Original article

Leukocyte toll-like receptor expression in pathergy positive and negative Behçet's disease patients

Tim B. van der Houwen [ID] [1,2], Willem A. Dik[2,*], Marco Goeijenbier[3], Manizhah Hayat[1], Nicole M. A. Nagtzaam[2,*], Martin van Hagen[1,2] and Jan A. M. van Laar[1,2]

Abstract

Objectives. To investigate whether the auto-inflammatory nature and the pathergic reaction in Behçet's disease (BD) are driven by a disturbed toll-like receptor (TLR) response.

Methods. We compared both TLR expression by flow-cytometry and TLR response by stimulation assay in 18 BD patients (both pathergy positive and pathergy negative) with 15 healthy controls.

Results. Expression of TLR1 and 2 was significantly elevated in B-lymphocytes of BD patients compared with healthy controls. TLR1, 2 and 4 were significantly more highly expressed in both $CD4^+$ and $CD8^+$ T-lymphocytes of BD patients. Granulocytes of BD patients displayed significantly higher expression of TLR1, 2, 4 and 6. TLR2, 4 and 5 expression was significantly increased on classical monocytes of BD patients. Intermediate monocytes of BD patients showed an increase in expression of TLR2. Furthermore, TLR2 and 5 were significantly more highly expressed in non-classical monocytes of BD patients. In pathergy positive patients, TLR5 was even more highly expressed compared with pathergy negative patients on B- and T-lymphocytes and granulocytes. Furthermore, TLR2 and 5 showed an elevated TNF-α response to stimulation with their cognate ligands.

Conclusion. Immune cells of BD patients overexpress TLR1, 2, 4, 5 and 6. Furthermore, after stimulation of TLR2 and 5, BD patients demonstrate a more potent TNF-α response. Although this is a small cohort, in the pathergy positive patients, TLR5 expression is even further augmented, suggesting that a microbial (flagellin) or damage (HMGB1) associated signal may trigger the exaggerated immune response that is characteristic for the pathergy phenomenon in BD. In conclusion, these results point to an exaggerated TLR response in the auto-inflammatory nature of BD.

Key words: Behçet's disease, TLR, pathergy, innate immune system, flagellin

Introduction

Behçet's disease (BD) is an auto-inflammatory vasculitis of unknown origin, affecting predominantly the orogenital mucosa, skin and eyes [1]. The clinical symptoms of BD relate to a disturbed T cell response; however, increasing evidence suggests an auto-inflammatory nature rather than an auto-immune nature [2]. Auto-inflammatory disorders are characterized by excessive innate immune responses, causing a hyper-inflammatory state [3].

In BD this hyper-inflammatory state is reflected by elevated serum cytokine levels, for instance TNF-α, IL-1β and IL-18 [4, 5]. Additional evidence of innate immune activation in BD is increased neutrophil activity [6] and elevated numbers of gamma delta T-lymphocytes, both in circulation and at inflammatory sites [7].

Furthermore, it has been proposed that HLA-B51 misfolding may cause endoplasmic reticulum stress thereby inducing an inflammatory response, comparable to HLA-B27 in spondylarthropathy [2, 8]. The BD-specific pathergy test [9] reveals an exaggerated inflammatory cutaneous response to sterile-needle-induced tissue damage. This suggests a role for innate pattern recognition receptors, including toll-like receptors (TLRs) [10].

TLRs are pathogen recognition receptors of which certain family members are expressed at the cell membrane surface (TLR 1, 2, 4, 5, 6 and 10), while others are typically expressed in the endosomal compartment

---

Rheumatology key messages
- Altered toll-like receptor (TLR) expression and activity could contribute to the inordinate innate immune activation in Behçet's disease.
- In pathergy positive Behçet's disease patients, augmented TLR5 expression suggests a microbial or damage signal triggering excessive inflammation.

---

within the cytosol (TLR 3, 7, 8 and 9) [11]. TLRs are present in virtually all immune cells and specialized in the recognition of conserved motifs present in pathogens [the so-called pathogen-associated molecular patterns (PAMPs)], and of endogenous molecules released upon tissue injury [the so-called damage-associated molecular patterns (DAMPs)]. Upon PAMP or DAMP binding, TLRs activate the transcription factor nuclear factor-κB (NF-κB) in a MyD88-dependent manner. Exceptions are TLR3, which uses TIR-domain-containing adaptor-inducing interferon-β (TRIF) to activate NF-κB, while TLR4 can signal through both pathways [12]. NF-κB activation stimulates production of pro-inflammatory molecules, including TNF-α, which is a key cytokine in the mechanistic pathway of BD [2, 13].

Data on TLR expression and activation in BD are inconsistent. Liu *et al.* demonstrated elevated TLR 2, 3, 4 and 8 expression in peripheral blood mononuclear cells (PBMCs) of BD patients compared with healthy controls [14]. Stimulation with specific TLR2 and 4 ligands results in elevated IL-1β and IL-23 production by monocytes of these patients as well as increased IL-17 production in T-lymphocytes and monocytes, compared with healthy controls. Seoudi *et al.* showed increased TLR2 and 4 mRNA expression in oral tissue from BD patients, but diminished activation of PBMCs upon stimulation with specific TLR1/2 and TLR4 ligands, compared with PBMCs from healthy controls [15]. Yavuz *et al.* described decreased TLR6 expression in granulocytes from BD patients, while expression of TLR1, 2 and 4 in granulocytes and monocytes was comparable to healthy controls [16]. Besides these discrepant data on TLR expression, no other data on TLR expression in relation to the pathergy test response in BD is available.

