

CCR4 and CCR5 expression in conjunctival specimens as differential markers of T_H1/ T_H2 in ocular surface disorders

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Background: Accurate inflammatory mechanisms in chronic ocular surface diseases (OSDs) cannot routinely be assessed. New techniques for investigating ocular surface inflammatory pathways are of major importance.

Objective: To investigate the expressions of CCR4 and CCR5, known to be related to the T_H2 and T_H1 systems, respectively, and HLA-DR in conjunctival impression cytology specimens from patients with chronic OSDs.

Methods: In this case-controlled study, impression cytology specimens were taken in a series of patients with vernal keratoconjunctivitis (n = 21), giant papillary conjunctivitis (n = 6), or keratoconjunctivitis sicca (KCS; n = 17), or receiving topical antiglaucoma treatments (n = 31), and from 20 normal subjects. Conjunctival cells were incubated with mAbs to CCR4, CCR5, CD45, and HLA-DR to quantify conjunctival inflammation in a masked manner using flow cytometry.

Results: HLA-DR was higher in the glaucoma and KCS groups than in allergic and normal eyes. CCR4 was overexpressed in allergy and glaucoma, whereas CCR5 was higher in the KCS and glaucomatous groups. CD45 was expressed by only few cells in all groups, with almost no significant differences. CCR4 expression was negatively correlated with CCR5 and HLA-DR, whereas CCR5 was positively correlated with HLA-DR.

Conclusion: This study confirms the overexpression of chemokine receptors by the conjunctival epithelium in OSDs. CCR4 and CCR5 expression may vary according to the immune pathway involved. Accurate mechanisms in ocular surface inflammatory reactions—that is, those related to the

T_H1 or T_H2 systems—could be differentiated by CCR4/CCR5 profiles. Our results also suggest that long-term use of topical treatments may stimulate both systems. (*J Allergy Clin Immunol* 2005;116:614-9.)

Key words: CCR4, CCR5, conjunctiva, flow cytometry, glaucoma, HLA-DR, impression cytology, Sjögren syndrome, T_H1, T_H2

Allergic eye diseases (AEDs) constitute a heterogeneous group of clinicopathological disorders that can cause a large variety of clinical manifestations, from simple intermittent symptoms of itching, tearing, or redness to severe sight-threatening corneal impairment.^{1,2} These diseases may involve quite different mechanisms, cytokines, and cellular populations as central actors. Mast cell degranulation, histamine release, and eosinophils thus play key roles in seasonal and perennial conjunctivitis, whereas vernal keratoconjunctivitis (VKC) or atopic keratoconjunctivitis (AKC) are characterized by cellular infiltrates mainly composed of T lymphocytes.³⁻⁵ In severe, chronic allergy-related conditions, 2 main inflammatory pathways may be differentiated: the T_H1 and T_H2 inflammatory cascades, which involve different cytokines and are roughly considered inhibitory of each other when activated.⁶ In previous reports based on conjunctival biopsies in patients with allergy, cytokines belonging to the T_H1 or T_H2 systems were investigated, resulting in the recognition of T_H2 activation in VKC, whereas both T_H1 and T_H2 cascades could be found in AKC.⁴ Similar results were obtained by flow-cytometry investigation of lymphocyte populations released in the tear flow in allergic disorders³ or cytokine expression assessed by RT-PCR in brush conjunctival cytology specimens.⁶ The necessarily invasive character of conjunctival biopsies, which cannot be routinely proposed to patients with AEDs, as well as the major difficulty of collecting enough lymphocytes from the tear film among cell debris, desquamated epithelial cells, or polymorphonuclear cells, render these techniques highly difficult for routinely exploring allergic patients. Moreover, clinically well-defined allergic disorders are only a part of the wide range of inflammatory ocular surface diseases (OSDs), which also include keratoconjunctivitis sicca (KCS), meibomian gland dysfunction, ocular rosacea, and eyedrop toxicity. Clinical symptoms and signs lack specificity, and most of them leave physicians and patients with unanswered questions and

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Abbreviations used

AED: Allergic eye disease
AKC: Atopic keratoconjunctivitis
GPC: Giant papillary conjunctivitis
KCS: Keratoconjunctivitis sicca
OSD: Ocular surface disease
PC5: Phycoerythrin-cyanin 5
VKC: Vernal keratoconjunctivitis

unsatisfactory responses concerning the accurate mechanisms involved, appropriate treatments, clinical behavior, and symptom relief.

