LABORATORY STUDY

Langerhans' cells in vernal keratoconjunctivitis express the costimulatory molecule B7-2 (CD86), but not B7-1 (CD80)

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Abstract

Purpose **Vernal keratoconjunctivitis (VKC) is associated with T-helper 2 (TH2)-like cell response and increased immunoglobulin (Ig) E production. Recent studies have suggested that interactions between costimulatory molecules B7 on antigen-presenting cells and CD28 on T cells are critical for successful antigen presentation and the development of the TH2 immune response. The objective of this study was to examine the expression of costimulatory molecules CD28, B7-1 (CD80) and B7-2 (CD86) in conjunctival biopsies from patients with active VKC and normal controls.**
Methods **Conjunctival biopsy specimens from 15 subjects with active VKC, and 8 control subjects, were studied by immunohistochemical techniques using a panel of monoclonal and polyclonal antibodies directed against CD28, B7-1 and B7-2 molecules. The phenotype of inflammatory cells expressing costimulatory molecules was examined by sequential double immunohistochemistry.**
Results **In the normal conjunctiva, B7-2 was expressed on a few mononuclear cells in the epithelium and substantia propria in 5 of 8 specimens. There was no immunoreactivity for CD28 or B7-1. In VKC specimens, few B7-1$^+$ mononuclear cells were noted in the substantia propria in 7 of 15 specimens. B7-2 was expressed on mononuclear cells in the epithelium and substantia propria in all specimens. Compared with normal controls, VKC specimens showed significantly more mononuclear cells expressing B7-2 (30.5 $\pm$ 14.1 vs 1.88 $\pm$ 2.5; $p < 0.001$). In VKC specimens, the numbers of mononuclear cells expressing B7-2 were significantly higher than the numbers of mononuclear cells expressing B7-1 (30.5 $\pm$ 14.1 vs 2.3 $\pm$ 3.1; $p < 0.001$). CD28 was expressed on mononuclear cells in the epithelium and substantia propria in 14 specimens. Colocalisation studies revealed that the majority of mononuclear cells expressing B7-2 were CD1a$^+$ Langerhans' cells, and that the mononuclear cells expressing CD28 were CD3$^+$ T lymphocytes.**
Conclusions **B7-2 is more widely and prominently expressed by Langerhans' cells compared with B7-1. The interaction of B7-2 with CD28 may mediate the development of the TH2 immune response in VKC. Thus the manipulation of this pathway could be an important target for the development of future therapies in VKC.**

Key words B7, CD28, Conjunctiva, Langerhans' cells, Vernal keratoconjunctivitis

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Vernal keratoconjunctivitis (VKC) is a bilateral, chronic, recurrent ocular allergic disease that primarily affects children and young adults.[1] The main histological features of VKC are the conjunctival infiltration by eosinophils, basophils, mast cells, B lymphocytes organised as small lymphoid follicles, plasma cells, CD4$^+$ T lymphocytes, monocytes/macrophages and dendritic cells.[2–4] Recently, several studies have demonstrated that CD4$^+$ T lymphocytes in VKC express T-helper 2 (TH2)-type cytokines.[5–8] There is overwhelming evidence to suggest that activation of antigen-specific CD4$^+$ TH2 cells in allergic individuals plays a central role in the pathogenesis of the allergic inflammation. TH2 cells are known to produce interleukin (IL)-3, IL-4, IL-5, IL-10, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF), which are involved in B cell switching to immunoglobulin (Ig) E,[9,10] mast cell proliferation[11] and eosinophil activation and recruitment.[12]

T lymphocyte activation requires interaction between the T cell receptor and specific antigen, presented in the form of processed peptides in association with major histocompatibility

complex (MHC) class II molecules. However, for complete T cell activation, a second signal termed costimulation is required. The most widely studied costimulatory molecule is CD28, which is constitutively expressed on the surface of both $CD4^+$ and $CD8^+$ T cells. Ligation of CD28 on T lymphocytes with B7-1 (CD80) or B7-2 (CD86) on the antigen-presenting cells is the best-characterised mode of costimulation. In the absence of CD28-mediated signalling, a state of unresponsiveness or anergy develops.[13–16]

Recent reports have suggested that B7-associated signals regulate the strength of T cell responses as well as the differentiation of naive T cells into TH1 and TH2 cells.[17–19] To date, the costimulatory signals that are required for the induction of a TH2 immune response in VKC have not been examined. The purpose of this study, therefore, was to examine the expression of the costimulatory molecules CD28, B7-1 and B7-2 in conjunctival biopsies from individuals with VKC and normal controls.