The goal of this work was therefore to thoroughly study the relationship between TLR expression/activity and to explore the relationship between TLRs and the pathergy test response in patients with BD. Expression levels of TLR 1, 2, 4, 5 and 6 by peripheral blood cells from pathergy test positive and pathergy test negative BD patients were studied. In addition, TNF-α production response by PBMCs upon stimulation with specific TLR ligands was examined. Our study reveals increased expression of TLR1 and 2 on B cells, T cells and granulocytes, increased expression of TLR2 on different monocyte subsets (classical, pro-inflammatory/intermediate and non-classical monocytes), increased expression of TLR4 on B cells, T cells, granulocytes and classical monocytes and increased expression of TLR5 on granulocytes, classical and non-classical monocytes. In addition, elevated TNF-α secretion

upon stimulation with TLR2 and TLR5 ligands is shown. Moreover, pathergy test positive BD patients are characterized by higher expression of TLR5 on B cells, T cells and granulocytes compared with pathergy test negative BD patients. The data indicate that altered TLR expression and activity could contribute to the excessive innate immune activation observed in BD and may partly explain the pathergy phenomenon observed in some BD patients.

Methods

Patients

Diagnostic work-up and detailed examination of blood from 18 patients with BD (diagnosed according to the International Study Group of BD Criteria [17]) and 15 healthy controls (age, gender and ethnicity matched) were performed following written informed consent according to the Declaration of Helsinki. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC-2011-172). Disease activity was measured using the BD Current Activity Form (BDCAF) [18]. A pathergy test was conducted in the diagnostic process according to International Study Group of BD Criteria [17]; both pathergy positive and pathergy negative patients were included.

Flowcytometric measurement of TLR expression

Heparinized whole-blood samples (200 μl) from BD patients and controls were lysed with ammonium chloride and washed with PBS containing 0.5% bovine serum albumin and incubated for 10 min with the following antibodies: CD45-PerCP, CD33-allophycocyanine (APC), CD16-PE-Cy7, CD8-APC-Cy7, CD14-APC-Cy7, CD20-APC-Cy7 (all from BD Biosciences, San Jose, CA, USA), CD19-PE-Cy7 (from Beckman Coulter, Fullerton, CA, USA), CD1c-FITC, TLR1-PE, TLR2-PE, TLR4-PE (all from eBioscience, San Diego, CA, USA), TLR5-FITC (from Abcam, Cambridge, UK) and TLR6-PE (from Biolegend, San Diego, CA, USA). Samples were measured on a flow cytometer (FACSCanto II machine; BD Biosciences) and analysis was performed with Infinicyt 1.7 flow cytometry software (Cytognos S.L., Salamanca, Spain).

Lymphocytes were defined as CD45 positive and low on sideward scatter. Within lymphocytes the following subsets were determined: B-lymphocytes (CD19$^+$CD20$^+$CD3$^-$), CD4 T-lymphocytes (CD3$^+$CD4$^+$) and CD8 T-lymphocytes (CD3$^+$CD8$^+$). Monocytes were

defined as CD45$^+$ with intermediate sideward scatter and subtyped based on CD14 and CD16 expression: classical (CD14^{++}CD16$^-$), intermediate (CD14^{++}CD16$^+$) and non-classical (CD14^{dim}CD16^{++}) monocytes. Granulocytes were determined on the basis of high sideward scatter and CD45dim expression. TLR expression level was measured using mean fluorescence intensity (MFI). The flow cytometer was calibrated according to a standardized instrument setting described in detail in the EuroFlow protocols [19]. The instrument setting used ensures that major leucocyte populations are gated in the window of analysis at a comparable location at each time and allows for MFI comparisons between samples.

Ex vivo stimulation of whole blood by TLR ligands

To determine the response to extracellular TLR activation, whole blood of therapy naïve BD patients ($n = 6$) and healthy controls ($n = 3$, ethnicity, age and gender matched) was stimulated with TLR-specific ligands, as described before [20]. Briefly, 50 µl of whole blood was stimulated with one of the following ligands for 6 h at 37°C, 5% CO_2: the TLR1 ligand Pam3CSK4 (100 ng/ml), the TLR2 ligand *Staphylococcus aureus* derived lipoteichoic acid (1 µg/ml), the TLR4 ligand lipopolysaccharide (LPS; 1 ng/ml *Escherichia coli* ultrapure), the TLR5 ligand flagellin (1 µg/ml, purified flagellin from *Salmonella typhimurium*) or the TLR6 ligand fibroblast stimulating lipopeptide 1 (FSL-1; 1 µg/ml). All TLR ligands were obtained from InvivoGen (San Diego, CA, USA). Hereafter, culture supernatant was collected and stored at -80°C until further analysis. TNF-α in the supernatants was determined by enzyme-linked immunosorbent assay (Biosource Europe SA, Nivelles, Belgium, human TNF-α CytoSets).