This dilemma emphasizes the need for simple, noninvasive or minimally invasive methods to achieve mechanism identification of chronic inflammatory OSDs and discriminate between allergic and nonallergic pathways. In the last few years, our group has developed the flow-cytometry technique in impression cytology specimens, which has provided extended knowledge of inflammation-related or apoptosis-related membrane antigens⁷⁻⁹ in OSDs, mainly those in relation with dry eye or antiglaucoma treatments. We therefore used the same technique in a series of patients with clinically well-defined allergic or nonallergic OSDs, with markers belonging to the chemokine receptor family and considered to be related to the T_H1 and T_H2 systems,¹⁰ CCR5 and CCR4, respectively, together with HLA-DR class II antigens as a hallmark for inflammatory assessment, and CD45, a molecule expressed on hematopoietic cells, used for identifying immune cells. The aim of this pilot exploratory case-controlled study was thus to explore new, promising markers and try to provide a useful tool for investigating OSDs and discriminating between the inflammatory pathways involved.

METHODS

Seventy-five patients with OSDs and 20 normal subjects underwent impression cytology collection for this case-controlled study conducted in compliance with the Declaration of Helsinki, Scotland amendment, 2000. Patient characteristics are given in Table I. Seventeen patients had aqueous-deficient KCS as assessed by Schirmer values less than 7 mm at 5 minutes and a positive fluorescein test (at least 2 on the Oxford scheme), associated ($n = 10$) or not ($n = 7$) with documented Sjögren syndrome. All of them had received only tear substitutes, with no steroid or topical cyclosporine, for at least 3 months. Twenty-seven patients were also diagnosed with chronic AEDs, 21 with VKC and 6 with giant papillary conjunctivitis (GPC) resulting from poorly tolerated contact lens wear. All patients with VKC had a history of atopy and characteristic features of the disease: limbal and/or tarsal involvement with papillae, follicles, and Trantas dots, and typical symptoms. Again, none of them at the time of impression cytology collection had received any steroid or topical cyclosporine, but only mast cell stabilizers and/or anti-H1 antagonists. All were highly inflammatory at the time of examination, demonstrating a poor effect of topical treatment in relieving allergic manifestations. A series of 31 glaucoma patients with chronic primary open-angle glaucoma was also investigated. They had been treated for at least 1 year with the same treatment, but they were included because they showed at least mild

symptoms and/or signs of poor tolerance of their eye drops: repeated stinging or ocular discomfort upon and between instillations; pronounced conjunctival hyperemia; a break-up time less than 8 seconds; a positive fluorescein staining of the cornea that could not be explained by another mechanism, such as aqueous deficiency; active blepharitis related to rosacea; or atopic manifestations. Patients with glaucoma were divided into 2 groups: 13 treated with a monotherapy, either a prostaglandin analogue or a β -blocker, all containing a preservative, and 18 receiving at least 2 topical treatments, including the latter 2 families and/or carbonic anhydrase inhibitors or α 2-agonists. Finally, 20 normal subjects who had no history of ocular disease or clinical ophthalmic abnormality, assessed after slit-lamp examination, and who had not received any topical treatment for at least 3 months, were also investigated, after approval by the Ethics Committee of Dijon University. Concerning the patients with OSDs, including symptomatic patients with glaucoma, the Ethics Committee of Paris 6 University had stated that the exploration of OSDs by using impression cytology collection did not require specific approval. Nevertheless, all patients received specific explanations on impression cytology principles and the study aims, and gave informed consent for the procedure and subsequent use of conjunctival specimens.

Impression cytology specimens

Only 1 eye was examined to avoid any bias related to immune between-eye dependency, especially in atopic patients. In most cases, the worse eye was chosen for impression cytology collection. In the case of total symmetry, the right eye was selected for impression cytology analyses. As previously described,^{7,9} specimens were collected in the superior bulbar conjunctiva by using 0.20- μ m polyether sulfone filters (Supor Membranes; Gelman Sciences, Ann Arbor, Mich) after instillation of 1 drop of 0.4% oxybuprocaine. Two pieces of filters, measuring 13 mm $\times$ 6.5 mm, were applied to the superior bulbar conjunctiva in areas covered by the upper eyelid to avoid cells exposed to desiccation and environment in the palpebral fissure. As we had previously validated this procedure,^{9,11} specimens were taken at least 15 minutes after the use of fluorescein eye drops. Membranes were immediately suspended and fixed in cold PBS containing 0.05% paraformaldehyde. Conjunctival cells were further extracted by gentle agitation and then centrifuged at 1600 rpm for 5 minutes before processing for flow cytometry.

