TIRAP Ser180Leu polymorphism is associated with Behçet’s disease

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Abstract

Objectives. The initiating cause of Behçet’s disease (BD) is unknown, but an aberrant response to infection has been suggested. In this study, single nucleotide polymorphisms in Toll-like receptors (TLRs) and associated molecules that have a sentinel function at mucosal surfaces were analysed in patients with BD.

Methods. TLR expression was determined by immunohistochemistry in buccal mucosal tissue from patients with BD, in tissue from patients with lichen planus (LP) or pyogenic granuloma (PG) as disease controls, or from healthy individuals. Using SSP-PCR we analysed SNP in CD14, TLR2, TLR4 and TIRAP (TIR domain-containing adaptor protein) in patients with BD from different geographical regions.

Results. TLR expression was increased in buccal lesions from patients with BD compared with healthy controls; however, a similar increase was seen in lesion tissue from patients with LP or PG, suggesting that this was a generalized inflammatory response as opposed to a BD-specific response. SNP analysis showed no association between CD14, TLR2 or TLR4 polymorphisms. However, TIRAP 180Leu was significantly associated with BD in UK, but not Middle Eastern, patients.

Conclusion. TLR expression showed no difference in tissue from patients with BD compared with either disease or healthy controls. Likewise, SNPs in TLR genes were no different from healthy controls. The association with the increased function variant of TIRAP suggests that encounter with a pathogen at mucosal sites will lead to increased cytokine production and tissue damage with persistence of mucosal lesions.

Key words: Behçet’s disease, Toll-like receptors, Toll/interleukin receptor 1 domain-containing adaptor protein, Gene polymorphisms, Inflammation, Infection.

Introduction

Behçet’s disease (BD) is an inflammatory disorder of unknown aetiology, characterized by oral and genital ulceration associated with uveitis, arthritis, skin lesions and neurological complications. The underlying pathological process in BD is a systemic vasculitis, and the breadth of organ involvement is extensive. The cause of BD is unknown, but an autoimmune aetiology has been suggested. In this scenario, bacteria or viruses infect mucosal surfaces inducing an immune response, which cross-reacts with self-proteins, leading to the organ-specific damage that is present in this disease [1].

Mucosal lesions have been shown to have intense lymphocytic and neutrophilic infiltration, and in older
lesions immunoglobulin and complement are deposited on the basement membrane. Evidence of chronic activation of the immune response in both mouth ulcers and peripheral blood T lymphocytes has been demonstrated in patients with BD [2]. Certain species of oral Streptococcus have been associated with disease, as has HSV [3, 4]. In support of this hypothesis, patients with BD, but not those with recurrent oral ulcers, were shown to respond to several peptides derived from bacterial HSP-65 that show strong homology with the human hsp60 [5].

An important part of the innate immune response are Toll-like receptors (TLRs) that recognize conserved molecules from pathogens and initiate an appropriate response to infection including the up-regulation of defensins and signalling to the adaptive immune response [6]. In patients with quiescent BD, the frequency of TLR6-expressing granulocytes compared with healthy controls was significantly reduced, but increased on in vitro stimulation with HSP-60. In comparison, TLR2/6+-expressing monocytes were similar in BD patients and healthy controls, but on challenge with lipopolysaccharide (LPS) or HSP-60 expression was significantly increased in controls, but not in patients with BD, suggesting a defect in this cell population [7]. In a second study, monocytes of patients with active BD showed increased expression of TLR2 and TLR4 that inversely correlated with serum 25-hydroxyvitamin D levels, and in vitro studies showed that 1,25(OH)2D3 down-regulated TLR2 and TLR4 mRNA and protein, suggesting a complex level of control of TLR expression [8].

The Arg753Gln mutation in TLR2 (rs574708) was shown to be linked to reduced responsiveness to bacterial peptides associated with a predisposition to staphylococcal infection and with the incidence of sepsis in a white population [9]. TLR2 Arg677Trp was associated with reduced production of IL-12 by monocytes and with patients with lepromatous leprosy in an Asian population [10]. TLR4 Asp299Gly (rs4986790) and Thr399Ile (rs498691) have been associated with systemic inflammatory hyporesponsiveness to inhaled LPS in human volunteers, and an increased risk of Gram-negative bacterial infection in critical care patients and patients with septic shock [11–13]. In addition, single nucleotide polymorphism (SNP) in CD14, a co-receptor for TLR4, has been associated with infection in critically ill patients [14]. MyD88 adapter-like protein (Mal), also known as Toll/interleukin receptor 1 (TIR) domain-containing adaptor protein (TIRAP), is an important molecule in the signalling cascade for TLR2 and TLR4. Recently, a SNP in TIRAP, Ser180Leu (rs8177374), has been linked to protection against infectious diseases, including both general and severe malaria in African and Vietnamese cohorts, and against developing tuberculosis or SLE in Columbus patients suggesting an important role of TIRAP in the pathogenesis of autoimmune diseases [15, 16].