Statistical analysis

Statistical analysis was performed using the Mann–Whitney test (SPSS Statistics version 21.0, IBM Corp., Armonk, NY, USA). A *P*-value <0.05 was considered significant.

Results

Patients

In total 12 patients [mean age 40 years (range 26–59 years), male: female 1:1.4] and 12 healthy controls [mean age 45 years (range 21–62 years), male: female 1:1] were included. Patients were at the time of study inclusion treated with colchicine ($n = 5$), adalimumab ($n = 4$) or colchicine and adalimumab ($n = 1$), or therapy naïve ($n = 2$). In addition to this, to test the peripheral blood TNF-α response after *ex vivo* stimulation with specific TLR ligands, we included six therapy naïve patients [mean age 32 years (range 23–43) male: female 1:5] and compared these with 3 healthy controls [mean age 28 years (range 26–30) male: female 1:2] (Table 1).

TLR expression in BD patients

B-lymphocytes of BD patients expressed TLR1 and 2 significantly more than healthy controls (both $P < 0.05$), and there was a trend towards increased expression of TLR4, 5 and 6 (Fig. 1A).

In both CD4$^+$ and CD8$^+$ T-lymphocytes of BD patients, TLR1 displayed the most significant increased expression compared with healthy controls ($P < 0.01$ in both CD4$^+$ and CD8$^+$ T-lymphocytes). Also TLR2 and 4 showed a significantly increased expression on CD4$^+$ and CD8$^+$ T-lymphocytes of BD patients (both $P < 0.05$). A trend towards increased expression of TLR5 and 6 was seen in CD4$^+$ and CD8$^+$ T-lymphocytes (Fig. 1B and C).

TLR1, 2, 4 and 6 were significantly more highly expressed ($P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.05$, respectively) on granulocytes from BD patients. In addition, granulocytes from BD patients displayed higher

TABLE 1 Patient characteristics

Characteristic	Patients ($n = 12$)	Therapy naïve patients ($n = 6$)	Healthy controls ($n = 15$)
Females, %	58	83	67
Age, mean (range), years	40 (26–59)	32 (23–43)	42 (21–62)
Clinical manifestations, %			
Oral ulceration	100	100	
Genital ulceration	92	67	
Uveitis	42	17	
Skin lesions	92	83	
Pathergy	67	67	
HLA-B51	42	67	
First-line medication	42	0	
Second-line medication	42	0	
Disease activity, median BDCAF	2	3	

First line medication is colchicine ($n = 5$). Second-line medication is adalimumab ($n = 5$). BDCAF: Behçet's Disease Current Activity Form.

Fig. 1 TLR expression in BD patients and healthy controls
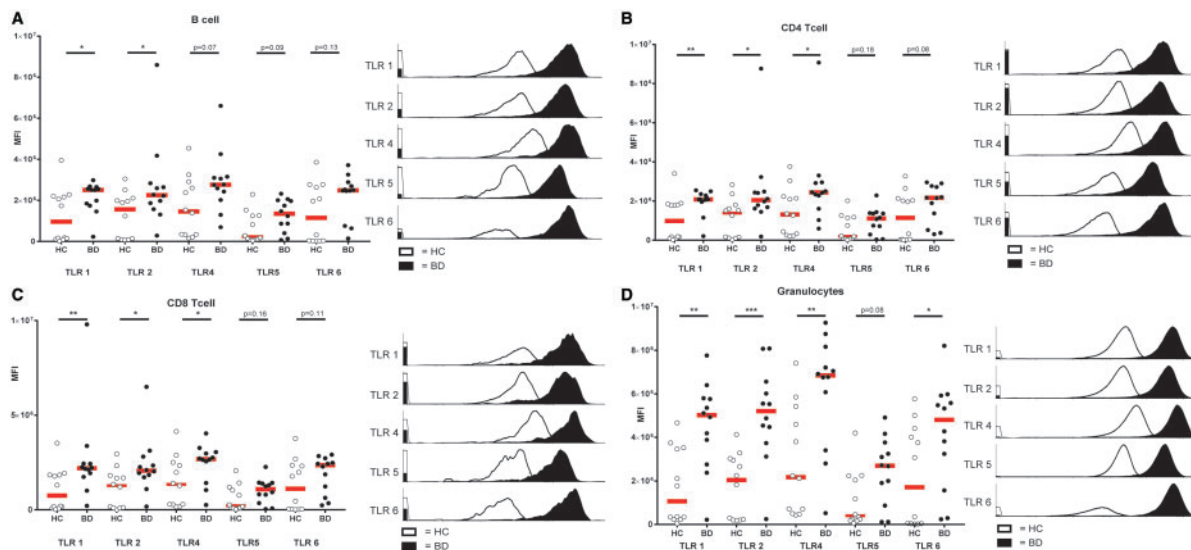
TLR expression in B-lymphocytes (**A**), CD4$^+$ (**B**) and CD8$^+$ (**C**) T-lymphocytes and granulocytes (**D**). Illustrated in black are BD patients; white represents healthy controls. Expression is shown as mean fluorescence intensity. The expression of the measured TLRs of a representative patient is shown in the curve on the right. Differences between controls and patient groups were statistically analysed using the Mann–Whitney test: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. BD: Behçet's disease; HC: healthy control; MFI: mean fluorescence intensity; TLR: toll-like receptor.

