Abnormal Cellular Reactivity to Microbial Antigens in Patients with Uveitis

Grégoire Jacques Noël Cozon,[1,2] *Florentin Martial Mbitikon-Kobo,*[1,2] *Fathi Fatoohi,*[1,2] *Myriam Spire,*[3] *Jean-Daniel Grange,*[3] *and Laurent Kodjikian*[3]

Purpose. The purpose of the present study was to evaluate the cellular response to microbial antigens in patients with idiopathic uveitis.

Methods. Blood lymphocytes from 31 patients with uveitis and 24 healthy controls were cultivated with microbial antigens and analyzed by flow cytometry after staining with monoclonal antibodies against CD3, CD4, and activation markers CD69 and CD25.

Results. Although no difference was noted in circulating lymphocytes, the activation of T cells, detected with CD69, was higher in 24-hour blood culture from uveitis patients with *Candida albicans* antigen (*Ca*-Ag) than from controls, especially in posterior uveitis and panuveitis. Moreover, late response, detected with CD25, to different microbial antigens was higher in patient with uveitis.

Conclusions. Such results suggest the role of *Ca*-Ag and microbial antigens in the pathogenic mechanisms of idiopathic uveitis. (*Invest Ophthalmol Vis Sci.* 2008;49:2526–2530) DOI: 10.1167/iovs.07-1454

Idiopathic uveitis is an inflammatory disease of unknown etiology affecting the inner eye, with a period prevalence of 115.3/100,000 persons in developed countries.[1] Autoimmune mechanisms are suggested by the homology of human diseases and experimental autoimmune uveitis (for a review, see Caspi[2]). The contribution of infectious agents in the pathogenesis of idiopathic uveitis has also been suspected in different clinical situations. Moreover, cross-reactivity between retina-specific autoantigens such as S-antigen (S-Ag) and IRBP (interphotoreceptor retinoid-binding protein) and environmental antigens, such as food antigens and microbial antigens, have already been suggested to explain autoimmune uveitis.[3] In the 1980s, Bloch-Michel and Timsit[4] showed abnormal skin reactivity to *Candida*-derived antigen (*Ca*-Ag) that can induce systemic reaction with a worsening or an improvement of uveitis, suggesting a role for *Ca*-Ag in the physiopathogenic mechanisms of idiopathic uveitis. In inflammatory bowel disease (IBD), often associated with uveitis, a role for yeast antigens such as *Saccharomyces* and *Candida* has been suggested by the presence of antibodies to mannan[5] and by abnormal cellular reactivity to mannan in T cells from IBD patients.[6] We already described abnormal cellular reactivity to *Ca*-Ag in patients with chronic fatigue syndrome.[7] In one patient with concomitant chronic fatigue syndrome and idiopathic panuveitis, we found abnormal reactivity to *Ca*-Ag (Kodjikian L, Cozon GJN, unpublished data, 2001). The present study was initiated to evaluate the cellular response to microbial antigens in the pathogenic mechanisms of idiopathic uveitis.

Methods

Patients with idiopathic uveitis treated at the ophthalmology department of Croix Rousse Hospital in Lyon were recruited from January 2002 to April 2004 after informed consent. They were classified according to uveitis nomenclature.[8] Among those with intermediate uveitis, scleral depression always precluded the presence of pars plana exudates. Broad hematologic and serologic workups, such as radiographic and functional tests, showed no abnormalities. The following were evaluated or performed in all patients except those with anterior uveitis: complete blood count with cell differentiation, angiotensin-converting enzyme (ACE) and lysozyme, serum calcium, liver enzymes, fluorescence treponemal antibody absorption, antinuclear antibodies, complement proteins, rheumatoid factor, *Bartonella*, *Toxoplasma*, *Toxocara*, *Rickettsia*, *Brucella*, Lyme, human immunodeficiency virus, Herpesvirus, *Candida* antigen titers, HLA typing, intracutaneous tuberculin testing, lumbosacral x-ray, chest x-ray, chest computerized tomography, pulmonary function, fluorescein and indocyanine green angiographies, and cerebrospinal magnetic resonance imaging. Results of this initial workup were always negative, and idiopathic uveitis was diagnosed in all patients. All patients had active uveitis and were included in the study before the prescription of any corticosteroids or immunosuppressive drugs. Healthy controls were recruited in the ophthalmology department among nurses, medical doctors, and residents. Four milliliters and 1 mL blood were withdrawn by venipuncture on lithium-heparin and ethylenediaminetetraacetic acid (EDTA) for culture and lymphocyte phenotyping, respectively, from patients and healthy controls. The study was approved by the institutional ethics committee (CCPPRB Lyon A) and was conducted in accordance with the tenets of the Declaration of Helsinki.

