Lack of association between Toll-like receptor 4 gene polymorphisms and giant cell arteritis

Lorena Alvarez-Rodriguez¹, Marcos Lopez-Hoyos², Ignacio Beares², Pedro Muñoz Cacho³, Cristina Mata⁴, Jaime Calvo-Alen⁴, Alfonso Corrales¹, Gaurav Tripathi¹,², Ricardo Blanco¹, Maite García-Unzueta⁵, Ignacio Villa⁴ and Victor M. Martínez-Taboada¹

Abstract

Objective. Coding variants in Toll-like receptor 4 (TLR4) have been reported to be associated with inflammatory diseases. The aim of this study was to determine whether two of these polymorphisms (+896 A/G and +1196 C/T) are associated with susceptibility and clinical features of GCA. We also attempted to correlate the functional consequences of these polymorphisms.

Methods. A total of 72 patients with GCA and 126 age-matched controls were genotyped using allele-specific PCR and restriction fragment length polymorphism analysis. TLR4 expression was studied on peripheral blood mononuclear cells by flow cytometry and TLR4 function was assessed by stimulating monocytes in vitro with a specific ligand.

Results. There was no significant difference in allele frequency or genotype of TLR4 (+896 A/G and +1196 C/T) between GCA patients and controls. The clinical characteristics of these patients were unrelated to the presence of these polymorphisms. Furthermore, we did not observe an association with TLR4 expression or a distinct phenotype of TLR4 response with the +896 A/G and +1196 C/T genotypes.

Conclusion. Our results do not support the association of these TLR4 variants with GCA. Studies including a larger number of patients and patient populations from different geographical origin are needed.

Key words: Toll-like receptor 4, Gene polymorphism, Giant cell arteritis.

Introduction

GCA is a granulomatous vasculitis affecting medium- and large-sized arteries that occurs exclusively in elderly people [1]. GCA is the most frequent primary vasculitis [2]; the incidence is 15–25 per 100 000 in at-risk populations. The disease is most prevalent in white persons, particularly those of Scandinavian descent. Women are more susceptible than men. The single most important risk factor is age. In this regard, susceptible persons are usually >50 years of age, and incidence rates increase with advancing age [3]. Around 50% of patients with GCA have PMR, a clinical syndrome characterized by pain and stiffness in the neck, shoulder and pelvic girdle [4]. The aetiopathogenesis of GCA remains unknown although genetic, autoimmune and environmental factors have been implicated [5–11].

Accumulating evidence has demonstrated that activation of innate immunity is a requisite to induce acquired immunity [12], and information about innate immunity on GCA is scarce and limited to the inflamed tissue and genetic analysis [13–16]. One of the most important elements of innate immunity, Toll-like receptors (TLRs), play an essential role in the activation and regulation of the innate and acquired immune responses through the recognition of specific molecular patterns of pathogens and
endogenous peptides [12]. TLR activation has been implicated in the loss of self-tolerance, which may lead to autoimmunity and chronic inflammation [17].

Besides the roles of TLRs in the immune response mentioned above, there are several facts that support the study of TLRs, and in particular TLR4, in patients with GCA. First, the initial events in the vessel wall of patients with GCA are mediated by the transition of immature adventitial dendritic cells (DCs) to the mature state that is triggered by TLR activation [18]. Secondly, TLR4 polymorphisms have been associated with the development of other chronic granulomatous conditions such as Crohn’s disease and sarcoidosis [19, 20]. Although with conflicting results, TLR4 polymorphisms have been associated with infections and cardiovascular diseases [21–23], and these two factors were also related to the pathogenesis of GCA [5]. Furthermore, two non-synonymous polymorphisms of TLR4 (Asp299Gly and Thr399Ile) have also been suggested to alter the function of the receptor [24, 25] and have been related to the development of chronic inflammatory conditions [19, 20, 26–32].

The aim of this study was to determine whether two of these polymorphisms in TLR4 (+896 A/G and +1196 C/T) are associated with susceptibility and clinical features in GCA. We have also attempted to correlate the functional consequences of these polymorphisms.