TLR5 expression than controls, although this was not statistically significant ($P = 0.08$) (Fig. 1D).

Classical monocytes of BD patients expressed significantly higher levels of TLR2 ($P < 0.05$), TLR4 ($P < 0.05$) and TLR5 ($P < 0.05$) compared with healthy controls. TLR2 was also expressed at higher levels by the intermediate ($P < 0.05$) and non-classical ($P < 0.05$) monocytes of BD patients. In addition to this, non-classical monocytes displayed a significantly higher TLR5 expression ($P < 0.05$). The distribution of monocytes in BD patients was comparable to healthy controls. Data are summarized in Table 2. Table 3 shows a summary of TLR expression in the examined leucocyte subsets.

TLR expression in pathergy positive patients

Pathergy positive BD patients displayed significantly higher expression of TLR5 in B-lymphocytes and CD4$^+$ and CD8$^+$ T-lymphocytes compared with pathergy negative BD patients ($P < 0.05$) (Fig. 2). In addition to this, a trend towards higher TLR6 expression by B-lymphocytes, CD4$^+$ and CD8$^+$ T-lymphocytes of pathergy positive patients was observed.

TLR5 was also expressed at significantly ($P < 0.05$) higher levels by granulocytes of pathergy positive BD patients than those of pathergy negative patients (Fig. 2). Moreover, TLR1 expression by granulocytes from pathergy positive BD was significantly ($P < 0.01$) lower than that of pathergy negative BD patients (Fig. 2). Classical monocytes of pathergy positive BD patients displayed a significantly ($P < 0.05$) higher expression of

TLR6 compared with pathergy negative BD patients (Table 2). Moreover, classical, intermediate and non-classical monocytes of pathergy positive BD patients displayed a trend towards increased expression of TLR5 (Table 2).

HLAB51 positivity or medication did not correlate with TLR expression by any of the leucocyte subsets analysed (data not shown). Table 3 shows a summary of TLR expression on the examined leucocyte subsets.

TLR response to stimulation in whole blood samples

Peripheral blood cells from BD patients produced significantly more TNF-α when stimulated with the TLR2 ligand lipoteichoic acid or the TLR5 ligand flagellin compared with healthy controls (Fig. 3, both $P < 0.05$). In contrast, upon stimulation with FSL-1, which activates the TLR2/6 complex, peripheral blood cells from BD patients tended to produce less TNF-α than healthy controls, although this difference was not statistically significant. Stimulation of peripheral blood cells with the TLR4 ligand LPS or the TLR1/2 ligand PAM3CSK4 revealed no differences in TNF-α production between patients and healthy controls.

Discussion

This study demonstrates increased expression of TLR1, 2, 4, 5 and 6 by lymphoid and myeloid cells of patients with BD compared with healthy controls. Moreover,

TABLE 2 TLR expression in subtypes of monocytes

	Patients	Healthy controls	*P*-value	Pathergy positive	Pathergy negative	*P*-value
Cell number, median (range), $\times 10^9$/l						
Monocytes	0.79 (0.12–2.17)	0.85 (0.32–1.71)	0.56			
Classical monocytes	0.44 (0.07–0.98)	0.58 (0.02–1.44)	0.52			
Intermediate monocytes	0.26 (0.01–1.25)	0.14 (0.05–0.23)	0.52			
Non-classical monocytes	0.08 (0.01–0.14)	0.08 (0.02–0.14)	0.85			
TLR expression in MFI, median (range), $\times 10^6$						
Classical monocytes						
TLR1	5.8 (0.5–7.4)	3.5 (0.2–9.0)	0.17	5.8 (0.5–6.3)	6.0 (4.0–7.4)	0.32
TLR2	5.6 (0.3–9.7)	2.6 (0.2–7.7)	**0.02**	5.2 (0.3–9.7)	6.2 (4.3–7.3)	0.53
TLR4	7.4 (0.7–10.5)	4.2 (0.4–11.0)	**0.03**	7.4 (0.7–10.5)	7.3 (6.2–8.4)	0.93
TLR5	3.1 (0.1–6.2)	0.4 (0.1–4.4)	**0.03**	4.4 (0.1–6.2)	1.5 (0.2–3.1)	0.16
TLR6	5.6 (0.5–10.1)	3.4 (0.7–9.2)	0.31	5.6 (1.4–10.1)	3.5 (0.5–7.4)	0.41
Intermediate monocytes						
TLR1	7.3 (0.4–18.0)	4.4 (0.0–15.1)	0.08	7.2 (0.4–18.0)	7.9 (5.8–9.3)	0.93
TLR2	7.1 (0.4–14.5)	3.2 (0.3–12.5)	**0.03**	8.2 (0.4–14.5)	6.7 (5.9–8.8)	0.68
TLR4	8.9 (1.0–13.5)	4.6 (0.6–17.1)	0.21	9.9 (1.0–13.5)	9.2 (6.7–10.0)	0.57
TLR5	5.7 (0.1–8.2)	3.7 (0.0–8.3)	0.05	6.7 (0.2–10.1)	1.4 (0.1–5.7)	0.07
TLR6	7.6 (0.5–13.2)	4.0 (0.1–14.5)	0.28	10.3 (0.6–13.2)	3.9 (0.5–8.7)	0.07
Non-classical monocytes						
TLR1	5.1 (0.3–9.1)	3.9 (0.0–9.4)	0.17	5.1 (0.3–9.1)	5.1 (4.4–5.8)	0.93
TLR2	4.8 (0.3–7.6)	3.0 (0.2–7.2)	**0.01**	5.3 (0.3–7.6)	4.5 (3.8–6.6)	0.79
TLR4	5.6 (0.7–8.5)	4.8 (0.6–10.7)	0.31	5.9 (0.7–8.5)	5.5 (5.2–5.6)	0.41
TLR5	2.4 (0.2–6.3)	0.3 (0.1–4.2)	**0.04**	4.0 (0.2–6.3)	1.2 (0.2–2.4)	0.11
TLR6	4.7 (0.4–8.3)	3.5 (0.1–9.3)	0.23	6.2 (1.9–8.3)	2.8 (0.4–4.6)	**0.02**