Flow cytometry

Expressions of HLA-DR, CCR4, CCR5, and CD45 were assessed by using flow cytometry and indirect or direct immunofluorescence procedures. Purified anti-HLA-DR (α -chain, clone TAL.1B5) was purchased from DakoCytomation (Glostrup, Denmark); phycoerythrin-conjugated anti-CCR4 (mouse IgG1, clone 1G1), fluorescein isothiocyanate-conjugated anti-CCR5 (mouse IgG2a, clone 2D7) from Pharmingen BD Biosciences (San Diego, Calif); and phycoerythrin-cyanin 5 (PC5)-conjugated anti-CD45 (clone Immu 19.2) from Beckman Coulter (Miami, Fla). Anti-HLA-DR was used at a 1:25 dilution and incubated 30 minutes in the dark at room temperature before being counterstained with a 1:25 dilution of fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin (Dako Cytomation) for 30 minutes. Direct immunostainings were performed for CCR4, CCR5, and CD45 for 30 minutes. Antibodies were used in a 1:50, 1:25, and 1:25 dilution, respectively. The corresponding isotypic negative controls were used, mouse IgG1 (Beckman Coulter) for HLA-DR, mouse IgG1-PE for CCR4, mouse IgG2a-PE for CCR5 (Pharmingen BD Biosciences), and mouse IgG1-PC5 for CD45 (Beckman Coulter). Cells were processed on a flow cytometer EPICS XL (Beckman Coulter). As previously described,^{9,11} cells were gated

TABLE I. Demographic characteristics of patients*

	Normal eyes	AED			KCS	Glaucoma		
		VKC	GPC	Total		Mono	Multiple	Total
Eyes, n	21	21	6	27	17	13	18	31
Age, y								
Mean	55.8	10.8	26.0	13.2	56.4	60.5	60.3	60.4
(SD)	(20.3)	(5.6)	(13.7)	(9.1)	(11.1)	(11.7)	(17.7)	(15.2)
Range	16-78	5-22	16-45	5-45	40-75	43-79	21-78	21-79
Sex ratio (M/F)	10/11	13/8	3/3	16/11	3/14	8/5	9/9	17/14

Mono, Patients with glaucoma receiving only 1 topical drug; *multiple*, patients receiving at least 2 topical drugs.

*No statistical difference for age among normal, KCS, and glaucoma groups (ANOVA test).

on a side scatter versus forward scatter histogram in logarithmic and linear modes, respectively. For each antibody, at least 1000 conjunctival cells were analyzed. Results were expressed in percentages of positive cells. In selected cases—those with high chemokine receptor expression—double immunostaining with CD45 and CCR4 or CCR5 was performed to identify the cell populations bearing these markers (Fig 1). All flow-cytometric analyses were performed in a masked manner for patient characteristics. Statistical comparisons were performed by using ANOVA to compare the immune marker levels between patient groups, and correlations were calculated by using the Spearman correlation test (Statview IV for Windows; Abacus, Berkeley, Calif).

RESULTS

Results are detailed in Fig 2 and Table II. As expected and described in previous studies,⁷⁻⁹ normal eyes showed low percentages of HLA-DR-positive cells amongst the conjunctival cell population, but also very few CCR4-positive or CCR5-positive cells (Table II). The difference between the apparently high percentage of HLA-DR positivity was explained by a known low-level expression of this marker by normal conjunctival epithelial cells,⁹ whereas chemokine receptors were most likely expressed in normal eyes by intraepithelial lymphocytes and/or inflammatory cells, as assessed by double immunostaining with anti-CD45 antibody, and the similarly low levels of CD45-positive cells found in the same specimens.

