

# Retinal S-antigen Th1 cell epitope mapping in patients with Behcet's disease

Changlin Zhao · Peizeng Yang · Hao He · Xiaomin Lin · Liping Du · Hongyan Zhou · Aize Kijlstra

Received: 17 July 2008 / Revised: 5 September 2008 / Accepted: 6 October 2008 / Published online: 29 October 2008  
© Springer-Verlag 2008

## Abstract

**Background** Retinal S-antigen (S-Ag) is a most characterized autoantigen of autoimmune uveitis. The recognized immunodominant epitope of human S-Ag in patients with uveitis has not been identified. In this study, we selected certain patients with active uveitis to map the Th1 cell epitope spectrum of human S-Ag in Behcet's disease (BD). **Methods** Blood samples were taken from eight active BD patients who showed an immune response to 40 mixed overlapping peptides spanning the entire sequence of human S-Ag. Peripheral blood mononuclear cells were isolated and stimulated with single S-Ag peptide at 5 µg/ml

or 20 µg/ml. Single-cell immune responses were measured by IFN-γ ELISpot assay.

**Results** BD patients heterogeneously responded to the S-Ag peptides at two concentrations. In general, the responses to 5 µg/ml peptides were slightly stronger than those to 20 µg/ml peptides, while the maximum SFC frequency to single peptide at the two concentrations was similar. Several peptides including P31, P35 and P40 induced a prominent response, with the frequency of S-Ag specific cells being about 0.007%. Significant reactivity pattern shift was noted in patients with different disease courses.

**Conclusions** Certain active BD patients have S-Ag specific Th1 cells with a low frequency. The S-Ag epitope specificity between patients is highly heterogeneous, and varies with the uveitis course.

There is no financial relationship with any organisation that sponsored the research. The authors have full control of all primary data, and agree to allow Graefe's Archive for Clinical and Experimental Ophthalmology to review our data upon request.

C. Zhao · P. Yang · H. He · X. Lin · L. Du · H. Zhou  
Zhongshan Ophthalmic Center, Sun Yat-sen University,  
Guangzhou, People's Republic of China

C. Zhao  
Department of Ophthalmology,  
454 Hospital of People's Liberation Army,  
Nanjing, People's Republic of China

P. Yang (✉)  
The First Affiliated Hospital, Chongqing Medical University,  
Chongqing 400016, People's Republic of China  
e-mail: peizengy@126.com

A. Kijlstra  
Department of Ophthalmology, Eye Research Institute Maastricht,  
University Hospital Maastricht,  
Maastricht, The Netherlands

A. Kijlstra  
Animal Sciences Group,  
Wageningen, UR, The Netherlands

**Keywords** Behcet's disease · Epitope mapping · Retinal S antigen · Uveitis

## Introduction

Uveitis is a group of clinically heterogeneous intraocular inflammatory diseases often leading to severe impairment of visual acuity. It has been presumed to be mediated by autoreactive CD4<sup>+</sup> T cells recognizing specific retinal antigens [1]. The most persuasive evidence stems from the observation that autoantigens such as retinal S-antigen (S-Ag) can induce experimental autoimmune uveitis (EAU), similar to human uveitis clinically and histologically in susceptible animals [2]. Furthermore, oral administration of bovine S-Ag or immunizing rats with human S-Ag-Ig fusion protein can induce immune tolerance, thereby preventing uveitis [3, 4]. Usually a typical immune response against a self protein is focused on one or two

epitopes within that protein [5]. Immunodominant epitopes of human S-Ag have been investigated in a variety of uveitis entities through lymphocyte proliferation assay [6–8]. However, no peptide was found to induce a predominant immune response in all or most responders.

Behcet's disease (BD) is one of the common uveitis entities in China [9], and lymphocytes in certain BD patients proliferated upon S-Ag stimulation [10]. Enzyme-linked immunospot assay (ELISpot) has been shown to be the most specific and sensitive technique for assessing low-frequency antigen specific T cells. In this study, we adopted the ELISpot assay to map the T cell epitope peptide spectrum of human S-Ag to identify the dominant peptides in BD patients.