Cell numbers are depicted as $\times 10^9$/l, as median (range). *P*-values <0.05 in bold indicate statistical significance. TLR expression is displayed as the median MFI. MFI: mean fluorescence intensity; TLR: toll-like receptor.

TABLE 3 Summary of TLR expression on the examined leucocyte subsets

|	Patients compared with healthy controls					Pathergy positive patients compared with pathergy negative patients				
	TLR1	TLR2	TLR4	TLR5	TLR6	TLR1	TLR2	TLR4	TLR5	TLR6
B cells	++	++	ns	ns	ns	ns	ns	ns	++	ns
CD4+ T cells	++	++	++	ns	ns	ns	ns	ns	++	ns
CD8+ T cells	++	++	++	ns	ns	ns	ns	ns	++	ns
Granulocytes	++	++	++	ns	++	−	ns	ns	++	ns
Classical monocytes	ns	++	++	++	ns	ns	ns	ns	ns	ns
Intermediate monocytes	ns	++	ns	ns	ns	ns	ns	ns	ns	ns
Non-classical monocytes	ns	++	ns	++	ns	ns	ns	ns	ns	++

Significant increase in TLR expression is displayed as ++. Significant decrease in TLR expression is depicted as −. ns: no significant differences; TLR: toll-like receptor.

pathergy test positive BD patients display even higher TLR5 expression on B-lymphocytes, CD4+ T-lymphocytes, CD8+ T-lymphocytes and granulocytes than do BD patients with a negative pathergy test. Furthermore, PBMCs from BD patients produce more TNF-α upon activation of TLR2 or 5 than PBMCs from healthy controls.

We have demonstrated that BD patients with a positive pathergy test have a significantly higher TLR5 expression level on B-lymphocytes, CD4+ T-lymphocytes, CD8+ T-lymphocytes and granulocytes compared with

pathergy negative BD patients. TLR5 acts by recognition of bacterial flagellin. Higher expressed levels have not been demonstrated in BD so far. Overexpression of TLR5 on monocytes has been associated with inflammatory responses in elderly. In addition to this, overexpression of TLR5 has been found on monocytes and peripheral blood lymphocytes in SLE and acute graft-*vs*-host disease [21–23]. In another auto-inflammatory disease that is clinically related to BD, Crohn's disease, TLR5 activation is considered to contribute to the

Fig. 2 TLR expression in pathergy positive and pathergy negative patients
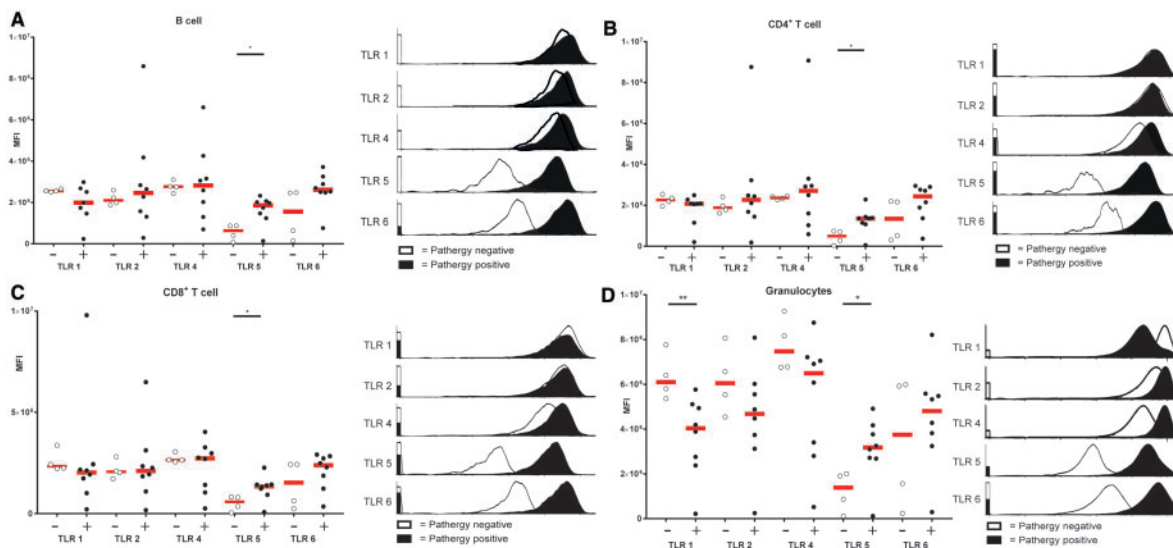
Differences in TLR expression in B-lymphocytes (**A**), CD4$^+$ (**B**) and CD8$^+$ (**C**) T-lymphocytes and granulocytes (**D**) between pathergy positive and pathergy negative patients. Illustrated in black are pathergy positive patients; white represents pathergy negative patients. Expression is shown in mean fluorescence intensity. The expression of the measured TLRs of a representative patient is shown in the curve on the right. Differences between patient groups were statistically analysed by using the Mann–Whitney test: *$P < 0.05$; **$P < 0.01$. MFI: mean fluorescence intensity; TLR: toll-like receptor.