Materials and methods

Study subjects

The present study included 72 patients with GCA and 126 age-matched healthy controls (HCs). The main demographic, clinical and laboratory characteristics of the study population are shown in Table 1. All patients with GCA fulfilled the 1990 ACR classification criteria for GCA [33], and 82% had characteristic findings of arteritis in the temporal artery biopsy (TAB) [34]. Both patients and controls were Caucasians of Spanish ancestry and lived in the same geographical area of North Spain, Cantabria. All the patients and controls gave signed informed consent according to the Declaration of Helsinki, and the study was approved by the regional ethics committee (CEIC Cantabria).

The clinical findings at diagnosis and during follow-up, the ESR and CRP values at diagnosis, as well as the initial prednisone dosage, were ascertained by reviewing the patients’ medical records. Patients were subgrouped according to the presence or absence of PMR [35] and the presence or absence of ischaemic complications. Ischaemic complications were defined as the presence of visual loss, jaw claudication, cerebrovascular accidents and/or aortic arch syndrome [36, 37]. For the analysis of some variables such as relapses/reurrences, duration of CS therapy and accumulated dose of prednisone, only patients with a follow-up of ≥2 years were included.

Genotyping TLR4 gene

Genomic DNA was extracted from 5 ml of whole blood using the DNAzol method according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Genotyping of the TLR4 Asp299Gly (+896 A/G; rs4986790) and TLR4 Thr399Ile (+1196 C/T; rs4986791) polymorphisms were performed using PCR-restriction fragment length polymorphism (PCR-RFLP) with primers described by Lorenz et al. [38]. Specifically, the primer sequences for TLR4 Asp299Gly were: forward 5’-GATAGCATCACTTGACTCACTCCATG-3’; reverse 5’-GATCAACCTTGAAAACATTCCCCAC-3’. And the primer sequences for TLR4 Thr399Ile were: forward 5’-GGTGTCTGGTTCTCAAAATGA TTTTGAGAAAG-3’; reverse 5’-ACCTGAAGACTGGAGAGT GATGTTAAATGCT-3’. Both forward primers were altered to generate a restriction site for the endonucleases NcoI and HinfI, respectively. PCR amplifications were performed in a 25 µl reaction containing 100 µM dNTP, 20 pmol each primer, 1 U of Taq polymerase (Promega, Madison, WI, USA) in PCR buffer 10× and 2 mM (2 mmol/l) MgCl₂.

PCR conditions for TLR4 Asp299Gly and TLR4 Thr399Ile were respectively:

- Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 66°C for 30 s, extension at 72°C for 30 s and final incubation at 72°C for 5 min.
- Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 66°C for 30 s, extension at 72°C for 30 s and final incubation at 72°C for 5 min.

PCR products were overnight digested using NcoI and HinfI restriction endonucleases (New England Biolabs, Ipswich, MA, USA) and electrophoresed in a 3% agarose gel. After digestion, fragment sizes for carriers of the polymorphic allele decreased from 249 bp (wild-type) to 223 + 26 bp (mutant) for the 299 residue, and from 406 bp (wild-type) to 377 + 29 bp (mutant) for the 399 residue. To improve the genotyping quality, all mutant and heterozygous samples were re-genotyped using a double-blind method, and the results were noted only for those samples that were reproducible without any discrepancy.

Table 1 Main demographic and clinical characteristics in patients with GCA and HCs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>126</td>
<td>72</td>
</tr>
<tr>
<td>Age, mean (s.d.), years</td>
<td>74.3 (11.0)</td>
<td>74.1 (7.3)</td>
</tr>
<tr>
<td>Sex: females, %</td>
<td>68.3</td>
<td>62.5</td>
</tr>
<tr>
<td>Time to diagnosis, mean (s.d.), months</td>
<td>–</td>
<td>2.4 (2.2)</td>
</tr>
<tr>
<td>PMR symptoms, %</td>
<td>–</td>
<td>43.1</td>
</tr>
<tr>
<td>Ischaemic manifestations, %</td>
<td>–</td>
<td>46</td>
</tr>
<tr>
<td>TAB positive, %</td>
<td>–</td>
<td>82</td>
</tr>
<tr>
<td>Pre-treatment ESR, mean (s.d.), mm/h</td>
<td>0.27 (0.2)</td>
<td>8.7 (5.6)</td>
</tr>
<tr>
<td>Pre-treatment CRP, mean (s.d.), mg/dl</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