## Materials and methods

### Synthetic peptides

Forty peptides spanning the entire length of human S-Ag sequence (pubmed locus P10523) were synthesized by conventional solid-phase chemistry and purified to at least 95% purity (Sangon Biological Engineering Technology & Services Company, Shanghai, China). Each peptide determinant measured 20 amino acids (aa) in length and overlapped the neighboring sequence by 10 aa, except for the last one, which measured 15 aa. The exact sequence and naming of the overlapping peptides are shown in Fig. 1.

### Subjects

Eight male BD patients with active uveitis with an average age of 31 years (ranged from 20 to 43 years) were included

	P1	P2	P3	P4	P5	P6
1	maasgktsks	epnhvifkki	srdksvtiyl	gnrdyidhvs	qvqpvdgvvl	vdpdlvkgkk
	P7	P8	P9	P10	P11	P12
	vyvtlcafr	ygqedvdvig	ltfirdlyfs	rvqvyppvga	astptklqes	llkklgsnty
	P13	P14	P15	P16	P17	P18
121	pflltfdpyl	pcsvmlqpap	qdsqkscgvd	fevkafatds	tdaeedkipk	kssvrylirs
	P19	P20	P21	P22	P23	P24
	vqhaplemgp	qpraeatwqf	fmsdkplhla	vslnreiyfh	gepipvtvtv	tnntektvkk
	P25	P26	P27	P28	P29	P30
241	ikacveqvan	vvlyssdyyv	kvpmeeaqe	kvppnstltk	ttllpllan	nrregriald
	P31	P32	P33	P34	P35	P36
	gkikhedtnl	asstiikegi	drtvlgilvs	yqikvkltsv	gflgelsse	vatevppfrlm
	P37	P38	P39	P40		
361	hpqpedpake	siqdanlvfe	efarhnlkda	geaegkrdk	ndade	

**Fig. 1** The sequence of 40 overlapping peptides that spanned the entire amino acid sequence of human S-Ag

in this study. The patients met the diagnostic criteria established by the International Behcet's Disease Study Group [11]. All patients showed cellular responses to mixed S-Ag peptides and were positive for human leucocyte antigen (HLA)-B51 in our previous study [12]. Their mean durations of uveitis were 27 months (ranging from 13 to 35 months). All the patients relapsed into panuveitis, manifested by dust-like keratic precipitates, flare and cells in the anterior chamber, hypopyon, vitreous cells and retinal vasculitis as disclosed by fluorescein angiography. Five patients were receiving low-dose prednisone (20 mg/day or less) therapy, and the remaining three BD patients had no systemic treatment at the time of blood sampling. No healthy donors showed positive cellular responses to mixed S-Ag peptide in our previous study; therefore we did not use normal individuals as a control. All procedures followed the tenets of the Declaration of Helsinki and were approved by the Zhongshan Ophthalmic Center Ethics Committee, and blood samples were obtained from the patients after they had given their written informed consent.

### IFN- $\gamma$ ELISpot

Peripheral venous blood (18 ml or 34 ml) was drawn in a heparinized tube, and peripheral blood mononuclear cells (PBMCs) were isolated immediately by Ficoll–Hypaque density gradient centrifugation (Pharmacia Biotech, Shanghai, China). The ELISpot assay kit for IFN- $\gamma$  was purchased from BD Pharmingen (BD Biosciences, San Diego, CA, USA) and utilized according to the manufacturer's instructions. Briefly, 96-well nitrocellulose plates (Millipore) were coated with 5  $\mu$ g/ml anti-human  $\ddagger$ IFN- $\gamma$  capture antibody at 4°C overnight. A total of  $2 \times 10^5$  fresh PBMCs were added to microwells in duplicate, and incubated with 40 single S-Ag synthetic peptides (5  $\mu$ g/ml or 20  $\mu$ g/ml for each peptide) for 20 h in the presence of 1  $\mu$ g/ml costimulatory anti-CD28 (BD Pharmingen, clone CD28.2) monoclonal antibody (mAb). PBMCs cultured without peptides acted as negative controls. PBMCs stimulated with 10 ng/ml anti-CD3 (BD Pharmingen, clone HIT3a) plus 1  $\mu$ g/ml anti-CD28 mAbs were used as positive controls. Two  $\mu$ g/ml biotinylated anti-human IFN- $\gamma$ , streptavidin–HRP at 1:100 dilution and AEC were used as detection antibody, biotinylated detection antibody and substrate reagent respectively. The spots were counted automatically using ImmunoSpot Image Analyzer (Cellular Technology Limited, Cleveland, OH, USA). Digitized images were analyzed for the presence of areas in which color density exceeds background by an amount set on the basis of the comparison of experimental wells and negative control wells. A spot called spot-forming cell (SFC) represented an IFN- $\gamma$ -producing cell. The number of spots in negative control wells was less than two.

