

# Characterization of dendritic cell phenotype in allergic conjunctiva: increased expression of FcεRI, the high-affinity receptor for immunoglobulin E

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LABORATORY STUDY

## Abstract

**Purpose** Dendritic cells (DCs) express the high-affinity receptor for IgE (FcεRI) on their surface, which may enhance their ability to capture and internalize antigens for presentation to T-lymphocytes. The aim of this study was to determine if expression of FcεRI<sup>+</sup> DCs is increased in the conjunctivae of vernal keratoconjunctivitis (VKC) patients compared with those of normal controls.

**Methods** Conjunctival biopsies were obtained from non-atopic and VKC patients. Double immunohistochemical staining was carried out using antibodies against FcεRI and the CD1a antigen, a DC marker. The double-positive cells were counted in five representative fields of view for each conjunctival sample.

**Results** FcεRI<sup>+</sup> CD1a<sup>+</sup> cells were present in significantly higher numbers in VKC conjunctivae compared with normal controls (mean cell count of 21.3 in VKC *vs* 5.0 in controls,  $P < 0.005$ ). In normal patients the FcεRI-expressing DCs tended to be confined to the epithelial layer or the superficial substantia propria, but in the VKC samples these FcεRI<sup>+</sup> cells were mainly concentrated in the deeper substantia propria.

**Conclusions** FcεRI<sup>+</sup> DC numbers are elevated in the conjunctivae of VKC patients, a finding consistent with the results of other studies focusing on atopic conditions. Elevated expression of FcεRI on DCs would facilitate antigen presentation and enhance T-cell priming, thereby contributing to ocular symptoms.

Eye (2009) 23, 2099–2104; doi:10.1038/eye.2008.372; published online 19 December 2008

**Keywords:** allergy; dendritic cells; eye/ocular; Fc receptors

## Introduction

Dendritic cells (DCs) are part of the growing family of bone marrow-derived antigen presenting cells (APCs), involved in the initiation and control of the immune response to antigens present at the interface with the environment. DCs are the most potent stimulators of naïve T cells and initiators of primary immune responses,<sup>1</sup> and are responsible for initiating and maintaining the T helper-2 response to encountered allergens.

With the increasing prevalence of allergic conjunctivitis, whether as a disease entity by itself or in association with generalized atopic conditions, there has been renewed interest in the role of IgE and its central role in allergic responses. The interaction of the allergen–IgE complex with the IgE receptors on the surface of DCs, and the subsequent processing of this complex and presentation to T cells, triggers the immune reaction. The consequences of this immune reaction, when disproportionate, cause the debilitating signs and symptoms associated with allergy. The importance of IgE in this response is highlighted by the success of omalizumab (Novartis Pharmaceuticals Ltd), an IgE-specific antibody, in the treatment of asthma and other allergic diseases.

To define the relationship between DCs and IgE responses, investigators have examined IgE

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Received: 20 August 2008  
Accepted in revised form: 19 November 2008  
Published online: 19 December 2008

levels and the phenotype of DCs in atopic conditions. In asthma, total serum IgE levels directly correlate with the severity of asthma symptoms and airway hyperresponsiveness.<sup>2</sup> This IgE binds to its specific receptors (FcεRI and CD23) on cell surfaces, thereby providing a trigger for the release of inflammatory mediators. It is known that Langerhans' cells (LCs), a tissue APC similar in phenotype to DCs, express the high-affinity receptor for IgE (FcεRI), and that this may enhance their ability to capture and internalize antigens for subsequent presentation to T lymphocytes. Using bronchial biopsy specimens from atopic asthmatic individuals and non-atopic, non-asthmatic control individuals, Tunon-de-Lara *et al*<sup>3</sup> showed that (i) the numbers of DCs are significantly higher in the airways of asthmatics compared with non-atopic healthy control individuals, and (ii) the proportion of these DCs expressing the α-subunit of the FcεRI receptor was significantly increased in the asthmatic group. Semper *et al*<sup>4</sup> examined the surface expression of FcεRI on LCs in epidermal sheets from the skin of normal and atopic donors (atopic dermatitis or asthma with allergic rhinitis). They detected surface FcεRI-bound IgE in the skin of individuals with active atopic dermatitis, asthma, or rhinitis, but not in non-atopic individuals or in patients whose atopic disease was in remission.

