1 **CHARACTERIZATION OF THE PHENOTYPE AND FUNCTION OF MONOCYTE-**
2 **DERIVED DENDRITIC CELLS IN ALLERGIC CONJUNCTIVA**

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20 **Key words:** dendritic cells, allergic conjunctivitis, flow cytometry, mixed leukocyte reaction
21 assay.

22 **Word Count:** 2574
23
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ABSTRACT

Background: Dendritic cells (DCs) are the most potent antigen-presenting cells involved in initiating the immune response, presenting antigens to T cells and leading to T cell proliferation. In an immature state, DCs lack accessory signals required for T cell stimulation but are highly specialized to capture antigens. Full DC maturation changes the cell surface phenotype and facilitates stimulation of T cell proliferative responses. To examine the degree of DC maturity associated with vernal keratoconjunctivitis (VKC), we examined the phenotype and antigen-presentation capability of blood derived DCs from VKC patients and from normal controls.

Methods: Flow cytometry was used to identify the cell surface expression of markers of DC maturity (CD83, CD86, MHC class II) and mixed leukocyte reactions to assess DC induction of T cell proliferation.

Results: DCs derived from VKC patients were of more mature phenotype than those from normal controls. However, these VKC DCs had reduced capability for induction of T cell proliferation compared to DCs from controls.

Conclusion: The increased maturity of DCs in VKC patients correlates with the heightened immune responsiveness associated with this disorder. A number of mechanisms may underlie the impaired ability of DCs in atopy to stimulate T cell proliferation. This impairment of DC induction of T cell activation is likely to be one factor which contributes to the modified inflammatory response seen in VKC patients and the recognised susceptibility of these patients to viral infection.

INTRODUCTION

Approximately 20% of the Western world suffers from allergic diseases, such as allergic rhinoconjunctivitis, allergic asthma and the atopic asthma / dermatitis syndrome.[1] DCs are the sentinel cells that ensure that an immune response is appropriate in specificity, intensity, and type to the nature of the threat posed by the antigen bearing entity. DCs are divided into 2 major subtypes in human: the myeloid DCs (including Langerhan's cells (LCs)) and plasmacytoid DCs. DCs are located in most tissues where they capture and process antigens, and where they exist in an 'immature' state: they lack the signals required for T cell stimulation but they are highly specialized to capture antigens. Following encounter with and uptake of an antigen, DCs process this antigen into smaller fragments, couple it to the major histocompatibility complex (MHC) class II molecule and present it on their surface to T cells. Additionally, a second co-stimulatory signal is also required to initiate T cell proliferation.[2] DCs that display the MHC class II molecule and the co-stimulatory molecules (CD83, CD86) on their surface are considered to be mature.

KleinJan *et al.* [3] studied nasal mucosal biopsy specimens taken from patients with perennial allergic rhinitis (AR) and found that the number of DCs in the nasal mucosa was higher and displayed a more mature phenotype in symptomatic patients than in healthy controls. This increase in LCs of the nasal mucosa was also observed during the pollen season in work done by Till *et al.* [4]. Allam *et al.* [5] performed detailed phenotypical comparison of nasal and oral myeloid DC (mDC) from atopic and non-atopic donors. In atopic donors, these mDCs showed elevated expression of the high affinity receptor for IgE (FcεRI) with receptor occupation by IgE but FcεRI was present only in low amounts in these cells in non-atopic donors. Semper *et al.* [6] studied the phenotype of DCs on bronchial biopsy specimens from normal and asthmatic airways and demonstrated the presence of FcεRI-bearing CD1a⁺ LCs in both types of specimens. Total expression of FcεRI on all cells was slightly higher in the airways of asthmatics compared to normal controls, but this failed to reach statistical significance. They did

find—in keeping with other studies [7, 8]—that the numbers of CD1a⁺ DCs was higher in asthmatics when compared to normal controls.

We have previously demonstrated that the number of DCs expressing FcεRI in the substantia propria of the conjunctiva in the atopic disorder vernal keratoconjunctivitis (VKC) patients is increased, indicating an increased ability of DCs to capture and process specific antigens for presentation to CD4⁺ T cells, thereby initiating the immune response.[9] In this study, we sought to further define the phenotypic and functional characteristics of DCs associated with VKC. We examined DCs derived from the peripheral blood of VKC patients and normal patients, testing the hypothesis that DCs in VKC are more mature, and have increased capacity for T cell proliferation.

METHODS

Selection of donors

Donors had a clinical history and signs of VKC, and were selected from patients attending clinic follow-up appointments. The control group donors did not have any history of atopy and did not display any signs of ocular allergy (table 1). A difference did exist between the mean age of the donors in the VKC group compared to the control group but this was unavoidable and was consequent to the difficulties in obtaining blood from children for the control group and in adequate volume quantities to perform the experiments. Local ethics committee approval was obtained for the research and each patient gave written informed consent. All research procedures adhered to the guidelines established in the Declaration of Helsinki.