## Statistical analysis

Data are expressed as mean  $\pm$  SD. Statistical analysis was performed using the Mann-Whitney *U* test and the linear mixed-effects model fit by the maximum likelihood method by statistical software SAS 9.1 (SAS Inc., Cary, NC, USA). A *P* value of less than 0.05 was considered statistically significant.

## Results

### Assessment of cellular immune response to S-Ag peptides by IFN- $\gamma$ ELISpot

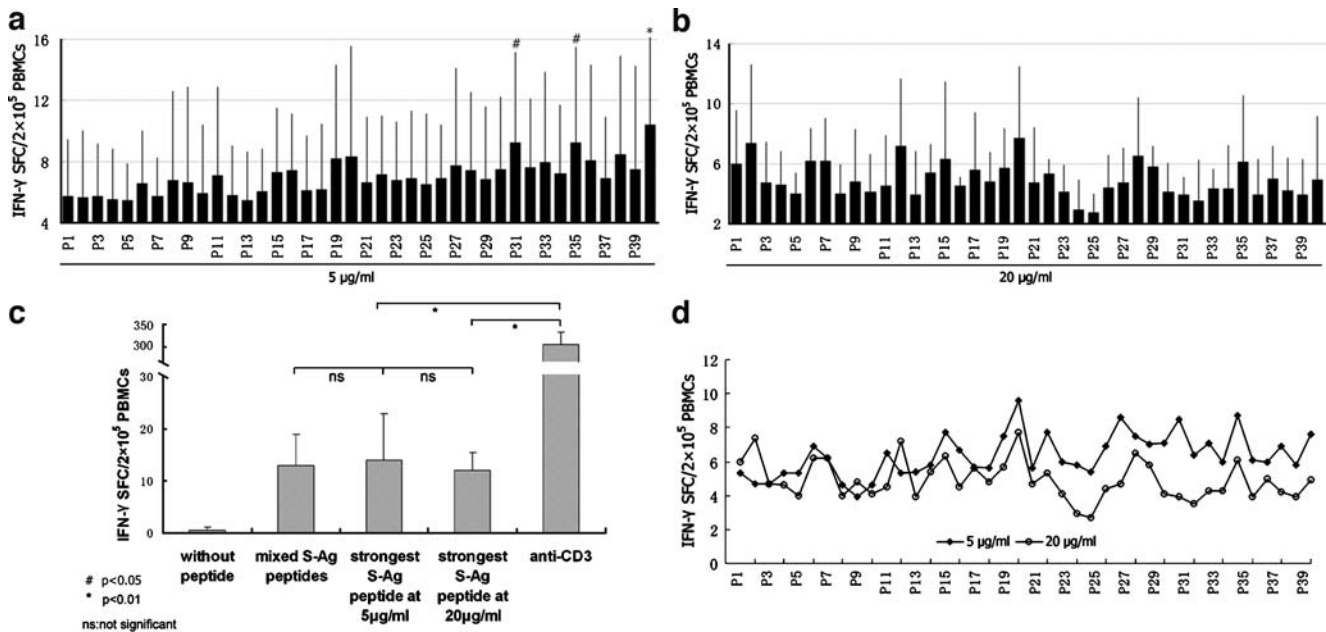
Our preliminary study found that 5  $\mu$ g/ml of mixed S-Ag peptides was an optimal concentration for ELISpot assay. Therefore, that concentration was used in this study. Since earlier studies showed that 20  $\mu$ g/ml of S-Ag peptides induced an optimal lymphocyte proliferative responses in patients with uveitis [7, 8], we simultaneously adopted this concentration for ELISpot assay in five out of eight patients. The results revealed highly heterogeneous responses against individual peptides at either concentration in all the patients (Fig. 2a,b). In general, each patient responded to more than one of the peptides in the panel, and the