Description of the Studied Antigens

Candida albicans–derived antigen (100 μg/mL) is a commercial antigen (Allerbio, Varennes-en-Argonne, France) used for skin reactions, as previously described.[7]

Supernatants of staphylococcal strains RN4220 (without superantigen) and FRIS6 (containing the staphylococcal enterotoxin B) were a generous gift from Gérard Lina.[9] Tetanus toxoid (TT; 5 μg/mL) was from Sanofi Pasteur (Marcy l'Etoile, France). *Toxoplasma* antigen (8.7 μg/mL) was a gift from François Peyron (Laboratoire de Parasitologie, Faculté Rockefeller, Lyon, France). Tuberculin purified protein derivative (PPD 1 mg/mL) was purchased from Statens Serum Institute (Tuberculin Department; Copenhagen, Denmark). Antigen S (100 μg/mL) was a generous gift from Yvonne de Kozak (Institut National de la Santé et de la Recherche Médicale U598, Centre Biomédical des Cordeliers, Paris, France). Phytohemagglutinin (PHA, 10 μg/mL; Sigma, St. Quentin Fallavier, France) was used as a positive control for nonspe-

From the [1]Laboratoire d'Immunologie and the [3]Service d'Ophtalmologie, Hôpital de la Croix-Rousse, Hospices Civils de Lyon, Lyon, France; and [2]INSERM U851, Université Lyon 1, Lyon, France.

Supported by grants from Hospices Civils de Lyon AO, Lyon, France.

Submitted for publication November 12, 2007; revised January 12, 2008; accepted April 15, 2008.

Disclosure: **G.J.N. Cozon**, None; **F.M. Mbitikon-Kobo**, None; **F. Fatoohi**, None; **M. Spire**, None; **J.-D. Grange**, None; **L. Kodjikian**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Laurent Kodjikian, Department of Ophthalmology, Croix-Rousse University Hospital, 103, Grande Rue de la Croix-Rousse, Lyon 69004, France; kodjikian.laurent@wanadoo.fr.

cific T-cell activation. RPMI 1640 culture medium (Sigma) was used as a negative control.

Blood Culture and Stimulation

As previously described, samples of 50 μL whole heparinized blood were incubated in sterile polypropylene (45 × 88 mm) tubes (Micronic System, Lelystad, Netherlands) with 50 μL different antigens in RPMI 1640 medium, or RPMI 1640 medium for the negative controls, at 37°C in 5% CO_2 for 24 hours or 7 days. Seven-day cultures were supplemented on day 1 with 500 μL RPMI 1640 medium. Incubation times for optimal cellular responses had previously been determined by kinetic assays.[10]

Lymphocyte Phenotyping

Peripheral blood cells and cultured cells were stained for three-color flow cytometry after erythrocyte lysis with a solution of NH_4Cl (155 mM), $KHCO_3$ (10 mM), and EDTA (0.1 mM) and were analyzed on a flow cytometer (Epics XL; Beckman-Coulter, Villepinte, France) using Beckman-Coulter software (Expo 32). T cells and T-cell subsets were identified by direct conjugation of monoclonal antibodies anti-CD3, anti-CD4, anti-CD8, anti-CD2, anti-γδ, and CD45; B cells by anti-CD19; and natural killer (NK) cells by anti-CD56, evaluated on $CD3^-$ lymphocytes; T-cell activation markers by anti-CD25, anti-CD69, and anti-HLA-DR. Directly conjugated murine IgG1 (control isotype) was used to ascertain background staining. All monoclonal antibodies were obtained from Immunotech Beckman-Coulter, with the exception of anti-CD45, phycoerythrin-conjugated anti-CD4, anti-CD25, and anti-CD69, which were obtained from Dako (Trappes, France).