Patients and methods

Patients

Fifteen consecutive patients with active VKC (12 males, 3 females; mean age 14.3 ± 2.8 years, range 10–19 years), seen at the outpatient clinic of King Abdulaziz University Hospital, were included in the study. The symptoms mentioned by all the patients were itching, redness, photophobia and tearing. All the patients had the limbal form of the disease characterised by broad gelatinous infiltrates of the limbus. Nasal or temporal limbal conjunctival biopsy specimens were obtained from each patient. None of the patients was on topical or systemic therapy before obtaining the biopsy. In addition, eight limbal conjunctival biopsy specimens were obtained from patients undergoing strabismus surgery without obvious inflammation and served as controls. The controls (5 males, 3 females) were from the same age group.

Immunohistochemical staining

The conjunctival biopsy specimens were immediately snap-frozen in Tissue-Tek optimum cutting temperature (OCT) compound (Miles Laboratories, IN, USA) and maintained at –80 °C until use. For immunohistochemistry, 5 μm serially cut cyrostat sections were dried overnight at room temperature, fixed in absolute acetone for 10 min, then treated with 2% hydrogen peroxide in methanol for 3 min to block endogenous peroxidase activity, and stained with a three step avidin-biotinylated peroxidase-labelled complex procedure. Rehydrated slides were incubated for 30 min with the monoclonal and polyclonal antibodies listed in Table 1, which were diluted to an optimum concentration. The sections were then incubated with the biotinylated secondary antibody, and reacted with the avidin-biotinylated peroxidase complex (Dakopatts, Copenhagen, Denmark). All incubations were carried out for 30 min at room temperature, then washed in three changes of phosphate-buffered saline (PBS) at pH 7.2 for 15 min. The reaction product was visualised by incubation for 10 min in 0.05 M acetate buffer at pH 4.9, containing 0.05% 3-amino-9-ethylcarbazole (Sigma) and 0.01% hydrogen peroxide, resulting in bright red immunoreactive sites. The slides were faintly counterstained with Harris haematoxylin. Finally, the sections were rinsed with distilled water and coverslipped with glycerol. Omission of the primary antibody or its substitution with an irrelevant antibody of the same species was used as a negative control. Positive controls were previously positive-staining tissue.

Table 1. *Monoclonal and polyclonal antibodies used in this study*

Primary antibody	Dilution	Source[a]
Anti-CD28 (H-93) (pc)	1:25	Santa Cruz Biotechnology Inc.
Anti-CD80/B7-1 (H-208) (pc)	1:25	Santa Cruz Biotechnology Inc.
Anti-CD86/B7-2 (BU63) (mc)	1:25	Dako
Anti-CD1a (010) (mc)	1:1	Immunotech
Anti-CD3 (UCHT1) (mc)	1:400	Dako

[a]Location of manufacturers: Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; Dako A/S, Glostrup, Denmark; Immunotech, Marseille, France.
pc, polyclonal antibodies; mc, monoclonal antibodies.

Double immunohistochemistry

To examine the phenotype of inflammatory cells expressing B7-2 and CD28 costimulatory molecules, cryostat sections were studied by sequential double immunohistochemistry. Colocalisation studies were performed in three VKC specimens. After rinsing the slides with PBS, they were incubated for 30 min with the appropriate monoclonal antibody to determine cellular phenotype (CD1a or CD3) and rinsed again with PBS. Subsequently, the sections were incubated for 30 min with Envision +, peroxidase, Mouse (Dako, CA, USA) and washed again with PBS. Then, the reaction product was visualised by incubation for 10 min in 0.05 M acetate buffer at pH 4.9 containing 0.05% 3-amino-9-ethylcarbazole (Sigma) and 0.01% hydrogen peroxide, resulting in red immunoreactive staining. Afterwards the sections were rinsed with PBS, washed with distilled water and incubated for 30 min with B7-2 or CD28 antibodies. After a wash with PBS, the sections were incubated for 30 min with the biotinylated secondary antibody, followed by a monoclonal anti-biotin–alkaline phosphatase conjugate (Sigma). The blue reaction product was developed using Fast Blue BB salt (4-benzoylamino-2,5-diethoxybenzene-diazonium chloride; Sigma-Aldrich, Bornem, Belgium) for 5 min.