Vernal keratoconjunctivitis (VKC) is an ocular inflammatory disease of the upper tarsal conjunctival surface that is severe, bilateral, recurrent, and chronic.<sup>5,6</sup> It is a potentially blinding condition if the cornea becomes involved. There are two forms of the disease, limbal or palpebral, depending on which portion of the conjunctiva is predominantly affected.<sup>7</sup> A common immunopathological reaction is elicited in patients with both forms of the disease, either with high or with normal production of IgE.<sup>8</sup> The characteristic inflammatory infiltrate of the conjunctival epithelium and stroma consists of eosinophils, degranulated mast cells, basophils, plasma cells, DCs, lymphocytes, and macrophages.<sup>6</sup> A substantial body of evidence suggests that VKC is a Th-2-mediated process as many of its clinical, histopathological and biochemical features are related to the release of Th-2 cytokines.<sup>7</sup>

Little is known about the phenotype of DCs in the conjunctiva of VKC patients. As LCs in lesional skin of patients with active atopic dermatitis or in the bronchial tree of asthmatic patients show increased cell-surface expression of the FcεRI receptor,<sup>9</sup> we decided to investigate the expression of the FcεRI receptor in the conjunctival DCs of VKC patients and normal controls. Double immunohistochemical staining was carried out using an antibody against the FcεRI receptor and the CD1a antigen, the latter being a widely used marker for tissue DCs of myeloid origin, including LCs. We

hypothesized that DC expression of FcεRI would be elevated in the VKC conjunctivae.

## Methods

### Selection of donors

Donors with ocular allergy showing a clinical history of VKC ( $n = 6$ ) were selected from ophthalmology clinics in Padua, Italy. Non-atopic donors lacking a history of atopy and not exhibiting any clinical signs of allergic eye disease ( $n = 6$ ) were randomly selected from patients undergoing cataract surgery in London. Both selections were made in accordance with the local ethics committee and each patient gave written informed consent after explanation of the nature and the purpose of the study. We certify that all applicable institutional and governmental regulations concerning the ethical use of human tissues were followed during this research.

### Reagents and antibodies

The following antibodies (Abs) directed against human antigens and isotype-matched controls were used for immunohistochemical staining: fluorescein isothiocyanate-conjugated (FITC-) anti-human CD1a and FITC-conjugated mouse IgG1 isotype control (both from Insight Biotechnology, Wembley, UK), mouse anti-human FcεRIα chain antibody 15-1 (a kind gift from Professor Jean-Paul Kinet), mouse IgG1 isotype control (Dakocytomation, Ely, UK), alexa 546-conjugated goat anti-mouse IgG (MolecularProbes<sup>®</sup>, Invitrogen, Paisley, UK), goat anti-human eosinophil major basic protein (EMBP), clone A19 (Autogen Bioclear, Calne, UK), alexa 488-conjugated donkey anti-goat IgG (MolecularProbes, Invitrogen, Paisley, UK), normal goat IgG (Calbiochem, Nottingham, UK), normal goat serum (NGS, Calbiochem, Nottingham, UK), normal mouse serum (NMS) and normal donkey serum (NDS) (both from Stratech Scientific, Newmarket, UK). The sera were diluted in phosphate buffered saline (PBS, 0.01 M, Gibco, Paisley, United Kingdom) with 1% bovine serum albumin (BSA, Sigma-Aldrich, Poole, United Kingdom), and 0.01% sodium azide solution (Fisher Biotech, Tustin, California, USA). Of note, the mouse anti-human FcεRIα chain antibody 15-1 recognizes only properly folded α chain, for example as present at the surface of FcεRI<sup>+</sup> cells.

### Sample collection

Conjunctival samples were obtained from VKC patients and from non-atopic control individuals at the time of cataract surgery. Both were embedded in OCT compound (Sakura Finetek Europe B.V. Zoeterwoude,

Netherlands), frozen immediately in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Sections  $5\text{--}10\ \mu\text{m}$  in thickness were cut with a cryostat, mounted on slides and air-dried for 30 min. For each specimen, one slide was stained with haematoxylin and eosin (H & E). The remaining slides were fixed in acetone for 10 min at  $4^{\circ}\text{C}$ , and then air-dried for 30 min. Serial sections were used for immunohistochemical staining.