Statistical Analysis

All statistical analyses were carried out and all figures were drawn with commercial software (StatView 5.0; Abacus Concepts, Inc., Berkley, CA). Unpaired *t*-test was used to compare mean values. $P < 0.05$ was considered statistically significant.

Results

Ophthalmologic Diagnosis

Thirty-one patients (19 females, 12 males) with uveitis were studied. Patients were classified as previously recommended.[8] Panuveitis, anterior uveitis, intermediate uveitis, and posterior uveitis type were present in, respectively, 16, 2, 4, and 9 patients. Twenty-our healthy controls (16 females) were students, laboratory technicians, and nurses from the ophthalmological unit. Ages were identical between patients and controls (44.6 ± 3.8 years vs. 40.5 ± 2.0 years [mean ± SEM]; $P = 0.38$). Patient ages ranged from 13 to 81 years.

Lymphocyte Phenotyping

Figure 1 shows that there were no significant differences in the percentages of the different lymphocyte subpopulations between patients with uveitis and healthy controls for constitutive or activation markers. A slight but not significant difference was noted in the percentages of $CD4^+CD25^+$ T cells between controls and patients with uveitis (12.9 ± 2.2 and 8.3 ± 2.4, respectively; $P = 0.167$).

Cellular Reactivity to Antigens

Early cellular reactivity to antigens was studied using flow cytometry detection of CD69 expression in a 24-hour blood culture with antigen preparation. Figure 2A shows that higher percentages of CD69-expressed $CD4^+$ T cells were present in *Candida*-stimulated blood culture from patients with uveitis

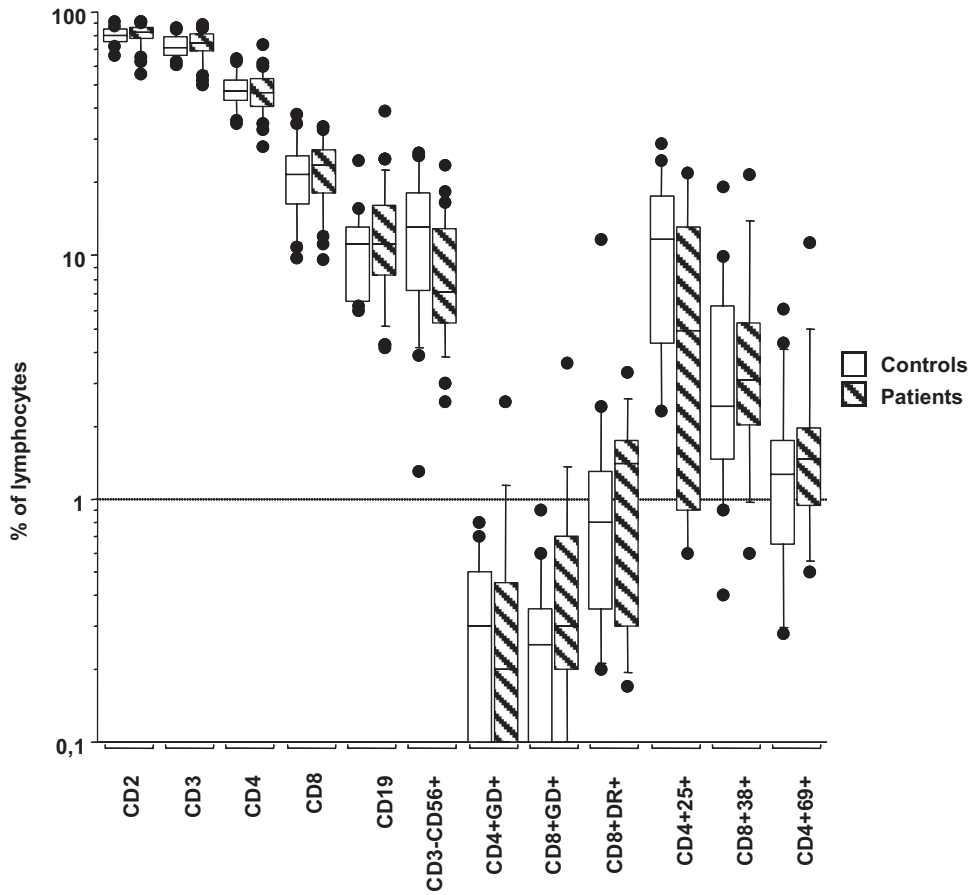

Figure 1. Distribution of lymphocyte subpopulations in blood from controls and patients with uveitis after staining with monoclonal antibodies and analysis in flow cytometry.