Cells were counted in five representative fields. We used an eyepiece calibrated grid with ×40 magnification. With this magnification and calibration we counted the cells present in an area of 0.33 × 0.22 mm. The mean ± SD was calculated for each cell type in VKC and control specimens. The Mann–Whitney *U*-test was used

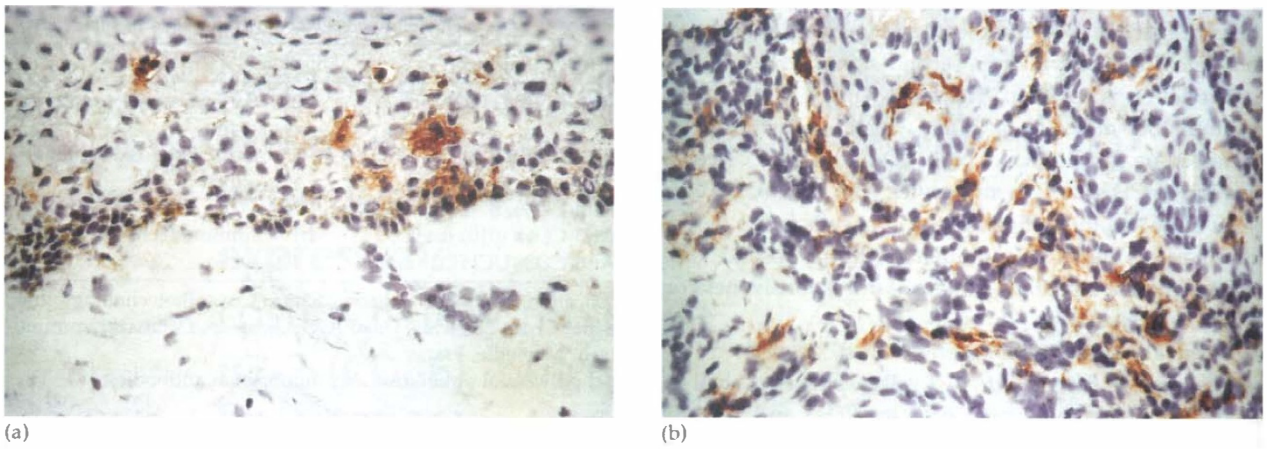

Fig. 1. *Immunohistochemical staining for CD1a showing CD1a$^+$ Langerhans' cells in conjunctiva from a normal control subject (a) and conjunctiva from a patient with vernal keratoconjunctivitis (b) (×500).*

