

ROLE OF TLR4 RECEPTOR POLYMORPHISMS IN BOUTONNEUSE FEVER

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The genetics of the interaction between host and microbes plays an essential role in the survival of the individual and attainment of longevity. The activation of toll-like receptor (TLR)4 plays a key role in natural and clonotypic immune responses. We evaluated whether TLR4 genotype is a component of genetic background protective versus rickettsiosis and whether this background influences longevity. We genotyped for +896A/G TLR4 polymorphism 78 patients affected by Boutonneuse fever, 78 age-matched controls and 78 advanced age individuals from Sicily. The +869G allele, that attenuates receptor signalling, was significantly overrepresented in patients in comparison with age-matched controls. By analyzing data according to gender, this allele was significantly higher in female patients when compared to advanced age women. Pro-inflammatory responses are programmed to resist fatal infections. So, it is not surprising that the genetic background of people that survive to an advanced age may be protective against infections. However, this seems to occur in women but not in men. In a previous study, the +896G TLR4 allele was overrepresented in advanced age men and underrepresented in men affected by myocardial infarction. Thus, previous and present results tend to agree with the suggestion that men and women may follow different trajectories to reach longevity. For men it might be more important to control atherogenesis, whereas for women it might be more important to control infectious diseases.

The activation of innate immunity constitutes the first line of host defence against invading pathogens, and provides instructive signals to the clonotypic immune system. Therefore, the expression of a limited number of highly active genes during the activation of innate immunity is able to induce rapid and efficient defensive immune responses (1-3). Several cell types contribute to innate immunity, but the mononuclear phagocyte lineage plays a pivotal role. Monocytes, macrophages and their tissue

differentiated derivatives express pattern recognition receptors, namely various scavenger and Toll-like receptors (TLR). These receptors induce transmembrane signals that activate NF- κ B and mitogen dependent protein kinase pathways. TLR activation induces the expression of a wide variety of genes encoding proteins, such as cytokines, with regulatory functions upon leukocyte activation and tissue inflammation (3-5). Therefore, the capacity of each individual organism to regulate the activation

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of innate immunity and local inflammatory responses is crucial for attaining defensive action against pathogens, limited tissue damages, fast recovery and tissue healing (1). The nonsynonymous adenine to guanine (A/G) transition at nucleotide +896 of TLR4 mRNA that occurs within the extracellular domain of TLR4 protein results in the substitution of asparagine with glycine at amino acid residue 299 (Asp299Gly). Carriers of the G allele at this single nucleotide polymorphism (SNP) exhibit reduced lipopolysaccharide (LPS) responsiveness as well as an increased risk and susceptibility to gram-negative infections (6-11).

Human Boutonneuse fever (BF), a rickettsiosis caused by *Rickettsia conorii* (short gram-negative bacillary microbe), and transmitted by the dog-tick *Rhipicephalus sanguineus* (12), is an endemic disease in the Mediterranean basin characterized by fever, malaise, headache, and frequently by a local lesion (eschar or tache noir) at the site of the tick bite. So far, the mechanisms of host defence are not completely understood, although cell-mediated immunity is thought to play a crucial role (13).

The genetic control of the interaction between the host and the parasite certainly plays an essential role in the survival of the individual, as demonstrated by studies on human leukocyte antigens (HLA) and cytokines. Studies have suggested that molecules involved in resistance to pathogens, such as HLA-DR11 and interferon- γ , positively influence women's longevity (14-16). The aim of this study was to evaluate whether TLR4 genotype is a component of genetic background that is protective versus rickettsiosis and whether this background may influence longevity positively. To this purpose we genotyped for +896A/G TLR4 SNP patients affected with BF, age-matched controls and advanced age individuals from the same geographic area.

MATERIALS AND METHODS

Subjects

Seventy-eight Sicilian patients with confirmed BF (42 females and 36 males ranging in age from 30 to 60 years), 78 healthy age-related medical and laboratory staff Sicilians (42 females and 36 males; age range: 30-60) and 78 advanced age Sicilians (42 females and 36 males; age range: 96-104) participated in the study. The Sicilian ethnicity of all subjects was confirmed by having all four

grandparents born in Sicily; immigration and intermarriage has historically been rare. The BF patients had characteristic signs and symptoms of active BF (presence of fever, eschar at the site of tick bite, and maculopapular rash). The diagnosis was confirmed by serological data (high levels of anti-*R. conorii* antibodies assayed by ELISA and indirect immunofluorescence) (13). The 78 young and the 78 advanced age controls were selected at random from our databases to match for gender, age (as regards younger controls) and domicile (town or country) of our study population. Good health of controls was assessed at the time of recruitment, taking into account their clinical history over the last 5 years. In particular serology excluded the occurrence of rickettsiosis in the past. The study was approved by the Hospital Ethics Committee and informed consent was obtained from all subjects.

Genotyping

DNA was genotyped for +896A/G (Asp299Gly) TLR4 SNP according to published methods (17). Briefly, we mixed 3' and 5' allele specific primer in a 25 μ l total volume that contained DNA template, 1.50 mM magnesium chloride, 9.8 mM ammonium sulphate, 39.6 mM Tris, 200 μ M dNTPs, and 0.2U Taq-Gold polymerase (Applera Italia, Monza, Italy). Cycling was performed at 95°C for 10 minutes, followed by thirty cycles at 96°C for 30 seconds, 64°C for 30 seconds, 72°C for 30 seconds. Products of polymerase chain reaction were digested with endonuclease NcoI (New England Biolabs, USA) to evaluate restriction patterns of the two alleles (+896A and +896G), and were detected by electrophoresis on 2% agarose.