#### Double immunohistochemical staining

The slides were washed with PBS, and one slide from each section was mounted to serve as the negative control. The remaining sections were incubated with 10% NGS at room temperature (RT) for 30 min to block non-specific binding of the antibodies. Sections were then stained with either mouse anti-human  $\text{Fc}\epsilon\text{RI}\alpha$  at the titrated optimal dilution or the isotype-matched control at the same concentration and incubated overnight at  $4^{\circ}\text{C}$ . The next day, each section was washed with PBS and stained with the diluted secondary antibody (goat anti-mouse alexa 546) for 1 h at RT. After further washes with PBS, the sections were then incubated with 10% NMS for 30 min at RT. The final incubation was with FITC-conjugated anti-CD1a or FITC-conjugated mouse IgG1 for 90 min at RT. Finally, the slides were washed in PBS, then distilled water, and mounted. Immunofluorescence was examined using a confocal microscope (Carl Zeiss Meditec, Ltd., Welwyn Garden City, UK), and serial Z-sections were collected and combined to give a single projection. Cells were manually counted in five representative  $40\times$  ( $230\ \mu\text{m}\times 230\ \mu\text{m}$ ) fields of view per sample. The mean  $\pm$  standard deviation (SD) was recorded for each sample and the unpaired student's *t*-test was performed to compare the means from the normal and VKC samples. The difference in the means was considered to be significant if the *P*-value was  $\leq 0.05$ . The percentage of DCs expressing  $\text{Fc}\epsilon\text{RI}$  was also calculated for the normal and VKC tissue samples, and the significance of the result was calculated.

#### Eosinophil staining

To confirm the clinical diagnosis of VKC, each of the conjunctival samples was stained for eosinophils, as eosinophilia is a hallmark of VKC. Conjunctival sections from VKC and control patients were rehydrated, and washed with PBS. Each section was blocked using 10% NDS for 30 min at RT before being incubated with anti-EMBP at the optimal dilution or the isotype control (normal goat IgG) at the same concentration for 1 h at RT. After incubation, the samples were washed with PBS and then each specimen was incubated with alexa

488-conjugated donkey anti-goat IgG for a further 1 h at RT. Finally, the samples were washed with PBS followed by a final wash in distilled water before mounting. Pictures were taken using a fluorescence microscope (Olympus UK, Southhall, UK) connected to a digital (CCD) camera.

#### Results

Atopic status and the diagnosis of VKC were made purely on clinical signs and symptoms. IgE levels were determined for patients from both normal and VKC groups (data not shown), but the IgE levels were not consistently high in VKC patients and indeed this is a recognized feature of VKC—IgE levels are not always elevated.