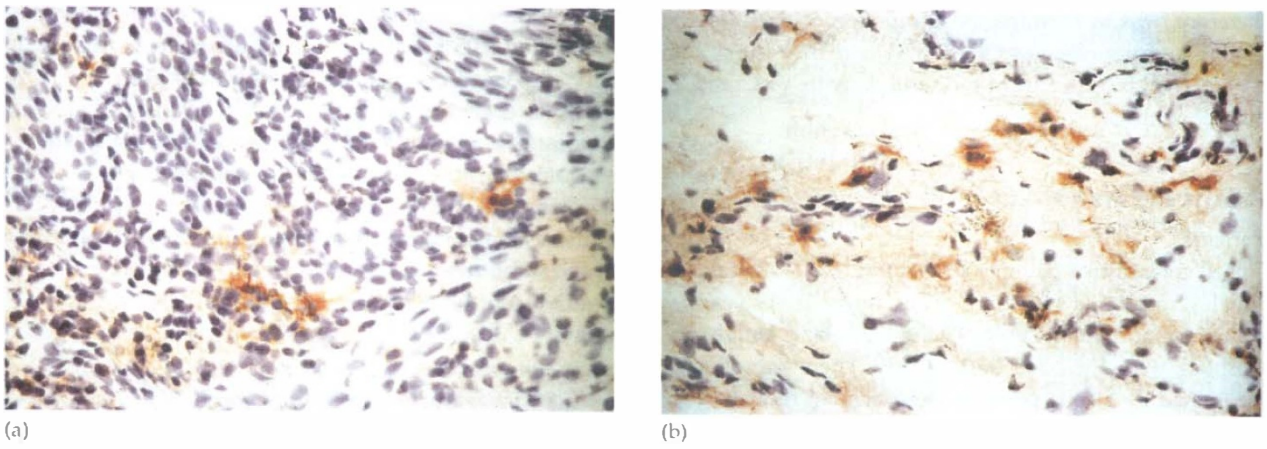

Fig. 2. *Vernal keratoconjunctivitis. Immunohistochemical staining for B7-2 showing B7-2$^+$ cells in the superficial substantia propria (a) and in the deep substantia propria (b). Note that most of the B7-2$^+$ cells had a dendritic morphology (×500).*

to compare the distributions of cell counts for two independent groups. The differences were considered significant if the p value was < 0.05. Program 9R from the BMDP Statistical Package was used to investigate the correlations between the numbers of cells expressing costimulatory molecules CD28, B7-1 and B7-2.

Results

There was no staining in the negative control slides. In normal conjunctiva, few CD1a$^+$ Langerhans' cells were noted in the basal epithelial layers and in the superficial substantia propria in 7 of 8 specimens. The majority of CD1a$^+$ Langerhans' cells were located in the basal epithelial layers (Fig. 1). A few B7-2$^+$ mononuclear cells were noted in the basal epithelium and in the superficial substantia propria in 5 of 8 specimens. The majority of B7-2$^+$ mononuclear cells were found in the superficial substantia propria. There was no immunoreactivity for CD28 or B7-1.

In VKC specimens, CD1a$^+$ Langerhans' cells were noted in the epithelial and stromal inflammatory infiltrate in all specimens. Most of the CD1a$^+$ cells had a dendritic morphology (Fig. 1). B7-2 was expressed on mononuclear cells in the epithelial and stromal infiltrate. Most of the B7-2$^+$ mononuclear cells had a dendritic morphology and were located in the substantia propria (Fig. 2). A few B7-1+ mononuclear cells were noted in the

Table 2. *Number[a] of immune cells in VKC and control specimens*

Patient no.	CD1a	B7-1 (CD80)	B7-2 (CD86)	CD28
VKC				
1	67	8	39	60
2	42	0	15	17
3	104	2	46	11
4	53	0	14	6
5	44	0	18	10
6	66	9	34	52
7	61	0	22	9
8	40	0	26	19
9	70	0	22	7
10	26	0	22	0
11	ND	3	26	48
12	50	4	26	75
13	36	3	38	54
14	42	5	66	18
15	47	0	44	45
Control				
1	8	0	1	0
2	16	0	7	0
3	4	0	0	0
4	0	0	2	0
5	1	0	0	0
6	4	0	4	0
7	8	0	1	0
8	11	0	0	0

VKC, vernal keratoconjunctivitis; ND, not done.
[a]Cells counted in an area of 0.33 × 0.22 mm.