In contrast, patients with KCS showed significantly higher percentages of cells expressing HLA-DR ($P = .013$ compared with normal eyes) and CCR5 ($P < .0001$), but not CCR4, which was even slightly lower than in normal eyes (not significant). Again, CD45-positive cells remained at very low percentages, even lower than in normal eyes ($P = .02$), and double-immunostaining confirmed a CCR5 expression by both CD45-positive cells and by a larger population of CD45-negative cells gated with epithelial cells. Although results of HLA-DR and CCR5 were higher in the Sjögren group, no statistically significant difference could be found between the 2 groups.

Conversely, patients with allergy considered as a whole showed inverted results compared with those with KCS eyes. HLA-DR and CCR5 levels found were not significantly different from those obtained in normal eyes.

However, CCR4 showed significantly higher levels in the allergy group ($P < .0001$ compared with both normal and KCS eyes). CD45 remained very low, not significant compared with normal eyes, and double-immunostaining confirmed that CCR4 could be expressed by CD45-negative cells (Fig 1). Interestingly, when discriminating between the VKC and GPC subgroups, a significant difference was found: CCR4 was higher in the GPC eyes than in the VKC eyes ($P = .0022$). Nevertheless, the difference between VKC and normal or KCS eyes remained highly significant ($P < .0001$ for both comparisons).

The glaucoma group showed an inflammatory profile different from those of the KCS and allergy groups. As previously reported,^{7,9} HLA-DR was found positive in a high percentage of conjunctival cells ($P < .0001$ compared with normal and allergic eyes, not significant compared with KCS eyes). Although the multitherapy group showed even higher levels than the monotherapy group (63.8% vs 51.8%), the difference was not significant. However, the glaucoma group showed high levels of both CCR4 and CCR5, and CCR4 levels were significantly higher than in normal subjects and KCS ($P < .0001$ for both comparisons), and CCR5 was significantly increased compared with normal and allergic eyes ($P = .0002$ and $P < .0001$, respectively). CD45 remained at low, nonsignificantly increased levels compared with normal eyes. The 2 subgroups—monotreatments or multiple treatments—did not show any significant differences for CCR4 or CCR5. Interestingly, 21 of 31 glaucoma eyes simultaneously expressed both markers at high levels, which showed that their globally increased expression did not result from 2 separate subgroups of patients with glaucoma expressing only 1 of the 2 markers.

We found significant negative and positive correlations, respectively, between HLA-DR and CCR4 ($z = -2.394$; $P = .017$; Spearman correlation test) and between HLA DR and CCR5 ($z = 3.96$; $P < .0001$). In the whole population, however, the correlation between CCR4 and CCR5 was not significant, most likely because of the presence of patients with glaucoma, roughly positive to both markers. We therefore excluded this group, and a significant negative correlation was found between CCR4 and CCR5 ($z = -2.92$; $P = .003$), reaching even higher significance when also removing the normal eyes, because

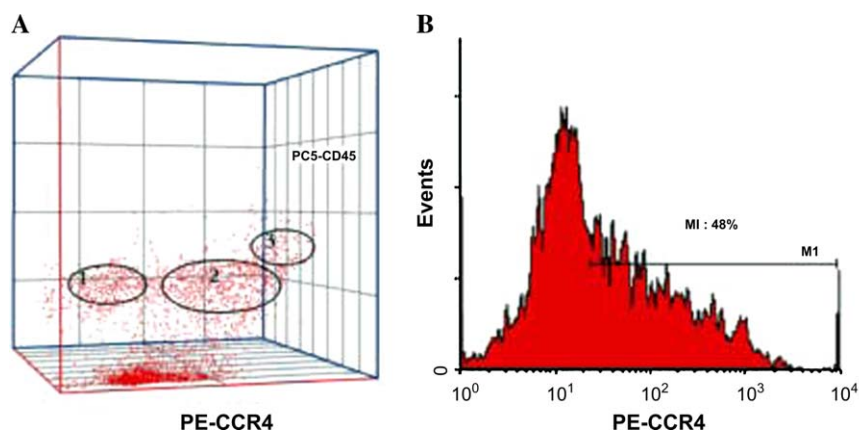


FIG 1. A, Example of double immunostaining (CCR4/CD45) of a conjunctival imprint taken from a patient with VKC. Phycoerythrin-CCR4 vs PC5-CD45; *gate 1*, negative epithelial cells; *gate 2*, CCR4-positive/CD45-negative epithelial cells (45.5%); *gate 3*, CCR4-positive and CD45-positive lymphoid cells (2%). PC5-negative isotypic control was less than 0.5%. B, Fluorescence histogram of phycoerythrin-CCR4 gated on all cells (epithelial and lymphoid cells); the M1 cursor shows 48% of positive cells for CCR4, corresponding to gates 2 and 3, with less than 2% for the phycoerythrin-negative isotypic control.