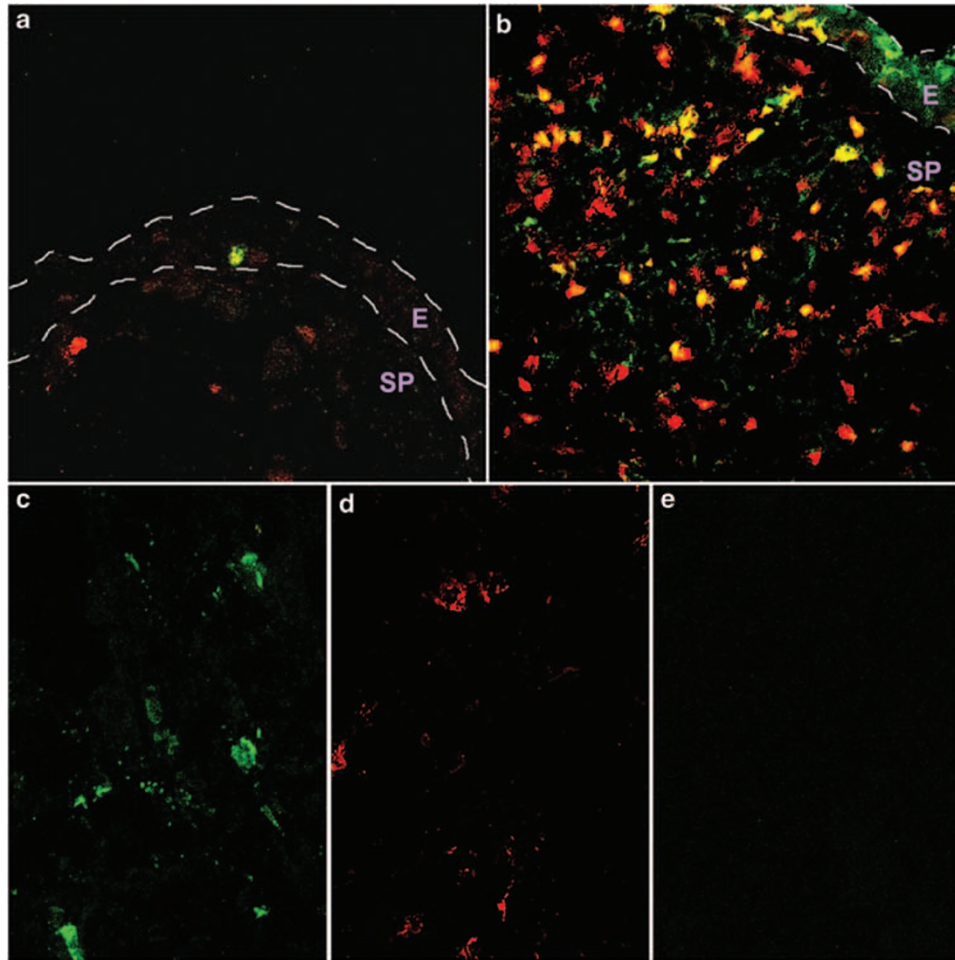
Conjunctival sections were incubated with an antibody to EMBP, a marker for eosinophils. As expected, normal conjunctival samples contained no eosinophils, but eosinophils were present in the substantia propria in all six VKC samples, with the mean eosinophil count being 15. The presence of eosinophilia is a characteristic hallmark of VKC.

Double immunohistochemical staining for CD1a and  $\text{Fc}\epsilon\text{RI}\alpha$  showed that the VKC conjunctivae (Figure 1b) contained more  $\text{CD1a}^+ \text{Fc}\epsilon\text{RI}^+$  cells than did the normal controls (Figure 1a). Furthermore, these cells tended to be confined to the epithelial layer or in the superficial substantia propria. Compared with normal conjunctival control samples, VKC specimens showed a statistically significant ( $P < 0.005$ ) increase in the numbers of  $\text{Fc}\epsilon\text{RI}\alpha^+ \text{CD1a}^+$  DCs.  $\text{CD1a}^+ \text{Fc}\epsilon\text{RI}^-$  cells and  $\text{CD1a}^- \text{Fc}\epsilon\text{RI}^+$  cells were also observed (Figure 1c and d respectively), most likely indicating non- $\text{Fc}\epsilon\text{RI}$ -expressing DCs and non-DC, IgE-recognizing cells, such as mast cells, respectively. No fluorescence was observed in the negative control slides (Figure 1e).

The mean number of DCs was increased in the VKC samples as compared with normal samples (28.2 *vs* 9.9 cells/field of view,  $P = 0.005$ ) (Figure 2). To determine if the increase in  $\text{Fc}\epsilon\text{RI}$  expression was solely due to an increase in DC numbers or also due to an increased proportion of the  $\text{Fc}\epsilon\text{RI}^+$  DCs, the percent of DCs expressing  $\text{Fc}\epsilon\text{RI}$  was determined for each sample. Whereas in normal sections 46.5% of the  $\text{CD1a}^+$  cells expressed  $\text{Fc}\epsilon\text{RI}$ , in the VKC sections 74.4% of  $\text{CD1a}^+$  cells were also  $\text{Fc}\epsilon\text{RI}^+$ , a statistically significant increase ( $P = 0.02$ ) (Figure 3).