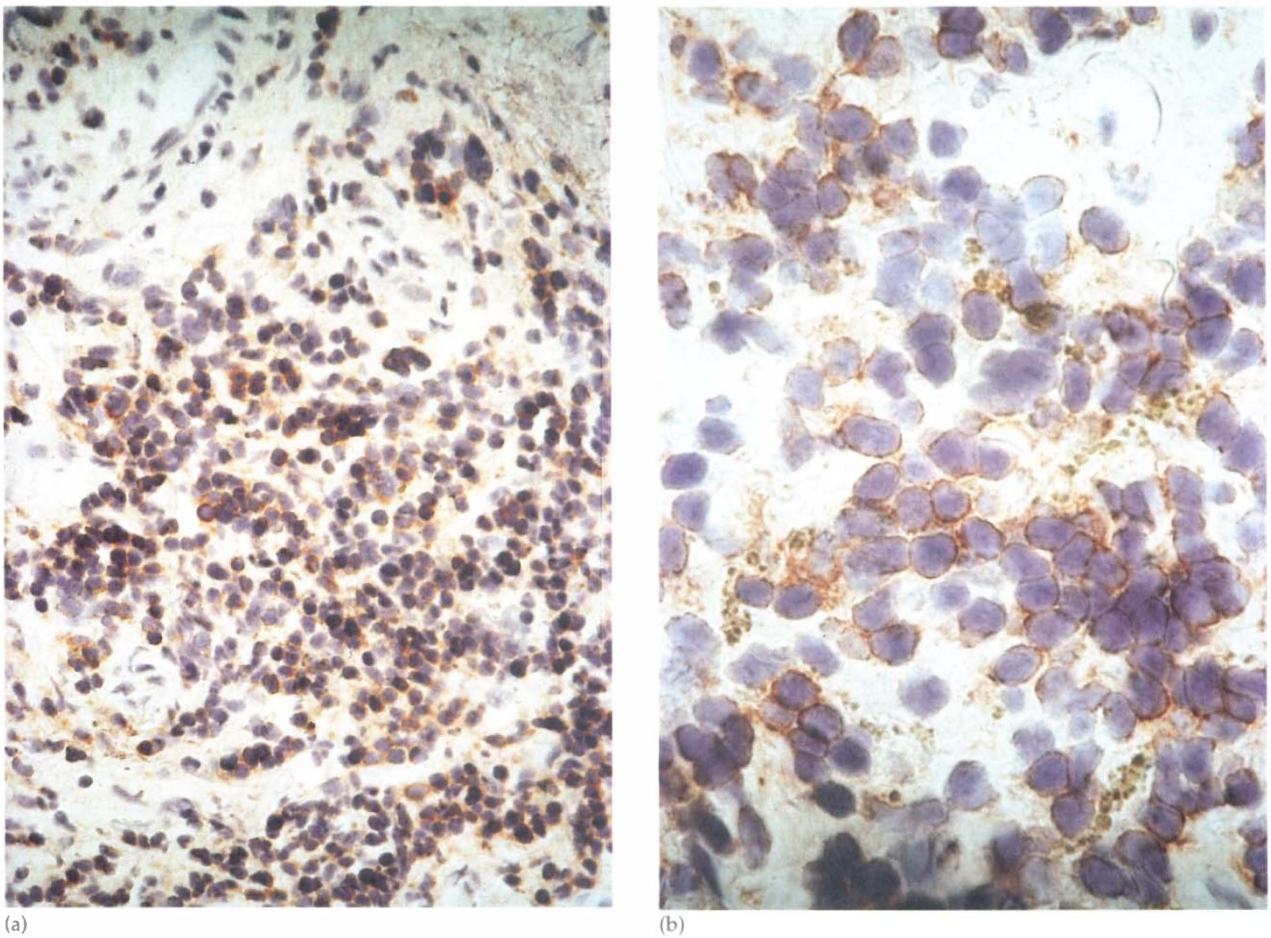

Fig. 3. *Vernal keratoconjunctivitis. Immunohistochemical staining for CD28 showing CD28$^+$ mononuclear cells in the diffuse inflammatory infiltrate in the substantia propria (a) (×500). (b) High magnification of CD28 expression on mononuclear cells (×1200).*

substantia propria in 7 of 15 specimens. CD28 was expressed on epithelial and stromal mononuclear cells in 14 of 15 specimens. The majority of CD28$^+$ mononuclear cells were located in the diffuse inflammatory infiltrate just underneath the epithelium (Fig. 3).

The cell counts are presented in Tables 2 and 3. Compared with controls, VKC specimens showed statistically significantly more CD1a$^+$ Langerhans' cells and B7-2$^+$ cells. In VKC specimens, the numbers of mononuclear cells expressing B7-2 were significantly higher than the numbers of mononuclear cells expressing B7-1 ($p < 0.001$). The number of cells expressing B7-1 and B7-2 were positively correlated with the numbers of cells expressing CD28 ($r = 0.743$ and 0.248, respectively). In addition, there was a positive correlation between the numbers of cells expressing B7-1 and B7-2 ($r = 0.588$).