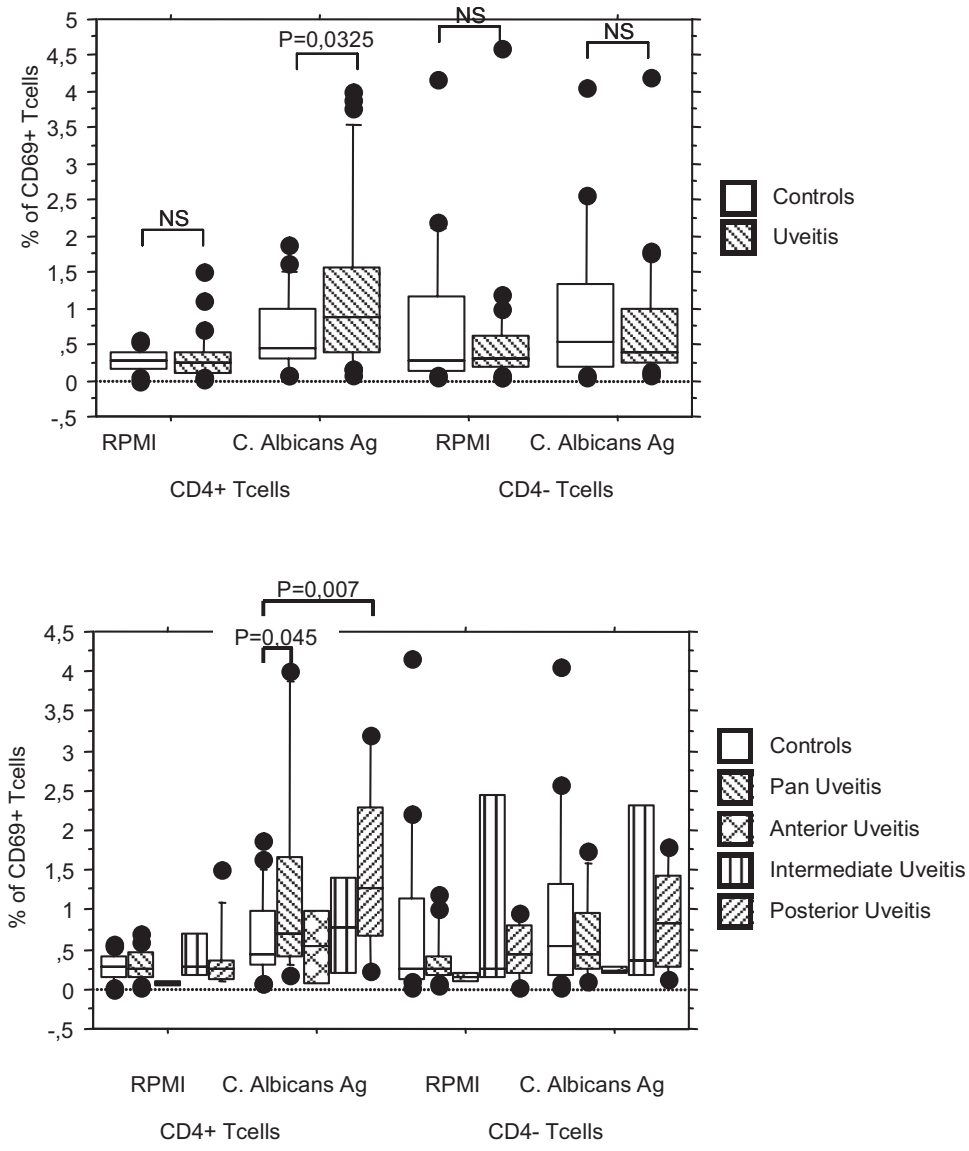

FIGURE 2. Percentages of activated ($CD69^+$) $CD4^+$ and $CD4^-$ T cells in 24-hour blood culture from controls and patients with uveitis in the presence of culture medium (RPMI) or *C. albicans* antigen. Results were separated with regard to subtype of uveitis.

than from healthy controls (1.24% ± 0.22% vs. 0.67% ± 0.11%, respectively; $P = 0.0325$). No difference was detected for $CD4^-$ T cells. There were no significant differences in nonactivated culture (RPMI) or in culture stimulated with *Staphylococcus*, tetanus toxoid, *Toxoplasma*, or PPD antigens (data not shown). Moreover, when subtypes of uveitis were studied, higher reactivity was detected in cultures from patients with panuveitis and posterior uveitis (Fig. 2B).