Fig. 3 TNF-α response to stimulation with TLR agonists in whole blood
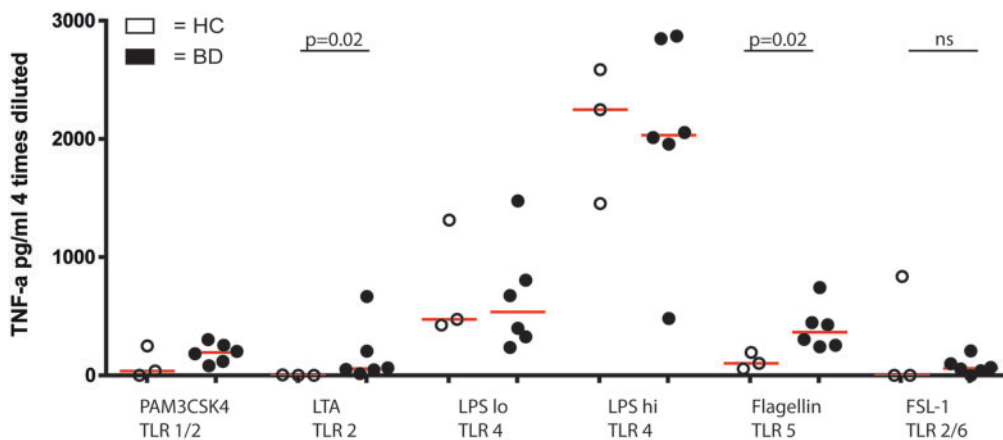
TNF-α response to stimulation with TLR ligands. Black scatter dots represents BD patients; white scatter dots illustrates TNF-α response in healthy controls. Differences between controls and patient groups were statistically analysed by using the Mann–Whitney test. BD: Behçet's disease; FSL-1: fibroblast stimulating lipopeptide 1; HC: healthy control; hi = high concentration, 10000 ng/ml; lo = low concentration, 1000 ng/ml; LPS: lipopolysaccharide; ns: not significant; TLR: toll-like receptor.

inflammatory state since antibodies against flagellin are found in 50% of Crohn's disease patients. Interestingly, in a Crohn's disease mouse model, worsening of colitis occurs after rectal infusion of flagellin [24, 25]. The elevated response to TLR5 stimulation that we show in our current study supports the hypothesis that a similar

mechanism might be associated with the immunopathogenesis of BD. Inflammatory infiltrates are dominated by T-lymphocytes, neutrophils and monocytes/macrophages in BD characteristic pathergic lesions [26–28]. In these cell types higher levels of TLR5 are expressed in the pathergy positive patients. Despite the

difference in TLR5 expression, we have not observed any difference in TNF-α production between peripheral blood cells from pathergy positive BD and pathergy negative BD patients upon TLR5 stimulation, yet this might be related to the small number of patients tested ($n = 4$ pathergy positive and $n = 2$ pathergy negative). Nevertheless, considering the clear differences in TLR5 expression between pathergy positive and pathergy negative BD patients, we hypothesize that strongly enhanced TLR5 expression in these cells reflects the local inflammatory response to danger molecules in BD patients.

Recently, high mobility group box 1 (HMGB1) was identified as a TLR5 agonist [29]. HMGB1 is a non-histone nuclear protein that is known as a DAMP able to activate the innate immune system via TLR2, 4 and 5. In pathergy positive BD patients, HMGB1 released from necrotic cells could act as danger molecule stimulating the local inflammatory response via the upregulated TLR5 expression. This hypothesis is further strengthened by the elevated HMGB1 levels in serum of BD patients as found by de Souza et al. [30]. Because of the known flagellin–TLR5 interaction, it is also possible that bacterial components contribute to the pathergy reaction. This is supported by the observed decline of pathergy positivity after cleaning the skin prior to testing with 100% chlorhexidine [31]. It can thus be hypothesized that the exaggerated inflammatory cutaneous response as seen in a positive pathergy test can be triggered via the upregulated TLR5 in leukocytes, by skin-derived flagellin and/or HMGB1 released from necrotic cells.