Table 3. *Number[a] of immune cells in VKC and control specimens (mean ±SD)*

Cell type	Control ($n = 8$)	VKC ($n = 15$)	p value
CD1a	6.5 ± 5.3	57.7 ± 19.5	<0.001
B7-1 (CD80)	0.00	2.3 ± 3.1	–
B7-2(CD86)	1.88 ± 2.5	30.5 ± 14.1	<0.00
CD28	0.00	28.7 ± 24.1	–

Values are mean ± SD.
VKC, vernal keratoconjunctivitis.
[a]Cells counted in an area of 0.33 × 0.22 mm.

Double immunohistochemistry to confirm the phenotype of B7-2$^+$ and CD28$^+$ mononuclear cells revealed that the mononuclear cells expressing B7-2 were CD1a$^+$ Langerhans' cells (Fig. 4), and that the mononuclear cells expressing CD28 were CD3$^+$ T lymphocytes (Fig. 5).

Discussion

The major findings of the present study are that: (a) VKC specimens showed statistically significantly higher counts than control specimens for CD1a$^+$ Langerhans' cells and B7-2$^+$ cells; (b) in contrast to B7-2, B7-1$^+$ cells were minimally detected in VKC conjunctiva and were negative in all controls. The latter finding corresponds with the results of Tesavibul *et al.*,[20] who were unable to detect B7-1 in normal conjunctiva; (c) All controls were negative for CD28$^+$ cells. In contrast, CD28 was expressed on T lymphocytes in VKC conjunctiva; (d) The B7-2$^+$ mononuclear cells were CD1a$^+$Langerhans' cells. These data collectively suggest that the CD28/B7-2 costimulatory pathway may influence the development of conjunctival inflammation in VKC.

Our data show that Langerhans' cells, which are potent antigen presenting cells, expressed the costimulatory molecular B7-2. These results are consistent with the work of Agea *et al.*,[21] who

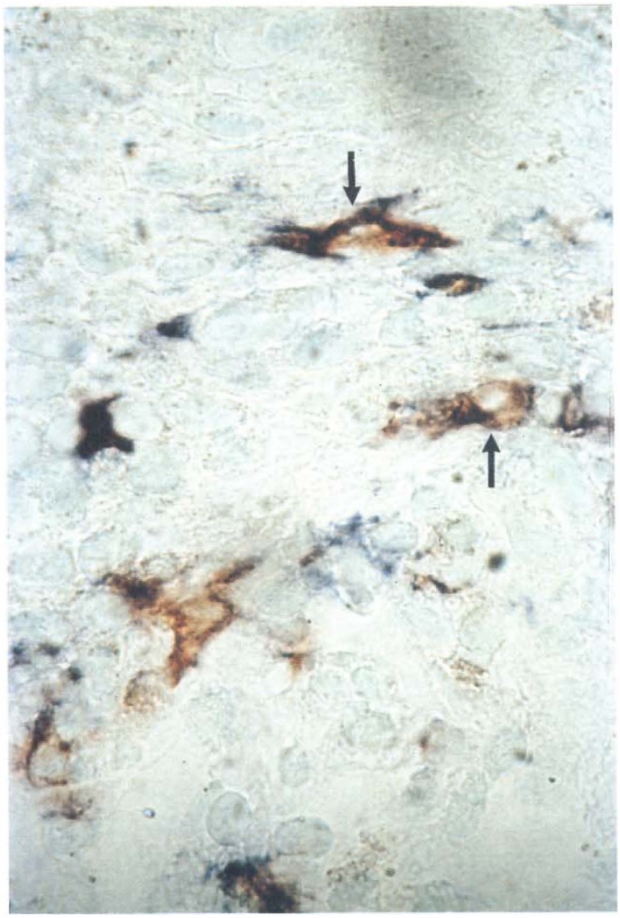

Fig. 4. *Vernal keratoconjunctivitis. Double immunohistochemical staining for B7-2 (blue) and CD1a (red), showing B7-2$^+$ cells coexpressing CD1a marker (arrows) (×1200).*