they were almost negative to both markers ($z = -4.33$; $P < .0001$). Conversely, in the glaucoma group, CCR4 and CCR5 were positively correlated ($z = 2.8$; $P = .005$), thus confirming the coactivation of both systems.

DISCUSSION

As most clinical symptoms and signs largely lack specificity, simple clinical examination often makes differential diagnosis among OSDs very difficult.¹² Laboratory investigations aimed at discriminating between the different possible inflammatory mechanisms impairing the ocular surface have major practical implications for improving clinical diagnosis and treatment management. The T_H1 and T_H2 pathways constitute the 2 major mechanisms involving T cells and controlling cell-mediated immune reactions.^{13,14} AEDs are regarded as predominantly T_H2-dependent immune responses. Indeed, elevation of IL-4 was reported in the tears and conjunctiva of patients with VKC and AKC,¹⁵ and high levels of IL-4, but not IFN- γ , were reported in brush cytology specimens of patients with seasonal allergic conjunctivitis.⁶ AKC and VKC tissues were already proven to express T_H2-type cytokines dominantly and to be infiltrated by T lymphocytes mainly belonging to the T_H2 system.^{3-5,15}

T_H1 cells would play a less important role in allergic pathologies, although AKC seemed to be a mixed pathology involving the 2 systems.⁴ IFN- γ , the major product of T_H1 lymphocytes, was found weakly expressed in AEDs, whereas this cytokine and TNF- α were shown to be overexpressed in Sjögren syndrome and experimental models of dry eye,¹⁶ with little or no T_H2-type IL-4 mRNA, demonstrating a T_H1-type cell reaction in dry eye syndromes.

Until now, there has been no useful way to assess the mechanisms involved at the ocular surface level other than

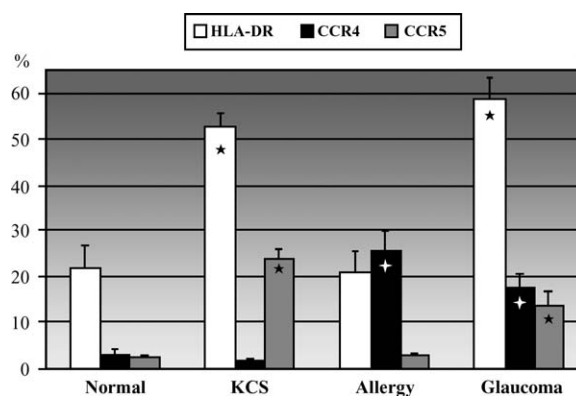


FIG 2. Expressions of HLA-DR, CCR4, and CCR5 in impression cytology specimens (means $\pm$ SEs). Allergy and glaucoma groups represent pooled data from the subgroups listed in Table II. $\star P < .001$ compared with normal and allergy groups, $\star P < .0001$ compared with normal and KCS groups (ANOVA test).

by using invasive procedures, such as conjunctival biopsies, or performing tear sample examination of rare, often altered, inflammatory cells or lacrimal cytokines, with the risk of falsely dosing proteins passively transudated from the serum to the tear film. Consequently, none of these techniques is routinely used. In the current study, we used a widely validated technique of flow cytometry in impression cytology specimens,^{7,8} which makes it possible to examine the expression of a large variety of markers on conjunctival cells. We previously showed that in allergic eyes, high levels of tear IgE could be found, contrasting with low levels of HLA-DR expression, whereas other inflammatory OSDs showed the inverted pattern: no IgE and high conjunctival levels of HLA-DR.¹⁷ Because HLA-DR expression in conjunctival cells is considered to result from IFN- γ and/or TNF- α secretion,¹⁸ and thus is associated with the T_H1 system, this was strongly suggestive of a reciprocal inhibition of the T_H1 and T_H2

TABLE II. Values of inflammatory markers in subgroups of patients with allergy or glaucoma (mean $\pm$ SE)