Increased expression of TLR 1, 2, 4 and 6 in BD patients has been shown previously, on several different cell types, including blood cells as well as oral epithelial cells [14–16, 32], and is further corroborated by our current study. Increased TLR expression on immune cells is not specific to BD, as this has been reported in other inflammatory diseases as well, including spondylarthropathy and IBDs [33, 34]. In IBD, TLR2 is more highly expressed in peripheral blood monocytes; in addition to this, an increased TNF-α response after stimulation of TLR2 is found in PBMCs from IBD patients compared with healthy controls [35]. TLR2 and 4 are also upregulated in PBMCs of patients with spondylarthropathy, which can be reversed by the TNF-α neutralizing drug infliximab [36]. One could speculate about the cause of the increased TLR expression on immune cells from BD patients, but because of the inflammatory nature of BD, inflammation-driven enhancement is likely to be involved. Inflammation modulates TLR expression via cytokines such as TNF-α, as shown in spondylarthropathy [36]. Also, in intestinal macrophages in IBD there is an inflammation-induced enhancement of TLR2 and 4 expression [37]. Also, type I IFNs are known to regulate TLR expression [38]. Recently, in BD a gene array strategy identified upregulated genes participating in type I interferon and Janus kinase–signal transducer and activator of transcription signalling pathways [39]. In vitro,

exogenously administered IFN-α or γ downregulated TLR5 gene expression [40]. IFN-α treatment was shown to be effective in decreasing disease activity in BD [41], which could be explained by the downregulating effect of IFN-α on TLR5 expression. These data raise the question of whether a defect in the IFN-α pathway is contributive to the pathergy reaction; this should be investigated in further research.

Limitations to our study could be the use of immuno-suppressive medication (colchicine, adalimumab). The effect of colchicine on TLR expression is unknown, but adalimumab may downregulate TLR expression [36, 42]. In theory, this may lead to underestimation of the actual overexpression of TLRs in BD. However, we believe that the impact of such an effect on our findings is limited since TLR expression was comparable between our patients when stratified for medication, as is depicted in Supplementary Fig. 1, available at Rheumatology online.

In addition to this, our stimulation assay was used to gain insight into the function of the different TLRs, by measuring response to stimulation. Unfortunately, the stimulation data lack information regarding which subset is responsible for the TNF-α production. To gain more insight into this, co-staining for TNF-α and TLR levels in the different subsets could be useful.

In conclusion, increased expression of several TLRs in BD patients suggests a prominent innate driven inflammatory response in this disease. Especially TLR2 and TLR5 might implicate the severity of this response. Also, the relation with TLR5 and the pathergy test opens interesting new possibilities for future mechanistic studies, which might unravel the attributional factors that can be of interest for specific targeted therapy and diagnostic work-up.

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

Supplementary data

Supplementary data are available at *Rheumatology* online.

References

1 Sakane T, Takeno M, Suzuki N, Inaba G. Behçet's disease. N Engl J Med 1999;341:1284–91.

2 Gül A. Pathogenesis of Behçet's disease: autoinflammatory features and beyond. Semin Immunopathol 2015;37:413–8.

3 Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta 2014;1843: 2563–82.

4 Evereklioglu C. Current concepts in the etiology and treatment of Behçet disease. Surv Ophthalmol 2005;50: 297–350.

5 Musabak U, Pay S, Erdem H *et al*. Serum interleukin-18 levels in patients with Behçet's disease. Is its expression associated with disease activity or clinical presentations? Rheumatol Int 2006;26:545–50.

6 Ureten K, Ertenli I, Oztürk MA *et al*. Neutrophil CD64 expression in Behçet's disease. J Rheumatol 2005;32: 849–52.

7 Hasan MS, Bergmeier LA, Petrushkin H, Fortune F. Gamma delta ($\gamma\delta$) T cells and their involvement in Behçet's disease. J Immunol Res 2015;2015:1–7.

8 Ambarus C, Yeremenko N, Tak PP, Baeten D. Pathogenesis of spondyloarthritis: autoimmune or autoinflammatory? Curr Opin Rheumatol 2012;24:351–8.

9 Kappen JH, van Dijk EHC, Baak-Dijkstra M *et al*. Behçet's disease, hospital-based prevalence and manifestations in the Rotterdam area. Neth J Med 2015;73: 471–7.

10 Varol A, Seifert O, Anderson CD. The skin pathergy test: innately useful? Arch Dermatol Res 2010;302:155–68.

11 Chen J-Q, Szodoray P, Zeher M. Toll-like receptor pathways in autoimmune diseases. Clin Rev Allergy Immunol 2016;50:1–17.

12 Chi H, Li C, Zhao FS *et al*. Anti-tumor activity of toll-like receptor 7 agonists. Front Pharmacol 2017;8:304.

13 Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature 2000; 406:782–7.

14 Liu X, Wang C, Ye Z, Kijlstra A, Yang P. Higher expression of Toll-like receptors 2, 3, 4, and 8 in ocular Behçet's disease. Invest Ophthalmol Vis Sci 2013;54: 6012–7.