In this study, we have analysed TLR expression in buccal tissue from patients with BD, as well as diseased and healthy controls. SNPs in TLR2, TLR4, CD14 and TIRAP were tested for association with BD. The results show that while TLR expression is increased in buccal tissue in BD, it is similar to the increase seen in other oral diseases. SNPs in TLR and CD14 were not linked to disease, while TIRAP Ser180Leu was significantly associated with BD in UK, but not Middle Eastern patients.

**Materials and methods**

**Patients**

For buccal biopsies, patients with BD were recruited from the clinic at St Thomas’ Hospital, London. For SNP analysis, two cohorts of 265 unrelated BD patients (145 UK patients from St Thomas’ Hospital and Birmingham and Midland Eye Centre that are predominantly white Caucasian), and 120 Middle Eastern patients from St John’s Ophthalmic Hospital, East Jerusalem were identified. The diagnosis of BD was reviewed and confirmed by using the classification criteria of the International Study Group for BD [17]. Clinical examination included full ophthalmological, oral and general examination. Disease activity status and treatment regimens were recorded on a proforma. The ethical committees of all centres approved the study (Sandwell and West Birmingham Hospitals NHS Trust; St John’s Eye Hospital; West Bank Palestine and St Thomas’ Hospital Trust) after written informed consent by the patients and controls was obtained in accordance with the Declaration of Helsinki. Ethically matched healthy controls were recruited separately at the participating Middle Eastern centres (n = 81) and Dublin (n = 1102).

**Tissue samples**

Buccal epithelial punch biopsies obtained from patients with BD (n = 16), and from patients with lichen planus (LP) (n = 3) or pyogenic granuloma (PG) (n = 3), were fixed, processed and embedded in paraffin wax. Normal mucosa was obtained from four laboratory volunteers. For staining, 10 µm sections were used.

**Immunohistochemistry**

Formalin-fixed paraffin sections were prepared by dewaxing through xylene and alcohol baths, then subjected to antigen retrieval solution (DAKO Cytomation, UK) at 95–99°C for 30 min. Sections were stained using the Catalysed Signal Amplification System (DAKO Cytomation, UK), and positive cells identified by diamino-benzidine staining. The antibodies used were anti-TLR-2 (TIR2.1), anti-TLR4 (HTA125), anti-TLR6 (86B1153.2) and anti-TLR9 (26C593) (Biocarta, UK). Positive cell counts were performed blind by two of the authors (G.R.W. and P.I.M.) and mean values and s.d.s were calculated.

**TLR polymorphism analysis**

DNA was prepared by proteinase K digestion, and salt extraction [18] and stored at −70°C until use. Polymorphisms in the promoter regions 677 and 753 in TLR2, and Asp299Gly and Thr399Ile 49 SNPs in the TLR4 gene, CD14 and TIRAP Ser180Leu were detected.
by a PCR-sequence-specific primer (SSP) assay using appropriate primer mixes (Table 1). Primers mixes were prepared that identified possible combinations of TLR2 and TLR4 SNPs or single CD14 and TIRAP SNPs using Taq Polymerase (Abgene, Epsom, UK). The PCR products were visualized by electrophoresis through a 1% agarose gel in 0.5× Tris buffered ethylenediaminetetraacetic acid (TBE) buffer containing 1 μg/mL ethidium bromide. The relative size of the PCR products was determined by comparison of the migration of a 100–1000 bp DNA molecular weight ladder (Abgene).

### Statistical analysis and data analysis

Associations with disease were sought between genotype and haplotype frequencies. Chi-squared analysis was carried out using EpiStat, with a Bonferroni correction factor applied. TLR expression was tested by Kruskal-Wallis as a non-parametric analysis of variance (ANOVA), and individually by Mann-Whitney test.

### Results

**TLR expression in oral mucosal tissue**

Expression of TLR2, TLR4, TLR6 and TLR9 was detected in mouth ulcers of patients with BD, with staining present throughout the lesion, with similar staining on biopsies from patients with PG, LP and normal healthy mucosa. As seen for TLR2 and TLR4, there was increased numbers of cells staining positive in all the inflammatory samples. Quantitative analysis was carried out on four representative biopsies of each of the four conditions stained as a batch on the same day. The results showed that TLR2 and TLR6 expression was significantly increased in biopsies from patients with BD and LP compared with normal control tissue. TLR4 and TLR9 expression was increased in all disease groups compared with healthy tissue, particularly BD, but did not reach significance (Fig. 1).