Late reactivity was detected after 7-day culture with different antigens and staining of activated CD4 T cells with the use of CD25-specific antibodies and flow cytometry. Figure 3 shows that higher percentages of $CD25^+$ T cells were detected in the blood cultures of uveitis patients with PHA, staphylococcal antigens from RN420 and FRIS6 strains, tetanus toxoid, tuberculin antigen (PPD), and *Toxoplasma* antigen than in the cultures of healthy controls. There was no difference in AgS-stimulated cultures.

DISCUSSION

Idiopathic uveitis is an ocular inflammatory disease of unexplained etiology. In Behçet disease, a systemic disorder of recurrent acute inflammation characterized by major symptoms of oral aphthous ulcers, uveitis, skin lesions, and genital ulcers, extrinsic pathogenic factors have been identified, including bacterial (*Streptococcus sanguis*, *Mycoplasma fermentans*) and viral (human herpesvirus) antigens (for a review, see Zouboulis and May[11]). The role of the herpesvirus has been recently reported in the etiology of nonnecrotizing retinopathy.[12] In the 1980s, Bloch-Michel and Timsit[4] showed that skin tests with candidin induced an obvious and transient change of the course of the ocular disease in 19 of 21 patients with uveitis and led to systemic symptoms in 15 patients. These observations suggest a cause-and-effect relationship in the pathogenesis of uveitis between microbial antigens and uveitis.

To better understand the pathogenic mechanisms of idiopathic uveitis and the role of microbial antigens, we analyzed the specific blood T-cell responses against different microbial antigens in patients with diagnoses of uveitis. The present study shows that patients with uveitis have an early cellular response to *Ca*-Ag and a higher late response to different microbial antigens.

We first analyzed lymphocyte subpopulations and activation markers on T cells in circulating leukocytes. We found no difference in the circulating lymphocyte subpopulations be-