www.rheumatology.oxfordjournals.org
TLR4 protein expression in PBMCs

Cell surface expression of TLR4 was assessed on distinct peripheral blood mononuclear cell (PBMC) subpopulations (T cells, B cells and monocytes) by flow cytometry. Cells collected into sodium heparin tubes were incubated with FITC-conjugated anti-human CD19, PerCP-conjugated anti-human CD3 and APC-conjugated anti-human CD14 to identify B, T and monocyte populations, respectively, and with PE-conjugated anti-human TLR4 or PE mouse immunoglobulin (Ig) G2a isotype control (eBiosciences, San Diego, CA, USA) for 20 min in the dark. Then, red blood cells were lysed with FACS lysing solution (BD Biosciences) for 10 min. After washing, the cells were resuspended in 1% paraformaldehyde. Expression of TLRs was gated and analysed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) as mean fluorescence intensity (MFI).

TLR4 function assessment in circulating monocytes

Cells collected in sodium heparin tubes were polyclonally stimulated for 18 h with lipopolysaccharide (LPS) (InvivoGen, San Diego, CA, USA) in polypropylene tubes. As controls, cells were incubated in identical medium without stimulus. After culture, cells were stained with FITC-conjugated anti-human CD14 (BD Biosciences, San Jose, CA, USA) to identify the monocyte population for 20 min in the dark. Then, red blood cells were lysed with FACS lysing solution (BD Biosciences) for 10 min. After washing, cells were permeabilized with FACS permeabilizing solution (BD Biosciences) and intracellularly stained with PE-labelled mAbs against cytokines (IL-1β, TNF-α, IL-6) (BD Biosciences) and analysed by flow cytometry (FACSCalibur; BD Biosciences). Levels of intracellular cytokine-producing monocytes were analysed using Cell Quest Pro Software (BD).

Statistical analysis

All statistical analysis of data was carried out using SPSS 12.0 (Chicago, IL, USA). Arlequin v3 software was used to determine the Hardy–Weinberg equilibrium and haplotype analysis. The actual sample size has a power of 80% for detecting odds ratio (OR) ≥ 2.7. The strength of the association between GCA and alleles or genotypes of the TLR4 gene was estimated using ORs and 95% CIs. Levels of significance were determined using contingency tables by either chi-square or Fisher’s exact test analysis. For expression and functional studies, statistical comparisons of data between GCA and controls were performed using the Mann–Whitney U-test. Differences were considered statistically significant when \( P < 0.05 \).

Results

Association between TLR4 gene polymorphisms and disease susceptibility in GCA

To evaluate the association between two polymorphisms of TLR4 (+896 A/G and +1196 C/T) and GCA, we conducted a case–control study of Spanish population samples. The distribution of TLR4 allele frequencies and genotypes in GCA patients and controls is shown in Table 2. The studied population was found to be in Hardy–Weinberg equilibrium for the TLR4 gene polymorphisms (see supplementary table 1, available as supplementary data at Rheumatology Online).

The haplotype analysis of TLR4 +1196C/T and +896A/G revealed that none of the haplotypes was significantly associated with disease risk among patients or controls (see supplementary table 2, available as supplementary data at Rheumatology Online). However, given the sample sizes (the actual sample size has a power of 80% for detecting OR ≥ 2.7) and the allele frequencies of the polymorphism studied, we cannot rule out a genetic risk in carriers of the polymorphism. In the case of allele frequency of TLR4 (+896 A/G), there was a risk of 0.79 (95% CI 0.34, 1.73) for GCA.

TLR4 gene polymorphisms are not associated with disease severity in GCA patients

The severity of GCA was addressed by analysing the presence of at least one relapse/recurrence, number of relapses, duration of CS treatment and cumulative prednisone dose. As patients had different follow-up and the average duration of CS treatment is ~2 years, only patients with at least 2 years of follow-up were included. The presence of ischaemic manifestations and PMR symptoms at any time was also analysed. No significant association between T gene polymorphisms and any of these variables was observed.