**Single nucleotide polymorphisms**

SNPs at positions 677 and 753 in TLR2, positions 299 and 399 in TLR4 and position –159 in CD14, were analysed in patients and healthy controls. The results showed that there was no association with any TLR SNPs and BD. Allele frequency of the TLR SNPs was extremely low: TLR2 677T (0%), TLR2 753A (4%), TLR4 399C (0.7%), TLR4 299G (2%) and no homozygotes for either SNP were identified. For CD14 SNP, the expression of the two alleles was not significantly different from controls (data not shown).

The TIRAP 180Leu variant was significantly associated with BD in UK patients compared with UK controls [odds ratio (OR) 1.5; \( \chi^2 = 7.32, P = 0.007 \)], due to an increased number of both variant homozygote and heterozygote individuals. By comparison, TIRAP 180Leu was not associated with BD in the Middle Eastern cohort (\( \chi^2 \leq 0.29, P = 0.5 \)) (Table 2). UK BD patients also showed significantly higher frequency of TIRAP 180Leu than Middle Eastern BD patients (\( \chi^2 = 7.77, P = 0.005 \)).

### Discussion

An outstanding question in BD is what causes the persistent inflammation seen in mucosal tissue. In this study, analysis of mouth ulcer biopsies for the expression of TLR found increased expression in samples from patients with BD compared with normal tissue; however, a similar increase was found in other inflammatory conditions, LP and PG, and therefore is not specific to inflammation seen in BD patients. This similarity with other oral diseases is supported by increased expression of IL-12, IFN-γ, TNF-α and the chemokine receptors, CCR5 and CXCR3, in oral ulcer biopsies from patients with BD and patients with recurrent aphthous stomatitis (RAS) [19]. In a second study, numbers of CD3 T cells and \( \gamma\delta \) T cells, and expression of adhesion molecules including CD44, CD58 vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were up-regulated in oral ulcers from patients with BD compared with healthy control tissue [20]. These findings are supported by data showing that \( \gamma\delta \)-T cells were present only in ulcerated oral mucosa and not in non-ulcerated BD or healthy control tissue. Since \( \gamma\delta \)-T cells are important in early responses against microorganisms by identifying a wide variety of antigens, it would suggest that they have a key role in BD [21]. Taken together, the data strongly suggest that lymphoid cells infiltrating buccal tissue are the source of the increased expression of TLR and may be responsible for inducing tissue damage.

Current evidence suggests that similar immunological mechanisms are also involved in the pathogenesis of LP, a condition characterized by a subepithelial inflammatory cell infiltrate. Oral biopsies from LP patients showed increased numbers of CD4, CD8 and CD1a cells. CD4 and CD8 cells also expressed lymphocyte function-associated antigen-1 (LFA-1), which correlated with significantly higher expression of ICAM and VCAM [22]. Antigen presentation by basal keratinocytes and antigen-specific keratinocyte killing by CD8 T cells, and MMP activation have been shown in oral LP. It was

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**Table 1** Primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' - 3'</th>
</tr>
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<tbody>
<tr>
<td>TLR2-1F (677)</td>
<td>CTT CAA GTT GTG TCT TCA TAA GG</td>
</tr>
<tr>
<td>TLR2-2F (677)</td>
<td>CTT CAA GTT GTG TCT TCA TAA GT</td>
</tr>
<tr>
<td>TLR2-3R (753)</td>
<td>GTC TG TGG ACC ATT TTC C</td>
</tr>
<tr>
<td>TLR2-4 (753)</td>
<td>GTC TTG GTG TTC ATT ATC TTC T</td>
</tr>
<tr>
<td>TLR4-1 (299)</td>
<td>ACT TAG ACT ACT ACC TCG ATG A</td>
</tr>
<tr>
<td>TLR4-2 (299)</td>
<td>ACT TAG ACT ACT ACC TCG ATG G</td>
</tr>
<tr>
<td>TLR4-3 (399)</td>
<td>AGA TCT AAA TAC TTT AGG CTG G</td>
</tr>
<tr>
<td>TLR4-4 (399)</td>
<td>AGA TCT AAA TAC TTT AGG CTG A</td>
</tr>
<tr>
<td>CD14FC</td>
<td>AGA ATC CTT CCT GTT ACG GC</td>
</tr>
<tr>
<td>CD14FT</td>
<td>AGA TCT CTT CCT GTT ACG GT</td>
</tr>
<tr>
<td>CD14R</td>
<td>CG ACA GGC TCT TGA AGT G</td>
</tr>
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suggested that chronicity may be due in part to deficient TGF-β-mediated immunosuppression, as the normal oral mucosa may be immune-privileged and breakdown could result in oral LP (OLP) [23].