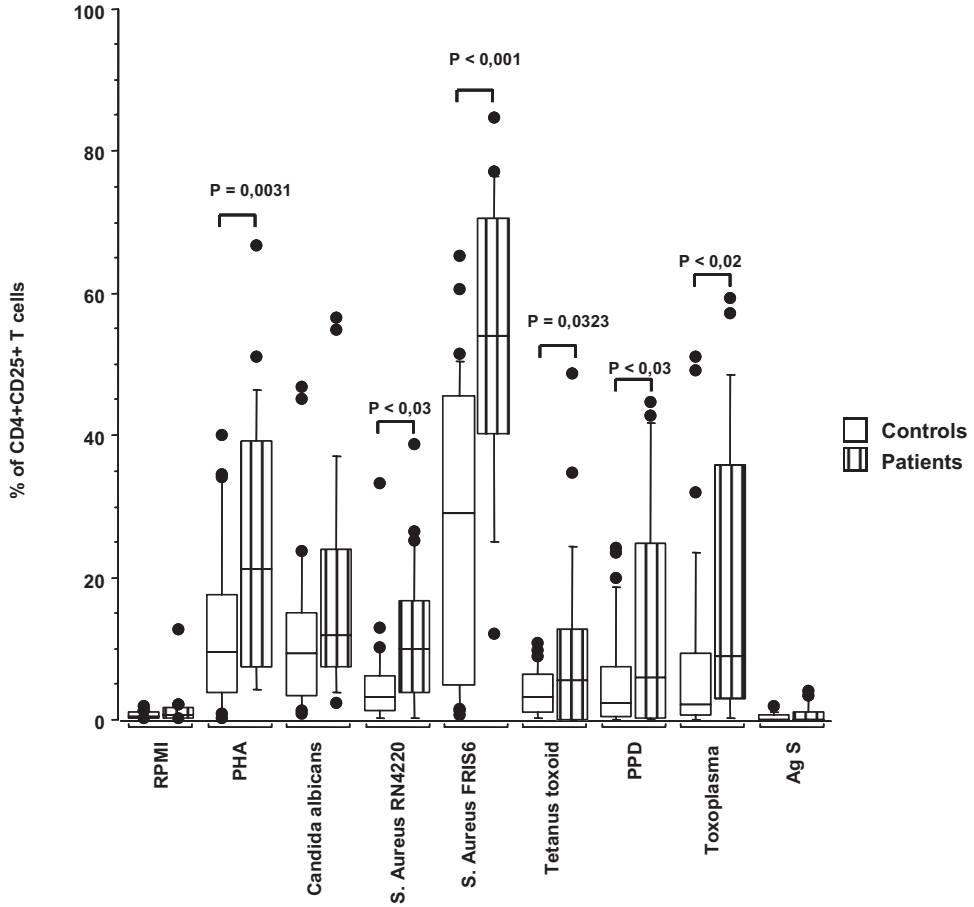

FIGURE 3. Percentage of activated ($CD25^+$) $CD4^+$ and $CD4^-$ T cells in 7-day blood culture from controls and patients with uveitis in the presence of culture medium (RPMI), PHA, *C. albicans*, *Staphylococcus aureus* strain RN4220, *S. aureus* strain FRIS6, tetanus toxoid, tuberculin (PPD), *Toxoplasma*, and AgS.

tween healthy controls and uveitis patients. This is in contrast to the subpopulations in patients with Behçet disease, in whom higher percentages of γδ T cells and CD56 NK cells were reported,[13] perhaps because of the heterogeneity of our patients. In contrast, patients with Behçet disease are more homogeneous, and most have a significant expression of HLA-B51.[11] Perhaps because of the small number of patients, the percentage of $CD4^+CD25^+$ T cells was lower in patient with uveitis than in healthy controls. $CD4^+CD25^+$ T cells are now known to be partially regulatory T cells (Treg) and to be involved in autoimmune disease (for a review, see Baecher-Allan and Hafler[14]). The slight decrease of $CD4^+CD25^+$ T cells in uveitis patients suggests a lower number of Tregs. Further studies using a specific marker for this T-cell population, Foxp3,[15] are necessary to validate this hypothesis.

No activation detected by CD69 or CD38 on circulating lymphocytes was present in healthy controls or in uveitis patients, in contrast to observations in previous studies.[16,17] This discrepancy might be explained by the difference in the anticoagulant used (heparin vs. EDTA) or by the difference in lymphocyte isolation. In our hands, the percentage of $CD4^+CD69^+$ T cells in peripheral EDTA-treated blood of healthy controls or patients never reached 5% (Fig. 1; Lina et al.[9]).

The early lymphocyte response was evaluated by detecting $CD69^+$ T cells after whole blood culture in the presence of different microbial antigens for 24 hours. The method has already been used to detect reactivity to polyclonal activation,[18] superantigens,[9] and mannan from *Saccharomyces cerevisiae* in patients with IBD.[6] Early activation of $CD4^+$ T cells, detected by CD69 expression, was greater in culture with *Ca*-Ag from uveitis patients than from healthy controls (1.24% ± 0.22 vs. 0.67 ± 0.11; $P = 0.0325$). This activation was predominant in patients with posterior uveitis or panuveitis (Fig. 2B) and was selective to *Ca*-Ag. No increase of CD69 expression was detected after culture with the other antigens except for the staphylococcal enterotoxin B–containing FRIS6 antigen and the PHA (data not shown). In patients with chronic fatigue syndrome, we have already described such an increase of CD69 expression on $CD4^+$ T cells after culture with *Ca*-Ag. Moreover, the increase of CD69 correlated with systemic reactions after skin testing with *Ca*-Ag.[6] Given that we observed an increase of urine neopterin, an interferon-induced macrophage product (Perret-Liaudet A, Cozon GJN, unpublished data, 2003), in patients with reactive chronic fatigue syndrome, systemic reactions to skin testing with *Ca*-Ag might have been caused by the production of γ-interferon. Further studies will evaluate neopterin in the urine of uveitis patients after skin tests with *Ca*-Ag. Our preliminary unpublished data (2005) show an increase of neopterin in the urine after skin testing to *Ca*-Ag in one patient with uveitis.