Fig. 5. *Vernal keratoconjunctivitis. Double immunohistochemical staining for CD28 (blue) and CD3 (red) showing CD28$^+$ cells coexpressing CD3 marker (arrows) (×1200).*

demonstrated that alveolar macrophages, which are of the same lineage as Langerhans' cells, from atopic asthmatics, but not control subjects, overexpressed B7-2, and to lesser extent B7-1, surface molecules. In addition, B cells from atopic patients with asthma,[22,23] dermatitis[24] and rhinitis[25] expressed higher levels of B7-2 than controls and the B7-2 levels were higher than B7-1 levels. In contrast, there was no difference in B7-1 expression among patients and controls. Moreover, a significant increase in the expression of B7-2 but not B7-1 was observed after allergen stimulation.[22,23,25] Similarly, experiments performed in the mouse model of asthma showed that allergen exposure upregulated expression of B7-2 but not B7-1 on B cells from the lung.[26]

Inflammatory cytokines may be involved in regulating B7-2 expression in VKC. Several studies have demonstrated that the TH2-derived cytokines IL-4, IL-13 and GM-CSF induced surface expression of B7-2, but not B7-1, on mononuclear cells.[21,22]

Several studies have demonstrated that the CD28/B7 costimulatory pathway is required for antigen-induced eosinophil recruitment, TH2 cytokine production, and local production of IgE in allergic inflammation. In an experimental model of allergic airway inflammation CD28 knockout mice, unlike wild-type controls, did not produce systemic IgE or eosinophilic airway inflammation after antigen challenge.[27] Furthermore, in murine models of allergic airway inflammation[26,28,29] and allergic rhinitis,[30] blocking the CD28/B7 costimulatory pathway effectively inhibited antigen-induced hyper-responsiveness, reduced serum levels of antigen-specific IgE and tissue eosinophilia, and decreased TH2 cytokines. Blocking CD28/B7 interaction, *in vitro*, inhibited antigen-specific proliferation as well as cytokine mRNA production of peripheral blood T lymphocytes of allergic asthma patients.[31] These experiments suggest that T cell costimulation plays a major role in the development of allergic inflammation.

There is accumulating evidence to suggest that B7-2, rather than B7-1, mediates the development of TH2 immune responses and plays a major role in regulating allergic disease. B7-2 cosignalling preferentially induces naive T cells to develop into TH2 cells, whereas B7-1 induces TH1 differentiation. Kuchroo *et al.*[7] demonstrated that CD28 ligation with B7-1 in mice was required for the generation of a TH1 response, while engagement of B7-2 promoted the development of T cells to a TH2 response. Other studies also support an important role for B7-2 in the signalling of IL-4 production and the development of TH2 cells.[18,19] Bashian *et al.*[32] have recently demonstrated that blocking B7-2 molecules with an anti-B7-2- monoclonal antibody (mAb) but not B7-1 down-regulates antigen-induced proliferation and cytokine gene expression of human peripheral blood mononuclear cells from allergic

individuals. Similarly, Jaffar *et al.*[23] demonstrated that allergen-induced secretion of IL-5 and IL-13 by peripheral blood mononuclear cells from patients with atopic asthma was inhibited by anti-B7-2 mAb but was not affected by mAb to B7-1. In addition, in atopic asthmatic patients, anti-B7-2 but not anti-B7-1 inhibited allergen-induced proliferation and IL-5 production of bronchoalveolar lavage T cells and antigen presenting cell activity of bronchoalveolar lavage-adherent cells to autologous T cells.[31,33] In the murine model of allergic asthma, B7-2 mAb but not B7-1 inhibited the recruitment of eosinophils into the lungs, IgE production, TH2 cytokine production and bronchial hyper-responsiveness.[26,28,29] Moreover, mice lacking B7-2 exhibited a significantly diminished contact hypersensitivity response to epicutaneous antigens while mice lacking B7-1 responded normally.[34]

In conclusion, our data suggest that interaction of B7-2 on Langerhans' cells with CD28 on T lymphocytes mediates the development of the TH2-type immune response and plays a major role in regulating the inflammatory reaction seen in VKC. These observations open up the attractive therapeutic potential that inhibiting B7-2/CD28 interactions in the conjunctiva of VKC patients may lead to the development of novel therapies for the treatment of VKC.

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