15 Seoudi N, Bergmeier LA, Hagi-Pavli E *et al*. The role of TLR2 and 4 in Behçet's disease pathogenesis. Innate Immun 2014;20:412–22.

16 Yavuz S, Elbir Y, Tulunay A, Eksioglu-Demiralp E, Direskeneli H. Differential expression of toll-like receptor 6 on granulocytes and monocytes implicates the role of microorganisms in Behçet's disease etiopathogenesis. Rheumatol Int 2008;28:401–6.

17 International Study Group for Behçet's Disease. Criteria for diagnosis of Behçet's disease. Lancet 1990;335: 1078–80.

18 International Society for Behçet's Disease. Behçet's disease current activity form. Leeds: University of Leeds, 2006.

19 Kalina T, Flores-Montero J, van der Velden VHJ *et al*. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. Leukemia 2012;26:1986–2010.

20 Goris MGA, Wagenaar JFP, Hartskeerl RA *et al*. Potent innate immune response to pathogenic leptospira in human whole blood. PLoS One 2011;6: e18279.

21 Qian F, Wang X, Zhang L *et al*. Age-associated elevation in TLR5 leads to increased inflammatory responses in the elderly. Aging Cell 2012;11:104–10.

22 Wong CK, Wong PTY, Tam LS *et al*. Activation profile of Toll-like receptors of peripheral blood lymphocytes in patients with systemic lupus erythematosus. Clin Exp Immunol 2010;159:11–22.

23 Skert C, Fogli M, Perucca S *et al*. Profile of toll-like receptors on peripheral blood cells in relation to acute graft-versus-host disease after allogeneic stem cell transplantation. Biol Blood Marrow Transplant 2013;19: 227–34.

24 Targan SR, Landers CJ, Yang H *et al*. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. Gastroenterology 2005;128:2020–8.

25 Rhee SH, Im E, Riegler M *et al*. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. Proc Natl Acad Sci USA 2005; 102:13610–5.

26 Gül A, Esin S, Dilsen N *et al*. Immunohistology of skin pathergy reaction in Behçet's disease. Br J Dermatol 2010;132:901–7.

27 Jorizzo JL, Solomon AR, Cavallo T. Behçet's syndrome. Immunopathologic and histopathologic assessment of pathergy lesions is useful in diagnosis and follow-up. Arch Pathol Lab Med 1985;109:747–51.

28 Ergun T, Gürbüz O, Harvell J, Jorizzo J, White W. The histopathology of pathergy: a chronologic study of skin hyperreactivity in Behçet's disease. Int J Dermatol 1998; 37:929–33.

29 Das N, Dewan V, Grace PM *et al*. HMGB1 activates proinflammatory signaling via TLR5 leading to allodynia. Cell Rep 2016;17:1128–40.

30 de Souza AWS, Félix Perazzio S, de França NR *et al*. High mobility group box 1 serum levels are increased in Behçet's disease, but not associated with disease activity or disease manifestations. Rheumatology 2015; 54:2151–5.

31 Fresko I, Yazici H, Bayramiçli M, Yurdakul S, Mat C. Effect of surgical cleaning of the skin on the pathergy phenomenon in Behçet's syndrome. Ann Rheum Dis 1993;52:619–20.

32 Do JE, Kwon SY, Park S, Lee E-S. Effects of vitamin D on expression of Toll-like receptors of monocytes from patients with Behçet's disease. Rheumatology 2008;47: 840–8.

33 Cario E. Toll-like receptors in inflammatory bowel diseases: a decade later. Inflamm Bowel Dis 2010;16: 1583–97.

34 Inman RD. Innate immunity of spondyloarthritis: the role of toll-like receptors. Adv Exp Med Biol 2009;649:300–9.

35 Cantó E, Ricart E, Monfort D *et al*. TNF alpha production to TLR2 ligands in active IBD patients. Clin Immunol 2006;119:156–65.

36 De Rycke L, Vandooren B, Kruithof E *et al*. Tumor necrosis factor alpha blockade treatment down-modulates the increased systemic and local expression of Toll-like receptor 2 and Toll-like receptor 4 in spondylarthropathy. Arthritis Rheum 2005;52:2146–58.

37 Hausmann M, Kiessling S, Mestermann S *et al.* Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology 2002;122:1987–2000.

38 Khoo JJ, Forster S, Mansell A. Toll-like receptors as interferon-regulated genes and their role in disease. J Interferon Cytokine Res 2011;31:13–25.

39 Puccetti A, Fiore PF, Pelosi A *et al.* Gene expression profiling in Behcet's disease indicates an autoimmune component in the pathogenesis of the disease and opens new avenues for targeted therapy. J Immunol Res 2018;2018:4246965.

40 Miettinen M, Sareneva T, Julkunen I, Matikainen S. IFNs activate toll-like receptor gene expression in viral infections. Genes Immun 2001;2:349–55.

41 Kötter I, Günaydin I, Zierhut M, Stübiger N. The use of interferon alpha in Behçet disease: review of the literature. Semin Arthritis Rheum 2004;33: 320–35.

42 De Pità O, Nardis C, Lupi F *et al.* Modulation of toll-like receptors in psoriatic patients during therapy with adalimumab. Int J Immunopathol Pharmacol 2011;24: 185–8.