Statistics

The data were tested for best match between the observed and expected genotype values and their match to Hardy-Weinberg equilibrium (HWE) by chi-square test. The frequencies of genotypes and alleles +896A/G TLR4 SNP in BF patients were compared to those in controls by Pearson chi-square test.

RESULTS

Table I shows the genotypic and allelic frequencies for +896A/G TLR4 SNP in BF patients, age-related healthy controls and advanced age controls from Sicily. The distribution of genotypes was in HWE in all the groups. The +869G SNP, known to attenuate receptor signalling (6-11), was overrepresented in BF patients (11.5%). However, significant differences in the frequency of

Table I. Genotype distribution and allele frequency of Asp299Gly (+896A/G SNP) TLR4 gene polymorphism in 78 age-matched controls, 78 BF patients and 78 advanced age senior controls from Sicily.

Subjects	Genotypes			Alleles	
	AA	AG	GG	+896A allele	+896G allele
Age-matched Controls	70	6	2	146 (93.6%)	10(6.4%)
BF Patients	63	12	3	138(88.5%)	18(11.5%)
Advanced age senior controls	66	11	1	143 (91.7%)	13 (8.3%)

Pearson chi-square test was performed to calculate significant different genotype and allele distribution between patients and controls. Significant differences in the frequency of TLR genotypes and alleles between the various groups were found only between BF patients and age matched controls ($P = 0.027$ and respectively $P = 0.0089$).

TLR genotypes and alleles between the various groups were found only between BF patients and age-matched controls ($P = 0.027$ and respectively $P = 0.0089$). By analyzing data according to gender, the frequency of +896G SNP was significantly higher in women BF patients when compared to that of advanced age women ($P = 0.0032$). No other significant differences were observed (Table II).

DISCUSSION

TLRs are one subset of a diverse group of molecules referred to as pattern recognition receptors.

TLR4 is well known as the receptor for LPS from gram-negative bacteria and its activation initiates a signal transmitted to a series of adaptor molecules and protein kinases resulting in nuclear translocation of NF- κ B and a subsequent cascade of inflammatory cytokines and chemokines. LPS initiates the activation of NF- κ B and other signalling pathways through the adaptor protein MyD88, and is crucial for the development of T-helper cell type-1-dependent immune responses (18-22). Indeed, several investigations have shown that MyD88-deficient mice cannot generate type-1 immune responses and in certain instances the concentration of

Table II. Allele frequency of Asp299Gly (+896A/G SNP) TLR4 gene polymorphism analysed according by gender in 78 age-matched controls, 78 BF patients and 78 advanced age senior controls from Sicily.

Subjects	Female		Male	
	+896A allele	+896G allele	+896A allele	+896G allele
Age-matched controls	78 (92.9%)	6(7.1%)	68 (94.4%)	4(5.6%)
BF Patients	76(90.4%)	8(9.6%)	62((86.2%)	10(13.8%)
Advanced age senior controls	81 (96.4%)	3 (3.6%)	62 (86.2%)	10 (13.8%)

Pearson chi-square test was performed to calculate significant different allele distribution between patients and controls. Significant differences in the frequency of TLR and alleles between the various groups were found only between women BF patients and advanced age senior women ($P = 0.0032$).

LPS may determine whether Th1 or Th2 response will develop (22-23). So, TLR is critical for the response to gram-negative bacteria and its activation seems crucial for the development of a type-1 immune response against these bacteria (3,6, 9,19,21-22). Therefore, the TLR4 receptor may be involved in the cellular clonotypic immune clearance of *Rickettsia conorii* in BF patients (13,24-25).

Carriers of the G-allele at this +896 SNP, exhibit reduced LPS responsiveness, and therefore have an increased susceptibility to gram-negative infections (6-11). Indeed, in a large cohort of 810 randomly recruited adults, Kiechl et al. (9) observed that this SNP was associated with low levels of inflammatory mediators and conferred an increased risk of severe gram-negative bacterial infections. Transient transfection experiments in THP-1 cells indicated that the 299Gly allele was able to disrupt TLR4 signalling. Besides, adenoviral transfection of a wild-type TLR4 construct was able to rescue LPS responsiveness in airway epithelial cells and alveolar macrophages derived from individuals with TLR4 mutations (6). Finally, severe respiratory syncytial virus bronchiolitis was shown to be associated with +896G TLR4 SNP (26) and present results show an association of BF with +896G TLR4 SNP. These data confirm the critical role of TLR4 in protection from microbial infections.

Our immune system has evolved to control pathogens and pro-inflammatory responses are likely to be evolutionary programmed to resist fatal infections. So, it is not surprising that the genetic background of advanced age individuals may be protective versus infectious diseases. However, this seems to occur in women but not in men. In men, we have recently demonstrated that TLR-4 gene plays an opposite role in human longevity and myocardial infarction (MI). The +896G TLR4 SNP was underrepresented in males with MI and overrepresented in advanced age men (27). So, polymorphisms of TLR4 which attenuate receptor signalling enhance the risk of infections but have opposite effects on atherogenesis, by better controlling inflammatory responses involved in atherogenesis and reducing the risk of atherogenesis complication (27). Thus, previous and present results agree with the suggestion that men and women may follow different trajectories to reach

longevity (28).

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