reactivity of the same peptide varied between patients. Both concentrations of antigens induced a similar SFC frequency with 0.007% ( $14/2 \times 10^5$ ) vs 0.006% ( $12/2 \times 10^5$ ) for the strongest reactive peptide at 5  $\mu$ g/ml vs 20  $\mu$ g/ml (Fig. 2c). The maximum frequency of T cell response against single peptide at the two concentrations was the same as that against mixed peptides at 5  $\mu$ g/ml (0.0065% [ $13/2 \times 10^5$ ]). However, generally speaking, the responses to 5  $\mu$ g/ml of 40 single peptides were stronger than that to 20  $\mu$ g/ml of peptides (Fig. 2d). Additionally, the dominant sites of S-Ag at 5  $\mu$ g/ml obviously localized at the C-terminal region of this protein, whereas that at 20  $\mu$ g/ml was noted at the N-terminal.

When PBMCs were stimulated with polyclonal anti-CD3 plus anti-CD28, a high frequency of IFN- $\gamma$ -secreting cells (0.1525% [ $305/2 \times 10^5$ ]) was observed in all patients. It was much higher than that induced by S-Ag peptide ( $P < 0.01$ ,  $P < 0.01$ ) (Fig. 2c).

### Identification of immunodominant T cell epitopes of S-Ag

In view of the highly heterogeneous reactivity spectrum in different individuals, we applied the linear mixed-effects model fit by the maximum likelihood method with the number of cases as the random effect, to analyze the difference in SFC frequency between 40



**Fig. 2** Mapping of Th1 cell responses to individual S-Ag peptides. PBMCs were cultured with mixed S-Ag peptides at 5  $\mu$ g/ml, single peptides at 5 or 20  $\mu$ g/ml and anti-CD3 respectively. IFN- $\gamma$ -producing cells were determined by ELISpot and expressed as SFC. Data are expressed as means  $\pm$  SD and bars indicate mean values. A statistically significant difference was set at  $P < 0.05$  (#),  $P < 0.01$  (\*), ns: not significant. **a** Shows the Th1 cell response spectrum of S-Ag peptides at 5  $\mu$ g/ml ( $n = 8$ ). The SFC frequency was analyzed by the

linear mixed-effects model fit by the maximum likelihood method, and the dominant responses were marked. **b** Shows the Th1 cell response spectrum of S-Ag peptides at 20  $\mu$ g/ml ( $n = 5$ ). **c** The SFC frequency for stimulation with mixed peptides, strongest single peptide (5 and 20  $\mu$ g/ml respectively) and anti-CD3 were compared. Statistical analysis was performed using Mann-Whitney *U* test. **d** The SFC frequency of the single peptides screening at the two concentrations was compared ( $n = 5$ )

peptides at 5  $\mu\text{g/ml}$ . The results showed that the responses to P31 ( $P=0.013$ ), P35 ( $P=0.015$ ) and P40 ( $P=0.001$ ) were significantly stronger than to other peptides.

#### Shift in peptides recognition in patients with different disease course

To investigate whether the S-Ag peptides response spectrum was influenced by the disease course of uveitis, we divided the eight cases into two groups according to their different duration of uveitis. Group one included three patients with a uveitis course of no more than 2 years; the other included five patients having uveitis for more than 2 years. Their SFC frequency of response to the 40 peptides at 5  $\mu\text{g/ml}$  was compared by linear mixed-effects model fit by the maximum likelihood method. In general, the response in patients with a disease course less than 2 years was stronger than that in patients with a disease course of more than 2 years. The patients with uveitis duration less than 2 years dominantly recognized P40 ( $P=0.001$ ). When the uveitis course extended to around 3 years in BD patients, the dominant response to S-Ag peptides varied to P27 ( $P=0.009$ ), P29 ( $P=0.049$ ), P30 ( $P=0.030$ ), and P31 ( $P=0.001$ ) (Fig. 3).