#### Discussion

The aim of this study was to determine whether DCs in allergic conjunctiva, namely from VKC patients, expressed higher levels of the high-affinity IgE receptor  $\text{Fc}\epsilon\text{RI}$  compared with normal control subjects. We found

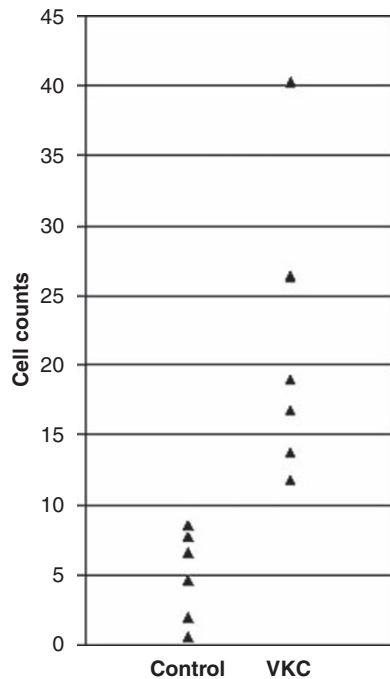


**Figure 1** Immunohistochemical staining for CD1a and FcεRI (yellow). (a) Normal conjunctiva; (b) VKC conjunctiva; and the isotype control slides (c) cell staining for CD1a alone (green), indicative of a tissue DC (d) cell staining positive for FcεRI alone, most probably a mast cell (red), and (e) double-negative staining ( $\times 40$  magnification). Dotted line represents the outline of the epithelial layer. E: epithelium, SP: substantia propria.

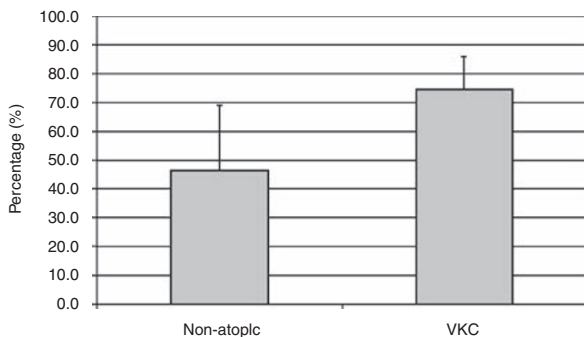
that the number of cells expressing both the DC marker CD1a and FcεRI is significantly increased in the conjunctivae of VKC patients compared with conjunctivae of control subjects. There is a relative paucity in the literature on the distribution and phenotype of DCs in the conjunctival tissues, whether normal or diseased. Yoshida *et al*<sup>10</sup> have found that the number of CD1a<sup>+</sup> cells present in the conjunctival epithelium of patients with atopic dermatitis significantly exceeded that of patients without the disorder, with most of these cells bearing IgE on their surface. Yamagami *et al*<sup>11</sup> characterized bone marrow-derived cells (BMCs) in the substantia propria of the human conjunctiva, and compared them with BMCs in the corneal stroma and epithelium. They identified cells of myeloid lineage (major histocompatibility complex (MHC) class II<sup>+</sup> CD68<sup>+</sup> CD11b<sup>+</sup> CD14<sup>+</sup>) that were different from the monocyte-lineage cells of the corneal

stroma and the DCs of the corneal epithelium. It was suggested that these newly characterized leukocytes, which were not DCs, may play a role in adaptive and immune responses in the human ocular surface. Ohbayashi *et al*<sup>12</sup> characterized the distribution, density, and localization of the different DC phenotypes during the course of allergic inflammation in a murine model of allergic conjunctivitis. The cell surface markers used in the study were purely for phenotype assessment (myeloid *vs* plasmacytoid DCs); surface IgE receptor expression was not examined. It is also important to note that the cell surface markers on murine DCs are slightly different from those on human DCs (eg both subsets of murine DCs express CD11c, but this marker is only found in human DCs of myeloid origin); these results might therefore not correspond to those for human tissues.

To our understanding, this is the first study directly looking at the expression of FcεRI on the surface of



**Figure 2** Chart outlining the difference in mean cell counts observed between the double-positive ( $Fc\epsilon RI\alpha^+ CD1a^+$ ) cells counted in the control and VKC samples.