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99 **Table 1: Background patient information.**

Patients for flow cytometry study							
Control subjects				VKC patients			
Patient number	Age (yr)	Sex	IgE level (kU/l)	Patient number	Age (yr)	Sex	IgE level (kU/l)
1	47	F	18	1	17	F	911
2	27	F	11	2	8	M	87
3	47	F	17	3	12	M	210
4	28	F	21	4	31	M	60
5	46	F	21	5	10	M	150
6	39	F	32	6	14	M	96
7	29	F	15	7	37	F	81
8	49	M	44	8	7	M	120
9	47	F	334	9	11	M	170
10	28	M	257	10	8	M	375
11	23	F	254	11	13	M	62
12	28	M	129	12	12	M	153
13	30	M	203				
Patients for MLR assays							
Control subjects				VKC patients			
Patient number	Age (yr)	Sex	IgE level (kU/l)	Patient number	Age (yr)	Sex	IgE level (kU/l)
1	41	M	11	1	10	M	80
2	21	F	31	2	29	M	16
3	50	M	13	3	13	M	69
4	42	F	18	4	9	M	216
5	49	F	37	5	31	M	10
6	38	F	9	6	14	F	42
7	21	F	212	7	11	F	48
8	25	F	55				

100

101 **Measurement of IgE concentration**

102 Serum (6 ml) was collected for measurement of IgE levels by a normal sandwich
 103 enzyme-linked immunosorbent assay (ELISA; ImmunoCAP 100, Phadia, Uppsala, Sweden).

MoDC isolation and culture

Peripheral blood mononuclear cells were isolated from EDTA-treated blood using Lymphoprep (Sigma-Aldrich, Poole, United Kingdom) centrifugation. Isolation of CD14⁺ monocytes from the resultant buffy coat layer was performed using anti-CD14 mAb conjugated to magnetic MicroBeads and the autoMACS system as described in the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). The derived pellet of purified monocytes was cultured into dendritic cells as per the method described by Sallusto and Lanzavecchia.[10] To generate monocyte-derived DCs (MoDCs), IL-4 (4 µg/ml, R & D systems, Abingdon, United Kingdom) and GM-CSF (6 µg/ml, GlaxoSmithKline Research Institute, Stevenage, United Kingdom) were added to each well of purified monocytes.

Flow cytometry

On day 6 or 7 the MoDC cultures from each well were combined for fluorescent staining. The cells were blocked for 10 minutes with human Fcγ fragments (Miltenyi Biotech) and then incubated on ice with the appropriate fluorescein isothiocyanate (FITC)- or phycoerythrin (PE) - conjugated antibody or with the respective isotype-matched control immunoglobulins.

Monoclonal antibodies directed against CD83 (FITC, clone HB15e), CD11c (PE, clone B-ly6), CD86 (FITC, clone 2331), HLA-DR (FITC, clone Tü39), CD23 (PE, clone M-L233), CD58 (PE, clone 1C3) and CD11c (PE, clone D12) were purchased from Becton Dickinson (Cowley, United Kingdom). The antibody against FcεRI-α (PE, clone AER-37) was purchased from eBioscience (Insight Biotechnology Ltd, Wembley, United Kingdom). FITC- and PE-conjugated isotype control mouse immunoglobulins (Igs; IgG₁, IgG_{2a}, IgG_{2b},) were obtained from Becton Dickinson and eBioscience. Additional FACS staining of T cells was performed using CD3 (FITC, clone HIT3a) (BD Pharmingen) and CD209 (DC-SIGN, PE, clone eB-h209, eBioscience). The αCD28/αCD3 (GlaxoSmithKline, Stevenage, United Kingdom) was used as a positive control.

Events were acquired using the Beckman Coulter flow cytometry machine and analyzed with Expo32 (Beckman Coulter Ltd, High Wycombe, United Kingdom). Cells were acquired and manually gated on the physical properties of the cell by FSC versus SSC. The measurements taken were the mean fluorescence intensity (MFI) and the percentage of positive cells as defined by the proportion expressing higher levels of fluorescence than those of the isotype controls.

T cell isolation and culture

T cells were isolated and purified from the buffy coat following layering of blood over Lymphoprep (Sigma-Aldrich) and centrifugation. The T cells were isolated from the resultant PBMC cell pellet using the pan T cell isolation kit II from Miltenyi Biotech (negative selection) according to the manufacturer's instructions. T cell purity was always greater than 99%.