#### Discussion

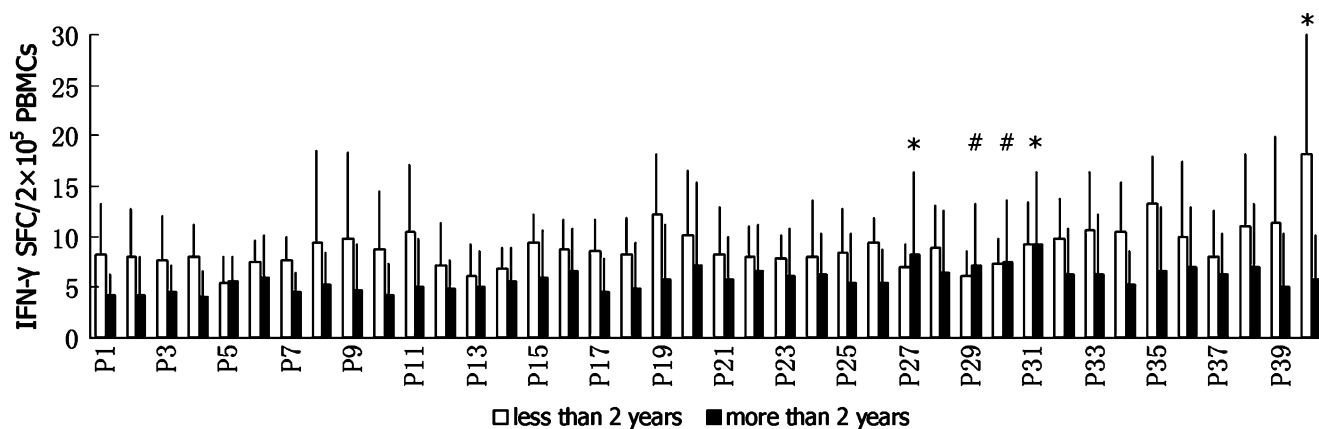
In this study, we applied the ELISpot assay to map the S-Ag Th1 cell epitopes response in a series of BD patients with similar disease characteristics. The results show that the peptides induced a low frequency of S-Ag specific Th1 cells, and that the T cell response against peptides is highly heterogeneous in different individuals. Several dominant peptides including P31, P35 and P40 were observed

statistically in certain patients. The reactivity pattern to the peptides varied in patients with different disease duration.

It has been shown that a greatly heterogeneous T-cell proliferative response spectrum against S-Ag peptides in uveitis may be attributed to variation in disease entities and different stages of disease activity of the examined patients [6–8]. To exclude the above factors in the antigenic peptide mapping, we chose BD patients with a similar intensity of uveitis at the time of blood sampling. In view of the fact that an efficiently processed specific antigen epitope is restricted by major histocompatibility complex (MHC) on the surface of an antigen-presenting cell, and that BD was associated with HLA-B51 in many ethnic groups [13, 14], we chose the patients expressing HLA-B51 as subjects in this study. The result showed that these patients also heterogeneously recognized the S-Ag peptides, suggesting that the S-Ag epitopes may be not associated with HLA-B51.

Although lymphocyte proliferation assay is a classic technique to assess cellular immunity, a non-proliferation measurement to characterize low-frequency antigen-specific T cell in autoimmune disease (AID) is needed to avoid overall clonal expansion and promiscuous T cell populations. The cytokine-specific ELISpot assay is able to display one reactive cell among one million T cells at the single cell level [15], and has been extensively applied to research on autoimmunity in various AID [16, 17]. Because an IFN- $\gamma$  mediated Th1 response has been implicated in the development of BD [18], we adopted the IFN- $\gamma$  ELISpot assay to map the S-Ag Th1 cell epitopes in BD patients.

To identify the immunodominant epitope of human S-Ag in uveitis, we used 40 overlapping peptides that are identical to the peptides used in earlier studies in uveitis patients [6–8]. The immune responses in those studies were



**Fig. 3** Shift in S-Ag peptide recognition in patients with different disease courses. According to the different duration of uveitis, the subjects were divided into two groups. Group 1 included patients with a uveitis course of less than 2 years ( $n=3$ ), group 2 included patients

with a disease duration of more than 2 years ( $n=5$ ). The SFC frequency was analyzed using the linear mixed-effects model fit by the maximum likelihood method



tested by lymphocyte proliferation assay. If a stimulation index (SI)  $\geq 2$  was considered a positive reaction, almost all of the S-Ag peptides except for P12 induced a positive response in different patients with various uveitis entities [6–8]. In BD patients, as shown in Table 2 of their paper, de Smet et al. [6] found that the responses to P18 and P40 were significantly higher than those in healthy controls. Similar peptides have been tested in the EAU model for their pathogenicity [19]. Ten peptides induced visible inflammation in the eye, and the most pathogenic sequences were P19, P35 and P36. Our ELISpot assay similarly showed a heterogeneous reactivity pattern in BD patients. The dominant peptide P35 coincided with the major pathogenic epitope in rat, and the prominent P40 was identical with the result of proliferation assays in BD. Therefore, P40 may be the most potential BD-associated S-Ag epitope peptide, and the immunodominant epitopes of S-Ag in uveitis may nest in the amino acid sequence of P35 and P40. However, it needs to be verified using smaller synthetic peptides in more uveitis patients.