	Normal	AEDs		KCS	Glaucoma treatments	
		VKC	GPC		Mono	Multiple
CD45	3.3 $\pm$ 0.5	3.3 $\pm$ 0.7	3.0 $\pm$ 0.8	1.1 $\pm$ 0.1***	2.9 $\pm$ 0.9	2.0 $\pm$ 0.7
HLA-DR	22.1 $\pm$ 0.5	22.9 $\pm$ 5.6	11.0 $\pm$ 4.5	52.4 $\pm$ 2.9* $\blacktriangle$	51.9 $\pm$ 7.7* $\blacktriangle$	63.8 $\pm$ 6.0* $\blacktriangle$
CCR4	3.5 $\pm$ 0.6	44.2 $\pm$ 7.5**/*	22.6 $\pm$ 3.8*	1.9 $\pm$ 0.2 $\blacktriangle$	19.8 $\pm$ 4.2* $\blacktriangle$ $\blacktriangle$	17.2 $\pm$ 4.4* $\blacktriangle$ $\blacktriangle$
CCR5	2.4 $\pm$ 0.5	1.9 $\pm$ 0.8	3.2 $\pm$ 0.6	24.1 $\pm$ 1.6*	13.0 $\pm$ 3.6**/*	14.8 $\pm$ 5.6**/*

Mono, Patients with glaucoma receiving only 1 topical drug; *multiple*, patients receiving at least 2 topical drugs.

* $P \leq .001$ compared with normal values; ** $P < .05$ compared with GPC; *** $P < .01$ compared with normal and VKC eyes; $\blacktriangle P \leq .0001$ compared with VKC and GPC; $\blacktriangle P < .02$ compared with KCS (ANOVA test).

pathways in the ocular surface, as in other mucosal systems.¹⁹ However, these techniques were based on different biological procedures, which made them difficult to perform and required further confirmation. In the current study, we chose to focus on conjunctival cell expression of 2 chemokine receptors respectively associated with the T_H2 and the T_H1 systems: CCR4 and CCR5.¹⁰

Little is known as yet on the expression of chemokine receptors in the normal or diseased conjunctiva. It was shown in conjunctival biopsies that some major chemokine receptors, including CCR4 and CCR5, were not or were very weakly expressed in normal eyes and found only on a few inflammatory cells in VKC, by using an immunohistochemical technique less sensitive than flow cytometry.²⁰ CCR4 is the main receptor for macrophage-derived chemokine and the thymus and activation-regulated chemokine. Conversely, CCR5 is expressed at high levels in T_H1 cells and is virtually absent from T_H2 lymphocytes. CCR5 ligands include the macrophage inflammatory proteins 1 α and 1 β , which recruit both macrophages and neutrophils and were recently found in conjunctival specimens.²¹ In a recent report on dry eye syndrome, a significant overexpression of CCR5 was found on conjunctival epithelial cells compared with normal eyes, confirming that CCR5 may be useful for assessing ocular surface inflammatory pathways.²²

In the current study, we used CCR4 and CCR5 expression in impression cytology specimens, together with HLA-DR as a standard for conjunctival inflammatory levels, as new tools for investigating OSDs. HLA-DR was most likely to be related to T_H1, as was CCR5, whereas CCR4 was expected to report T_H2-mediated reactions and to be expressed in AEDs. Because chemokine receptors are mostly considered to be expressed by lymphocytes, we also monitored CD45-expressing cells in this study. As expected, CCR4, CCR5, HLA-DR, and CD45 were only weakly expressed in normal, noninflammatory eyes. Percentages of CCR4 or CCR5 were consistent with those of CD45 and most likely corresponded to CD45-positive immune cells. CD45-positive cell levels in impression cytology specimens only weakly changed in OSDs, most likely because this technique removes only superficial cells and does not account for deeper inflammatory infiltrates, and most T cells are located in the subepithelial layers of the conjunctiva. CCR4, but not CCR5 or HLA-DR, was highly significantly increased in the 2 groups of