As functional differences in TLR could explain the differences in immunophenotypic expression and mucosal distribution of activation and inflammatory markers seen in BD, we analysed SNPs in TLR2 and TLR4, and found no

**Table 2** Single nucleotide polymorphisms in TIRAP Ser180Leu in patients with BD and healthy controls

<table>
<thead>
<tr>
<th>Group</th>
<th>TIRAP 180 genotype, %</th>
<th>( \chi^2 ) and P-value S vs L</th>
</tr>
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<tbody>
<tr>
<td>UK BD (n = 146)</td>
<td>SS 89 (0.62) SL 49 (0.33) LL 8 (0.05)</td>
<td>7.3; 0.007</td>
</tr>
<tr>
<td>UK controla (n = 1102)</td>
<td>SS 773 (0.70) SL 306 (0.28) LL 23 (0.02)</td>
<td>7.3; 0.007</td>
</tr>
<tr>
<td>ME BD (n = 120)</td>
<td>SS 91 (0.76) SL 27 (0.23) LL 2 (0.01)</td>
<td>0.29; 0.5</td>
</tr>
<tr>
<td>ME control (n = 81)</td>
<td>SS 64 (0.79) SL 16 (0.20) LL 1 (0.01)</td>
<td>0.29; 0.5</td>
</tr>
</tbody>
</table>

ME: middle eastern. aSee Khor et al. 2007 [15].
difference in allelic frequencies between patients and controls. Similarly, there was no association with SNPs in CD14, which encodes a co-ligand for LPS. In support of these findings, TLR2 Arg753G showed no association with BD in Turkish patients [24]. Similarly, TLR9 polymorphisms did not associate with BD in a Japanese cohort of patients [25]. Recent data from genome-wide screen analysis led to analysis of TLR4 that showed an association between a SNP (rs7037117) in the 3’-untranslated region and BD in Japanese patients compared with healthy controls. This SNP was associated with the incomplete, but not complete, type of BD, with age of onset >34 years, and with oral, genital, skin and ocular manifestations of the disease [26]. The association of this SNP and BD in other ethnic groups with patients fulfilling the international criteria, and the functional relevance of the polymorphism should be addressed. In a recent study, TLR4 Asp299Gly SNP was found in high prevalence in sub-Saharan Africa and was protective against malaria, whereas in Europe, where such a feature would be deleterious in response to bacterial infection, the TLR4 Thr399Ile SNP cancels out the loss of function induced by As299Gly [27].

The association of TIRAP 180Leu with BD is intriguing as this polymorphism is associated with reduced function in TLR signalling. This SNP has been associated with protection against infectious disease [15, 16]. The reason for 180Leu protection was suggested to be a less aggressive response to TLR ligand binding and therefore reduced immunopathology in response to pathogens based on an in vitro transfection assay. In contrast, challenge with LPS in human volunteers showed an increased cytokine response in TIRAP Ser180Leu heterozygous individuals compared with wild-type controls. Furthermore, in vitro stimulation of peripheral blood mononuclear cells with TLR2 ligand showed a similar response [28]. These data suggest that rather than a decreased response to TLR ligands, TIRAP Ser180Leu regulates a more potent cytokine response thereby clearing pathogens more effectively in heterozygotes. However, in 180Leu homozygotes this increased cytokine response could lead to the systemic inflammation seen in BD. The lack of variant homozygotes in the Middle Eastern population is also seen in African and Asian cohorts and may be related to the increased pathogen burden in these areas, where increased TLR signalling would be more dangerous. The increased frequency of TIRAP 180Leu in the UK cohort is probably due to selective pressure from infectious disease, an event suggested to have occurred in the early phase of human migration of humans into this region. Interestingly, analysis of TIRAP 180Leu prevalence in different populations showed the highest frequency in Bedouins from the Negev desert [28]. Why this Middle Eastern population demonstrates such a high prevalence compared with neighbouring populations is not clear. Therefore, the increased prevalence of 180Leu in European patients with BD may contribute to the increased cytokine production and mucosal tissue damage present in this disease.

**Rheumatology key messages**

- The Ser180Leu polymorphism in TIRAP is associated with BD.
- TLR expression is increased in BD buccal biopsies, but no greater than disease controls.
- Common SNPs in TLR2, TLR4 and CD14 do not associate with BD.

**Disclosure statement:** The authors have declared no conflicts of interest.

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