Discussion

One of the key cellular elements of the innate immunity system is the DC [40]. Ma-Krupa et al. [18] have shown that mature DCs are located at the adventitia–media junction of the temporal artery of patients with active GCA, and that these cells produce chemokines and are able to recruit and activate T cells to the inflammatory infiltrate. DCs in the inflammatory infiltrate express TLRs and the specific ligand of TLR4 have been found to induce activation of DCs and T-cell-driven arterial inflammation in an
Table 2 Distribution of TLR4 (+1196C/T and +896A/G) genotype, allele frequency and allele carriage frequency in patients with GCA and HCs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GCA</th>
<th>Controls</th>
<th>P-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4 +1196C/T (db SNP ID rs4986791) polymorphism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype frequency (n = 72)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>63/72 (87.5)</td>
<td>113/126 (89.7)</td>
<td>-</td>
<td>1.36 (0.54, 3.40)</td>
</tr>
<tr>
<td>CT</td>
<td>09/72 (12.5)</td>
<td>12/126 (8.5)</td>
<td>0.6321</td>
<td>-</td>
</tr>
<tr>
<td>TT</td>
<td>00/72 (0.0)</td>
<td>01/126 (0.8)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>135/144 (93.7)</td>
<td>238/252 (94.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T</td>
<td>09/144 (6.3)</td>
<td>14/252 (5.6)</td>
<td>0.8247</td>
<td>1.13 (0.48, 2.7)</td>
</tr>
<tr>
<td>Allele carriage frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C allele carriage</td>
<td>72/72 (100.0)</td>
<td>125/126 (99.2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T allele carriage</td>
<td>09/72 (12.5)</td>
<td>13/126 (10.3)</td>
<td>0.6447</td>
<td>1.24 (0.50, 3.07)</td>
</tr>
<tr>
<td>TLR4 +896A/G (db SNP ID rs4986790) polymorphism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>61/72 (84.7)</td>
<td>103/126 (81.7)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AG</td>
<td>11/72 (15.3)</td>
<td>22/126 (17.5)</td>
<td>0.8432</td>
<td>0.85 (0.39, 1.88)</td>
</tr>
<tr>
<td>GG</td>
<td>00/72 (0.0)</td>
<td>01/126 (0.8)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (reference)</td>
<td>133/144 (92.4)</td>
<td>228/252 (90.5)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>11/144 (7.6)</td>
<td>24/252 (9.5)</td>
<td>0.5237</td>
<td>0.79 (0.34, 1.73)</td>
</tr>
<tr>
<td>Allele carriage frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A allele carriage</td>
<td>72/72 (100.0)</td>
<td>125/126 (99.2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G allele carriage</td>
<td>11/72 (15.3)</td>
<td>23/126 (18.3)</td>
<td>0.6967</td>
<td>0.81 (0.37, 1.77)</td>
</tr>
</tbody>
</table>

Data are expressed as n/n (%), unless otherwise indicated.

Fig. 1 TLR4 expression on subpopulations of PBMCs according to TLR4 genotype at disease onset in patients with GCA and HCs. MFI expression of TLR4 in PBMCs of patients with GCA and HCs. Expression was measured by flow cytometry in 16 GCA patients and 60 HCs. (A) TLR4 Asp299Gly gene polymorphism. (B) TLR4 Thr399Ile gene polymorphism.
experimental model [18]. Therefore, a key role of TLR4 has been postulated in the pathogenesis of GCA [41].