AEDs, VKC and GPC, with the latter group showing even higher levels. In contrast, the inverse pattern was found in dry eye syndrome: high HLA-DR and CCR5 levels, contrasting with normal CCR4. This strongly suggests that mutually exclusive pathways are involved in these 2 categories of OSDs. When considering allergic eyes and KCS, a highly significant negative correlation was thus found between CCR4 and the 2 other markers, whereas CCR5 and HLA-DR were, as expected, positively correlated. CCR4 and CCR5 expressions largely exceeded the CD45 levels and most likely resulted from an epithelial expression, as found by double immunostaining with CD45 and as already demonstrated for CCR5 in conjunctival cells with the same arguments²² or in other mucosae.²³

Other OSDs would deserve further investigation, because some of them are probably more complex and may involve, either simultaneously or successively, cytokines of the 2 systems. This could be the case of AKC, as was reported the presence of both T_H1-related and T_H2-related cytokines in this disease.⁴

Indeed, our results also showed that patients with glaucoma treated for long periods clinically showing poor tolerance of their treatments could activate not only CCR4 but also CCR5 and HLA-DR, thus suggesting that T_H1 and T_H2 pathways were both involved in this iatrogenic OSD. Antiglaucomatous drug-induced toxicity has been the focus of great interest and debate because of its complexity, because it may involve the active compound, the preservative, or both, given that allergic and toxic mechanisms may combine. In previous studies, we showed that asymptomatic patients with glaucoma treated over the long term had increased conjunctival expression of HLA-DR,¹¹ IL-6, IL-8, and IL-10.⁹ The preservative benzalkonium chloride is toxic and may be responsible for a large part of these inflammatory reactions on the ocular surface,^{11,24} but the presence of CCR4 also suggests allergic T_H2-mediated involvement. Interestingly, when examining individual expression of the 2 markers by glaucomatous patients, we found that a majority of them expressed both CCR4 and CCR5. The long-term use of antiglaucoma drugs and/or cumulated doses of preservatives may thus stimulate both pathways and therefore resemble what was found with other techniques in AKC.⁴ Although patients with multiple treatments seemed to have higher inflammatory levels, the difference was not significant, most likely because all

patients included in this study were symptomatic and clinically inflammatory. They probably had high degrees of biological inflammation, in contrast with our previous studies investigating asymptomatic patients in whom we could detect subclinical inflammation.¹¹

The current work thus opens a very promising perspective toward a new, reliable, and minimally invasive way to discriminate between T_H1 or T_H2 involvement, according to the chemokine receptor profiles. It also shows that some OSDs are highly complex and do not respond to only 1 mechanism. Further studies will be necessary to understand better which pathway is involved in other ocular surface pathologies, such as AKC, rosacea, or contact lens wear, and to expand our knowledge on the reactions occurring under the influence of topical treatments and their preservatives. In the future, the aim could be to develop a simple test that would discriminate between the complex mechanisms involved in individual patients with a chronic ocular surface disorder when clinical criteria alone are unable to provide an accurate diagnosis and an effective therapy.

