumatoid factor positivity, anti-cyclic citrullinated peptide antibody positivity, methotrexate and anti-tumor necrosis factor use.
[c] Bolded p-values were considered to reach significance after Bonferroni correction ($p < 0.0167$).

of the intron region and does not appear to directly alter TLR4 structure, it is possible that it instead exerts its influence by preventing up-regulation of TLR4 by down-regulating gene expression either directly or through linkage disequilibrium with another SNP that does. This could potentially explain the strong relationship observed between rs1927911 and lower disease activity over time and the lack of association with rs4986790. However this hypothesis would need further testing for confirmation. An alternative hypothesis would be that those with the homozygous minor allele of rs1927911 might be more responsive to treatment, a hypothesis that also warrants further investigation.

There are limitations to our study. Although the study population was restricted to those self-reporting Caucasian race/ethnicity, this does not completely eliminate the possibility of population stratification. Ancestral informative SNP markers were not available, precluding a genotype-based approach in addressing this issue. It is possible that the results of our analyses are due to linkage disequilibrium with another SNP that more directly affects TLR4, a possibility particularly since TLR4 genotyping was limited to 3 SNPs identified during study planning as a means of reducing the chance of type I error. The rs1927911 SNP, for instance, is in linkage disequilibrium with at least 16 other variants within 20 kb of the TLR4 gene. The associations observed between this SNP and RA disease activity, could therefore be related to the functional consequences of one of these other variants. Despite our efforts to minimize type I error, it is still possible that our findings are a result of false discovery and estimation bias. However, we have taken a very conservative approach to avoid this by applying a Bonferroni correction and by using a bootstrapping approach which has been shown to be a preferred method to minimize the chance of false discovery in genetic analyses [36].

In summary, we have demonstrated that TLR4 rs1927911 is associated with lower disease activity over time using multiple outcome measures and the results do not appear to be meaningfully influenced by other covariates. In the future, more studies will be needed to confirm our findings in different populations and to identify potential mechanisms by which variation in rs1927911 affects TLR4 and RA disease progression.

Competing interests

The authors have no disclosures or competing financial interests.

Authors' contributions

MD participated in study design and coordination, performed statistical analysis, and drafted the manuscript. TL carried out molecular genetic sequencing. FY and HS contributed to the statistical analysis. JS performed immunoassays. KM contributed to study design and statistical analysis. GT performed immunoassays and participated in study design, TM conceived of the study, participated in study design, coordination, and manuscript preparation. All authors proof-read and approved the final manuscript.

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