The late lymphocyte response was evaluated by the detection of $CD25^+$ T cells after whole blood culture in the presence of different microbial antigens for 7 days. The method was shown to be as accurate as the measurement of [3H]-thymidine incorporation for detecting cellular immune responses to *Toxoplasma gondii*—specific antigens.[19] In our hands other markers, such as CD69 and CD71, were either uninformative or less accurate than CD25.[20] As previously shown, CD25 was detected at the surfaces of $CD4^+$ and $CD4^-$ T cells that are $CD8^+$.[10] The $CD4^+CD25^+$ T cells obtained after 7-day culture are different from regulatory $CD4^+CD25^+$ T cells, which are circulating T cells and do not proliferate in culture.[21] In antigen-stimulated blood culture, the percentages of $CD25^+$ T cells

doubled every day from day 4 to day 7 (Cozon GJN, et al., unpublished data, 1999). Moreover, as previously described,[22] activated $CD25^+$ T cells detected in the present study overexpressed $CD4^+$(data not shown). The absence of a detectable T-cell response to AgS, in contrast to previous studies, might have resulted from the difference in culture methods. In the present study, we used whole blood culture conditions, which might have underestimated the response to AgS because of specific antibodies in the sera of uveitis patients.[23] The present study shows that 7-day cultures with different microbial antigens induce higher percentages of activated $CD25^+CD4^+$ T cells in blood cultures of uveitis patients than of controls. In contrast, CD25 expression was low after culture with AgS in blood cultures from uveitis patients or from controls (Fig. 3). The higher reactivity to microbial antigens might be the consequence of an increase in contact with microbial antigens in uveitis patients or a slight decrease in regulatory cells such as T-reg cells. Dysbiosis that can be associated with uveitis has been described in patients with IBD.[24] Treatment of staphylococcal carriage can ameliorate intermediate uveitis (Cozon GJN, Kodjikian L, unpublished data, 2003), and ketoconazole plus cyclosporine (CsA) is more effective at preventing recurrences of uveitis than is CsA alone.[25] Moreover, the role of normal bacterial flora to induce T-regs has recently been suggested.[26] Further studies are necessary to explore this hypothesis. Another explanation of higher reactivity to microbial antigens would be the presence of multispecific autoreactive T cell that cross-react with microbial antigens by different mechanisms, such as molecular mimicry, bystander activation, or infection persistence with or without epitope spreading.[27]

In conclusion, the present study, initiated to evaluate the immune cellular reactivity against common microbial antigens in patients with idiopathic uveitis, shows that although circulating lymphocytes had similar phenotypes in uveitis patients and healthy controls, early reactivity to *Candida albicans* developed in patients with posterior uveitis and panuveitis. Another finding was the higher reactivity of circulating T cells to microbial antigens in patients with uveitis compared with controls. Further studies are necessary to understand the exact role of microbial antigens in the pathogenic mechanisms of idiopathic uveitis. Nevertheless such findings in uveitis patients outline the potential role of the mucosal flora, which can be a target of the treatment of patients with idiopathic uveitis.