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REFERENCES

- Bielory L. Allergic and immunologic disorders of the eye, part I: ocular allergy. *J Allergy Clin Immunol* 2000;106:805-16.
- Ono SJ, Abelson MB. Allergic conjunctivitis: update on pathophysiology and prospects for future treatment. *J Allergy Clin Immunol* 2005;115:118-2.
- Leonardi A, DeFranchis G, Zancanaro F, Crivellari G, De Paoli M, Plebani M, et al. Identification of local Th2 and Th0 lymphocytes in vernal conjunctivitis by cytokine flow cytometry. *Invest Ophthalmol Vis Sci* 1999;40:3036-40.
- Calder VL, Jolly G, Hingorani M, Adamson P, Leonardi A, Secchi AG, et al. Cytokine production and mRNA expression by conjunctival T-cell lines in chronic allergic eye disease. *Clin Exp Allergy* 1999;29:1214-22.
- Bonini S, Coassin M, Aronni S, Lambiase A. Vernal keratoconjunctivitis. *Eye* 2004;18:345-51.
- Fujishima H, Saito I, Takeuchi T, Shinozaki N, Tsubota K. Characterization of cytokine RNA transcripts in conjunctival cells in patients with allergic conjunctivitis. *Invest Ophthalmol Vis Sci* 1997;38:1350-7.
- Baudouin C, Brignole F, Becquet F, Pisella PJ, Goguel A. Flow cytometry in impression cytology specimens: a new method for evaluation of conjunctival inflammation. *Invest Ophthalmol Vis Sci* 1997;38:1458-64.
- Brignole F, Pisella PJ, Goldschild M, De Saint Jean M, Goguel A, Baudouin C. Flow cytometric analysis of inflammatory markers in conjunctival epithelial cells of patients with dry eyes. *Invest Ophthalmol Vis Sci* 2000;41:1356-63.
- Baudouin C, Hamard P, Liang H, Creuzot-Garcher C, Bensoussan L, Brignole F. Conjunctival epithelial cell expression of interleukins and inflammatory markers in glaucoma patients treated over the long term. *Ophthalmology* 2004;111:2186-92.
- Andrew DP, Ruffing N, Kim CH, Miao W, Heath H, Li Y, et al. C-C chemokine receptor 4 expression defines a major subset of circulating nonintestinal memory T cells of both Th1 and Th2 potential. *J Immunol* 2001;166:103-11.
- Pisella PJ, Debbasch C, Hamard P, Creuzot-Garcher C, Rat P, Brignole F, et al. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an ex vivo and in vitro study. *Invest Ophthalmol Vis Sci* 2004;45:1360-8.
- Bielory L. Ocular allergy and dry eye syndrome. *Curr Opin Allergy Clin Immunol* 2004;4:421-4.
- Umetsu DT, DeKruyff RH. T_H1 and T_H2 CD4+ cells in human allergic diseases. *J Allergy Clin Immunol* 1997;100:1-6.
- Matsuura N, Uchio E, Nakazawa M, Yago T, Matsumoto S, Ohno S, et al. Predominance of infiltrating IL-4-producing T cells in conjunctiva of patients with allergic conjunctival disease. *Curr Eye Res* 2004;29:235-43.
- Leonardi A. Vernal keratoconjunctivitis: pathogenesis and treatment. *Prog Retin Eye Res* 2002;21:319-39.
- Luo L, Li DQ, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates MAPK signalling pathways on the ocular surface. *Invest Ophthalmol Vis Sci* 2004;45:4293-301.
- Baudouin C, Bourcier T, Brignole F, Bertel F, Moldovan M, Goldschild M, et al. Correlation between tear IgE levels and HLA-DR expression by conjunctival cells in allergic and non allergic chronic conjunctivitis. *Graefes Arch Clin Exp Ophthalmol* 2000;238:900-4.
- Tsubota K, Fukagawa K, Fujihara T, Shimmura S, Saito I, Saito K, et al. Regulation of human leukocyte antigen expression in human conjunctival epithelium. *Invest Ophthalmol Vis Sci* 1999;40:28-34.
- Yanagita M, Hiroi T, Kitagaki N, Hamada S, Ito HO, Shimauchi H, et al. Nasopharyngeal-associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell-regulated IgA responses for the inhibition of bacterial attachment to epithelial cells and subsequent inflammatory cytokine production. *J Immunol* 1999;162:3559-65.
- Abu El-Asrar AM, Struyf S, Al-Mosallam AA, Missotten L, Van Damme J, Geboes K. Expression of chemokine receptors in vernal keratoconjunctivitis. *Br J Ophthalmol* 2001;85:1357-61.
- Miyazaki D, Nakamura T, Toda M, Cheung-Chau KW, Richardson RM, Ono SJ. Macrophage inflammatory protein-1a as a costimulatory signal for mast cell-mediated immediate hypersensitivity reactions. *J Clin Invest* 2005;113:1-9.
- Sood A, Argueso P, Michaud S, Tisdale A, Gipson IK, Dana R. Detection of chemokine receptor CCR5 on conjunctival epithelial cells in keratoconjunctivitis sicca. *Invest Ophthalmol Vis Sci ARVO Abstract* 2003;44(S):671.
- Yeaman GR, Asin S, Weldon S, Demian DJ, Collins JE, Gonzalez JL, et al. Chemokine receptor expression in the human ectocervix: implications for infection by the human immunodeficiency virus-type I. *Immunology* 2004;113:524-3.
- Debbasch C, Pisella PJ, De Saint Jean M, Rat P, Warnet JM, Baudouin C. Mitochondrial activity and glutathione injury in apoptosis induced by unpreserved and preserved beta-blockers on Chang conjunctival cells. *Invest Ophthalmol Vis Sci* 2001;42:2525-33.