The ELISpot assay quantified a low frequency of 0.007% ( $14/2 \times 10^5$ ) of S-Ag specific T cells in the peripheral circulation of BD patients with active uveitis. It was notably lower than that seen in animals with EAU [20]. The low frequency of SFC observed in our study is consistent with the weak proliferative response to S-Ag in uveitis patients [6–8].

Epitope spreading has been considered to be one of the mechanisms contributing to relapses in AID [21]. Deeg et al. [22] found that the epitopes recognized by uveitic horses could spread to different peptides of S-Ag or to the peptides derived from other uveitogenic autoantigens during the uveitic episodes. A shift in the specific proliferative response of S-Ag peptides was observed in two BD patients by testing the responses on two separate occasions [6]. Interestingly, we found that the BD patients with shorter uveitis course reacted more strongly to S-Ag and intensively responded to one dominant peptide, while those with a long disease course responded to others. This phenomenon seem to suggest that autoantigenic epitope spreading may be present in human uveitis.

Our patients showed a stronger response to low-concentration peptides than high-concentration ones, which was different from de Smet's finding [6]. In addition, the dominant responses induced by the two concentration antigens were not coincident. The discrepancy between the two concentration peptides may be due to different antigen doses affecting the development of T-cell repertoire to a given antigen in vitro, in compliance with the different avidity of T-cell-receptor for specific MHC-peptide complex [23]. We speculated that the 5  $\mu\text{g}/\text{ml}$  peptides preferred inducing an immune response mimicking the in vivo condition.

In summary, this is the first report concerning the analysis of T cell immunity against S-Ag by ELISpot assay wherein the S-Ag Th1 cell epitope response is measured in one uveitis entity. The peptide recognition pattern varied between BD patients with a mild reactive intensity. The responses to the dominant peptides and a possible shift reactivity pattern should be verified in more BD patients.

**Acknowledgements** The authors sincerely thank Ph.D. Qian Zhao, Department of Biostatistics, Zhongshan medical university, for her assistance in statistical analysis. This study was supported by the Natural Science Foundation for Research Groups of Guangdong Province (05200176), Key Project of Natural Science Foundation (30630064) and the National supporting project of P.R. China.

## References

- Murphy CC, Duncan L, Forrester JV, Dick AD (2004) Systemic CD4(+) T cell phenotype and activation status in intermediate uveitis. *Br J Ophthalmol* 88:412–416. doi:10.1136/bjo.2003.028506
- Nussenblatt RB, Kuwabara T, de Monasterio FM, Wacker WB (1981) S-antigen uveitis in primates. A new model for human disease. *Arch Ophthalmol* 99:1090–1092
- Torseth JW, Gregerson DS (1998) Oral tolerance in experimental autoimmune uveoretinitis: feeding after disease induction is less protective than prefeeding. *Clin Immunol Immunopathol* 88:297–304. doi:10.1006/clin.1998.4592
- Liang W, Karabekian Z, Mattapallil M, Xu Q, Viley AM, Caspi R et al (2006) B-cell delivered gene transfer of human S-Ag-Ig fusion protein protects from experimental autoimmune uveitis. *Clin Immunol* 118:35–41. doi:10.1016/j.clim.2005.08.007
- Vanderlugt CL, Miller SD (2002) Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol* 2:85–95. doi:10.1038/nri724
- de Smet MD, Bitar G, Mainigi S, Nussenblatt RB (2001) Human S-antigen determinant recognition in uveitis. *Invest Ophthalmol Vis Sci* 42:3233–3238
- Rai G, Saxena S, Kumar H, Singh VK (2001) Human retinal S-antigen: T cell epitope mapping in posterior uveitis patients. *Exp Mol Pathol* 70:140–145. doi:10.1006/exmp.2000.2338
- Tripathi P, Saxena S, Yadav VS, Naik S, Singh VK (2004) Human S-antigen: peptide determinant recognition in uveitis patients. *Exp Mol Pathol* 76:122–128. doi:10.1016/j.yexmp.2003.10.007
- Yang P, Zhang Z, Zhou H, Li B, Huang X, Gao Y et al (2005) Clinical patterns and characteristics of uveitis in a tertiary center for uveitis in China. *Curr Eye Res* 30:943–948. doi:10.1080/02713680500263606
- Yamamoto JH, Minami M, Inaba G, Masuda K, Mochizuki M (1993) Cellular autoimmunity to retinal specific antigens in patients with Behcet's disease. *Br J Ophthalmol* 77:584–589. doi:10.1136/bjo.77.9.584
- International Study Group for Behcet's Disease (1990) Criteria for diagnosis of Behcet's disease. *Lancet* 335:1078–1080
- Zhao C, Yang P, He H, Lin X, Li B, Zhou H et al (2008) S-antigen specific T helper type 1 response is present in Behcet's disease. *Mol Vis* 14:1456–1464
- Baricordi OR, Sensi A, Pivetti-Pezzi P, Perrone S, Balboni A, Catarinelli G et al (1986) Behcet's disease associated with HLA-B51 and DRw52 antigens in Italians. *Hum Immunol* 17:297–301. doi:10.1016/0198-8859(86)90281-8