References

1. Gritz DC, Wong IG. Incidence and prevalence of uveitis in Northern California: the Northern California Epidemiology of Uveitis Study. *Ophthalmology*. 2004;111:491–500.
2. Caspi RR. Ocular autoimmunity: the price of privilege? *Immunol Rev*. 2006;213:23–35.
3. Wildner G, Diedrichs-Mohring M. Autoimmune uveitis and antigenic mimicry of environmental antigens. *Autoimmun Rev*. 2004; 3:383–387.
4. Bloch-Michel E, Timsit JC. Uveitis with allergy to candidin. *Ophthalmologica*. 1985;191:102–106.
5. Standaert-Vitse A, Jouault T, Vandewalle P, et al. *Candida albicans* is an immunogen for anti-*Saccharomyces cerevisiae* antibody markers of Crohn's disease. *Gastroenterology*. 2006;130:1764–1775.
6. Konrad A, Rutten C, Flogerzi B, Styner M, Goke B, Seibold F. Immune sensitization to yeast antigens in ASCA-positive patients with Crohn's disease. *Inflamm Bowel Dis*. 2004;10:97–105.
7. Cozon GJ, Brunet JL, Peyramond D. Detection of specific T lymphocytes in systemic abnormal delayed type hypersensitivity to *Candida albicans*. *Inflamm Res*. 2000;49:S39–S40.
8. Jabs DA, Nussenblatt RB, Rosenbaum JT. Standardization of Uveitis Nomenclature (SUN) Working Group: standardization of uveitis nomenclature for reporting clinical data: results of the First International Workshop. *Am J Ophthalmol*. 2005;140:509–516.
9. Lina G, Cozon G, Ferrandiz J, Greenland T, Vandenesch F, Etienne J. Detection of staphylococcal superantigenic toxins by a CD69-specific cytofluorimetric assay measuring T-cell activation. *J Clin Microbiol*. 1998;36:1042–1045.
10. Kahi S, Cozon GJN, Greenland T, Wallon M, Gay-Andrieu F, Peyron F. Rapid flow cytometric method to explore cellular immunity against *Toxoplasma gondii* in humans. *Clin Diag Lab Immunol*. 1998;5:745–748.
11. Zouboulis CC, May T. Pathogenesis of Adamantiades-Behçet's disease. *Med Microbiol Immunol*. 2003;192:149–155.
12. Bodaghi B, Rozenberg F, Cassoux N, Fardeau C, LeHoang P. Nonnecrotizing herpetic retinopathies masquerading as severe posterior uveitis. *Ophthalmology*. 2003;110:1737–1743.
13. Freysdottir J, Lau S, Fortune F. Gamma delta T cells in Behçet's disease (BD) and recurrent aphthous stomatitis (RAS). *Clin Exp Immunol*. 1999;118:451–457.
14. Baecher-Allan C, Hafler DA. Human regulatory T cells and their role in autoimmune disease. *Immunol Rev*. 2006;212:203–216.
15. Sakaguchi S, Ono M, Setoguchi R, et al. $Foxp3^+$ $CD25^+CD4^+$ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev*. 2006;212:8–27.
16. Kilmartin DJ, Fletcher ZJ, Almeida JA, Liversidge J,. Forrester JV, Dick AD. CD69 expression on peripheral $CD4^+$ T cells parallels disease activity and is reduced by mycophenolate mofetil therapy in uveitis. *Invest Ophthalmol Vis Sci*. 2001;42:1285–1292.
17. Murphy CC, Duncan L, Forrester JV, Dick AD. Systemic CD4(+) T cell phenotype and activation status in intermediate uveitis. *Br J Ophthalmol*. 2004;88:412–416.
18. Maino VC, Suni MA, Ruitenberg JJ. Rapid flow cytometric method for measuring lymphocyte subset activation. *Cytometry*. 1995;20: 127–133.
19. Caruso A, Licenziati S, Corulli M, et al. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry*. 1997;27:71–76.
20. Kahi S, Cozon GJN, Pinon JM, et al. A switch towards Th2 during serological rebound in children with congenital toxoplasmosis. *Clin Exp Immunol*. 1999;117:524–528.
21. McHugh RS, Shevach EM. The role of suppressor T cells in regulation of immune responses. *J Allergy Clin Immunol*. 2002;110: 693–702.
22. Ridgway W, Fasso M, Fathman G. Following antigen challenge, T cell up-regulate cell surface expression of CD4 in vitro and in vivo. *J Immunol*. 1998;161:714–720.
23. Muiño JC, Juárez CP, Luna JD, et al. The importance of specific IgG and IgE autoantibodies to retinal S antigen, total serum IgE, and sCD23 levels in autoimmune and infectious uveitis. *J Clin Immunol*. 1999;19:215–222.
24. Tamboli CP, Neut C, Desreumaux P, Colombel JF. Dysbiosis in inflammatory bowel disease. *Gut*. 2004;53:1–4.
25. Ramadan AM, Nussenblatt RB, de Smet MD. Long-term follow-up of patients with chronic uveitis affecting the posterior pole treated with combination cyclosporine and ketoconazole. *Ophthalmology*. 1997;104:706–711.
26. Mazmanian SK, Kasper DL. The love-hate relationship between bacterial polysaccharides and the host immune system. *Nat Rev Immunol*. 2006;6:849–858.
27. Fujinami RS, von Herrath MG, Christen U, Whitton JL. Molecular mimicry, bystander activation, or viral persistence: infections and autoimmune disease. *Clin Microbiol Rev*. 2006;19:80–94.