**Figure 3** Chart outlining the percentage of  $CD1a^+$  cells expressing surface  $Fc\epsilon RI\alpha$  comparing the control and VKC samples. This result is statistically significant ( $P = 0.02$ ).

resident tissue DCs in human allergic eye disease. Our findings are consistent with those of other studies, in which enhanced  $Fc\epsilon RI$  expression on DCs/LCs was observed for atopic dermatitis, allergic rhinitis, and asthma.<sup>3,4</sup> Indeed Ebihara *et al*<sup>13</sup> have previously shown that  $Fc\epsilon RI^+$  cells are significantly increased in the giant papillae of VKC compared with normal conjunctivae. On observing under high magnification only, they concluded that as most of the  $Fc\epsilon RI^+$  cells were of dendritic shape, they were likely to be DCs. No staining was carried out to confirm this observation, however.

Using CD1a staining, we confirm that not only are DC numbers increased in VKC patients, but their surface expression of  $Fc\epsilon RI$  is also increased. As DCs capture and process antigen, and present it through the MHC class II molecules to T cells, increased  $Fc\epsilon RI$  expression by DCs in VKC may enhance capture, internalization, and presentation of allergens. This theory is supported by *in vitro* research showing that monocytes present allergen to T cells up to 1000-fold more efficiently if  $Fc\epsilon RI$  has mediated allergen uptake on these cells.<sup>14</sup>

The mechanism for increased expression of  $Fc\epsilon RI$  on the surface of DCs is unknown. It is unlikely that IgE binding to  $Fc\epsilon RI$  is solely responsible for this phenomenon, as some VKC patients display normal levels of serum IgE.<sup>15</sup> Other factors, such as the microenvironment of the conjunctival mucosal surface, may also contribute to stabilization and enhanced expression of  $Fc\epsilon RI$  on DCs. Transcriptional differences may also play a role in  $Fc\epsilon RI$  upregulation. The amount of  $Fc\epsilon RI\gamma$  chain in DCs correlates with the surface expression of this receptor,<sup>16</sup> and differential  $Fc\epsilon RI\gamma$  chain expression has been shown in atopic *vs* non-atopic donors.<sup>17</sup> If synthesis of the  $\gamma$  chain is upregulated in VKC patients, this might contribute to the increase in  $Fc\epsilon RI$  expression.

Our DC marker, CD1a, is a recognized marker for tissue LCs, which are of the myeloid DC subtype.<sup>18</sup> Use of this marker most likely underestimated the number of DCs in the conjunctival tissue, as CD1a is not expressed by plasmacytoid DCs. To clearly identify plasmacytoid cells, two additional markers would have to be used, namely CD123 and CD303 (BDCA-2). As CD123 is also present on other blood cells including monocytes, and CD303 is downregulated as plasmacytoid DCs mature, DC identification might still have been ambiguous as well as technically difficult given the number of antibodies that would have to be used simultaneously.

In summary, we have shown that the number of DCs expressing  $Fc\epsilon RI\alpha$  chain is increased in the conjunctiva of VKC patients, and that these cells are mainly found in the substantia propria. Upon ligation by IgE, the  $Fc\epsilon RI$  receptor controls the immunoregulatory signals within DCs. As the binding of the allergen-IgE complex to this receptor represents the very first event of the immune reaction, we believe that study of this receptor may provide further insight into the onset and propagation of the allergic cascade. This observed increased expression of the receptor is likely to increase the ability of DCs to capture and subsequently process specific antigens for presentation to  $CD4^+$  T cells, thereby initiating the immune response. Cellular activation by cross-linking of this surface receptor also triggers cytokine expression from DCs and the preferential induction of a T helper cell type 2 (Th2) form of T-cell activation, which is involved

in the allergic response. Therefore, blockade of DC activation through antagonists to this receptor may not only block activation of T cells at the beginning of the immune cascade, but also inhibit the migration of other inflammatory cells into the conjunctiva. Further understanding of the processes involved in the upregulation of FcεRI, as well as the cytokine milieu that promotes FcεRI expression, may offer potential targets for novel therapeutic strategies to combat VKC and other allergic disorders.

### Acknowledgements

We are very grateful to Professor Jean-Paul Kinet for the provision of the anti-FcεRI used in this study.

This research was carried out as part of a PhD degree funded by the Medical Research Council in the United Kingdom. A proportion of the funding has been received from the NIHR Biomedical Research Centre in Ophthalmology at Moorfields Eye Hospital and the UCL Institute of Ophthalmology.

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