14. Chang HK, Kim JU, Cheon KS, Chung HR, Lee KW, Lee IH (2001) HLA-B51 and its allelic types in association with Behcet's disease and recurrent aphthous stomatitis in Korea. *Clin Exp Rheumatol* 19:S31–S35
15. Guerkov RE, Targoni OS, Kreher CR, Boehm BO, Herrera MT, Tary-Lehmann M et al (2003) Detection of low-frequency antigen-specific IL-10-producing CD4(+) T cells via ELISPOT in PBMC: cognate vs. nonspecific production of the cytokine. *J Immunol Methods* 279:111–121. doi:10.1016/S0022-1759(03)00240-0
16. Nagata M, Moriyama H, Kotani R, Yasuda H, Kishi M, Kurohara M et al (2007) Immunological aspects of 'fulminant type 1 diabetes'. *Diabetes Res Clin Pract* 77:S99–S103. doi:10.1016/j.diabres.2007.01.041
17. Van der Aa A, Hellings N, Bernard CC, Raus J, Stinissen P (2003) Functional properties of myelin oligodendrocyte glycoprotein-reactive T cells in multiple sclerosis patients and controls. *J Neuroimmunol* 137:164–176. doi:10.1016/S0165-5728(03)00048-1
18. Sugi-Ikai N, Nakazawa M, Nakamura S, Ohno S, Minami M (1998) Increased frequencies of interleukin-2- and interferon-gamma-producing T cells in patients with active Behcet's disease. *Invest Ophthalmol Vis Sci* 39:996–1004
19. de Smet MD, Bitar G, Roberge FG, Gery I, Nussenblatt RB (1993) Human S-antigen: presence of multiple immunogenic and immunopathogenic sites in the Lewis rat. *J Autoimmun* 6:587–599. doi:10.1006/jaut.1993.1048
20. Prendergast RA, Iliff CE, Coskun NM, Caspi RR, Sartani G, Tarrant TK et al (1998) T cell traffic and the inflammatory response in experimental autoimmune uveoretinitis. *Invest Ophthalmol Vis Sci* 39:754–762
21. Vanderlugt CL, Begolka WS, Neville KL, Katz-Levy Y, Howard LM, Eagar TN et al (1998) The functional significance of epitope spreading and its regulation by co-stimulatory molecules. *Immunol Rev* 164:63–72. doi:10.1111/j.1600-065X.1998.tb01208.x
22. Deeg CA, Amann B, Raith AJ, Kaspers B (2006) Inter- and intramolecular epitope spreading in equine recurrent uveitis. *Invest Ophthalmol Vis Sci* 47:652–656. doi:10.1167/iovs.05-0789
23. Mazzanti B, Hemmer B, Traggiai E, Ballerini C, McFarland HF, Massaccesi L et al (2000) Decrypting the spectrum of antigen-specific T-cell responses: the avidity repertoire of MBP-specific T-cells. *J Neurosci Res* 59:86–93. doi:10.1002/(SICI)1097-4547(20000101)59:1<86::AID-JNR10>3.0.CO;2-U