TLR4 is an important pathogen recognition receptor that plays a major role in the innate immunity by binding exogenous and also endogenous ligands that are present in inflamed tissue [42]. Activation of TLR4 is followed by the secretion of pro-inflammatory cytokines and expression of co-stimulatory molecules that initiate the adaptive immune response [42]. Two co-segregating SNPs (+896 A/G and +1196 C/T) within the gene encoding TLR4 have been characterized and studied in several inflammatory conditions including GCA [15, 16]. With the present work, there are three different studies that examine TLR4 polymorphisms in GCA, two from different regions in Spain and a third one from Italy, another Mediterranean country. In the two initial studies [15, 16], an increased frequency of the G allele in the TLR4 (+896 A/G) gene polymorphism was found, although it was found to be significant only in the work of Palomino-Morales et al. [15]. The results of the present study did not show the same tendency. One of the major limitations of genetic studies on diseases with a low prevalence is the inclusion of patient populations with a low sample size as in the present study, where an OR \( \geq 2.7 \) would be needed to reach a statistical power \( \geq 80\% \). Therefore, larger multicentre studies or meta-analysis of published cohort results are necessary to clearly establish the association of TLR4 gene polymorphisms with GCA. Susceptibility to inflammatory or autoimmune diseases may be the result of the interaction of multiple genetic factors that regulate the threshold of autoreactivity, and this could be the case in GCA [43], where mutations in the TLR4 gene might also contribute to the development of a large-vessel vasculitis. TLR4 mutations have been implicated in susceptibility to develop other chronic granulomatous disorders [19, 20], suggesting that these genetic defects in the TLR4 gene may predispose to the development of an inflammatory granulomatous response. However, none of the three studies on GCA ([15, 16] and present results) found an association with the presence of specific clinical features such as PMR or ischaemic symptoms, or with the prognosis of the disease.

These TLR4 SNPs have been reported to impair the efficacy of LPS signalling [24, 25] and following the initial observations of Arbour et al. [24], several groups have studied the functional consequences of these mutations with contrasting results. To the best of our knowledge, there are no data on the potential influence of these polymorphisms in the expression and function of TLR4 in patients with large-vessel vasculitis. Here, we show that in both patients and HCs, there is no difference in expression and cytokine production in response to specific stimuli depending on the genotype of the TLR4 gene. Our results are in agreement with most of the studies done on stimulated whole blood and PBMCs where no distinct phenotype was reported for these TLR4 gene polymorphisms [25].

TLR4 is involved in innate and adaptive immune responses by binding to pathogens, microbial toxins or endogenous ligands such as HSPs, fragments of hyaluronic acid and fibronectin [44–46]. Formation of TLR–ligand complexes activates signal transduction pathways resulting in the release of inflammatory mediators [47]. In a previous study [48], we found that IgG hHSP60 Abs

---

**Fig. 2** TLR4 function according to TLR4 genotype at disease onset in patients with GCA and HCs. TLR function was assessed by measuring intracellular pro-inflammatory cytokine production of circulating monocytes after TLR4 stimulation with specific agonist (LPS) in 11 GCA patients and 35 HCs. (A) TLR4 Asp299Gly gene polymorphism. (B) TLR4 Thr399Ile gene polymorphism.
significantly increased in GCA patients in comparison with PMR patients and HCs. These data point to an initial immune response against an unknown infectious agent(s) that generates immune memory. Later on, due to the high interspecies homology of the HSP (molecular mimicry), self-HSP may be recognized by the immune system and produce hHSP60 Abs. Alternatively, it is possible that the vascular inflammation that occurs in GCA might be the inducer of a secondary humoral response against hHSP60. In summary, our results do not support the association of these TLR4 variants with GCA. However, studies including a larger number of patients and patient populations of different geographical origin are needed.

**Rheumatology key messages**
- The TLR4 (+896 A/G) gene polymorphism is not associated with GCA.
- The TLR4 polymorphism has no association with disease severity and with TLR4 expression and function.

**Acknowledgements**

We are especially grateful to Marta Gonzalez (supported by Fundación Marqués de Valdecilla-IFIMAV) and Carolina Santa Cruz (supported by a grant for Research Aid from Schering-Plough, Spain) for their helpful technical assistance. We would like to thank all the patients and controls included in the present study.

**Funding:** This work was supported by grants from Fundación Marqués de Valdecilla, Fondo de Investigación Sanitaria (PI050475 and PI080098), and Fundación Mutua Madrileña. L.A.-R., I.B. and G.T. were supported by a grant for Research Aid from Fundación Marqués de Valdecilla-IFIMAV.

**Disclosure statement:** R.B. has received consulting fees, speaking fees and/or honoraria from Roche, Abbott, and Schering-Plough (less than $10,000 each). All other authors have declared no conflicts of interest.

**Supplementary data**

Supplementary data are available at Rheumatology Online.

**References**


19. Browning BL, Huebner C, Petermann I et al. Has toll-like receptor 4 been prematurely dismissed as an...