Mixed leukocyte reaction (MLR) assays

MoDCs were harvested on day 6 and purity of population assessed with flow cytometry for CD11c +/- CD209. The MoDCs were washed and resuspended in T cell medium at a concentration of 1×10^4 DCs/100 μ l T cell medium. In all allogenic MLR assays, T cells from the same donor were used, thereby maintaining consistency between the MLR results obtained from the normal control MoDCs and the VKC-derived MoDCs. Each of the MLRs for each patient was performed in triplicate at five different concentrations of MoDC to T cells (1:10, 1:20, 1:40, 1:80 and 1:160). Graded doses of MoDC were added to T cells in 96-well U-bottom tissue culture plates (200 μ l/well)—the T cell number remained constant whilst serial dilution of MoDCs was performed to obtain the appropriate DC:T cell ratios for the MLR assays. The control wells, consisted of two sets of negative controls (1×10^4 MoDC only in T cell medium and 1×10^5 T cells in T cell medium) and one positive control (1×10^5 T cells only in T cell medium in wells lined with α CD28/ α CD3). The plates were incubated for 5 days at 37°C in 5% CO₂. T cell

proliferation was quantified by incubating the cells with 1 μ Ci of methyl-titrated thymidine deoxyriboside (10 μ l; Amersham Life Sciences, Buckinghamshire, United Kingdom) during the last 16 hours of the 5-day culture. Cells were harvested on a filter and radioactivity measured in a beta-plate liquid scintillation counter.

Statistical analysis

The Mann Whitney U test was used to determine if there was significant up/down regulation of the measured cell surface markers when comparing VKC MoDC to those derived from control subjects and to determine if the proliferation of T cells observed between VKC and control subjects at the different ratios tested was significant. A value of $p \leq 0.05$ was considered to be significant.

RESULTS

Flow cytometric analysis of circulating DC phenotype

MoDCs were isolated from the peripheral blood of 12 VKC patients and 13 normal controls. For each of the control group donors, a yield of $3 - 16.2 \times 10^6$ MoDCs was obtained after culture as compared with the VKC donors where a yield of $0.5 - 13.2 \times 10^6$ MoDCs was obtained. No morphological differences were readily identifiable between the two groups. DCs were identified as staining brightly for CD11c, the widely-used marker for human myeloid DCs. The cells were gated on their FSC and SSC properties with the MoDCs within the gate being $> 95\%$ CD11c⁺ (figure 1, (a)).

MoDCs of the control group individuals displayed constitutive cell surface expression of MHC class II, low expression of CD86, and no expression of CD83, a typical profile for DCs in the immature state. In MoDCs from VKC patients in comparison, there was a statistically significant upregulation of MHC class II, CD83 and CD86 (figure 1 (b) and table 2), an expression profile consistent with a more-mature state of development.

The cell surface adhesion proteins CD58, CD11b, and CD11c were constitutively expressed in the control MoDCs (figure 1 (c) and table 2). Of the two IgE receptors, only CD23 was constitutively expressed on control MoDCs, and little or no expression of FcεRI was detected on these cells. There was statistically insignificant upregulation of surface FcεRI on VKC-derived MoDCs, but significant upregulation of CD11b, CD11c and CD58. CD23 was down-regulated in VKC-derived MoDCs ($p = 0.02$).

Table 2: The mean fluorescent intensity (MFI) recorded for each marker measured during flow cytometric analysis. * Denotes a statistically significant result.

	Control subject MFI			VKC patient MFI			Fold change	p value
	Mean	SD	Range	Mean	SD	Range		
MHC class II	40.97	14.01	20.8-66.9	71.41	29.70	14.4-122	1.74	<0.01 *
CD86	2.42	1.85	0.45-5.68	5.98	3.40	1.71-11.7	2.47	<0.01 *
CD83	0.34	0.09	0.2-0.48	0.68	0.15	0.46-0.98	2.00	<0.001 *
CD58	4.18	2.59	1.97-12	9.28	3.17	4.77-15.6	2.22	<0.001 *
CD11c	7.86	4.29	3.63-18	12.09	6.77	5.07-30.7	1.54	0.05 *
CD11b	76.92	33.56	38.7-152	111.05	48.28	40.4-192	1.44	0.05 *
CD23	6.26	3.78	3.03-15.4	5.55	11.37	0.94-41.5	-1.13	0.02 *
FcεRI	0.52	0.24	0.27-0.92	0.54	0.18	0.23-0.91	1.04	0.77

Functional analysis of circulating DCs in VKC patients

Having determined that VKC patient-derived MoDCs had altered cell surface marker profiles compared to the controls, we then examined these cells for altered function by testing T cell stimulatory capacity. The responder T cells used were from a subject without any history of allergy or medication, and an IgE level of 61 kU/l (table 1).

For each of the control group donors, a yield of $2.4 - 7.5 \times 10^6$ MoDCs was obtained after culture as compared with a yield of $8 - 11 \times 10^5$ MoDCs for the VKC donors. No morphological differences were observed between the MoDCs from the VKC and the control donors. The MoDCs were labelled with anti-CD11c-PE and the T cells with anti-CD3-FITC. The yield of CD11c⁺ cells from both normal and VKC cultured MoDC populations was >90% (figure 2 (a)), whereas the yield of T cells from the T cell donor was consistently >99% pure (figure 2 (b)).

As expected, MoDCs from both the control group and VKC patients stimulated T cell proliferation. The mitogenic effect was statistically significantly greater with MoDCs from control group individuals than with MoDCs from VKC patients (figure 2 (c)) and was observed for all ratios of MoDC to T cells that were tested. Since all T cells used were from the same donor, the observed differences are likely to be explained by differing stimulatory capacity of MoDCs in the two groups. In a trial MLR using MoDCs derived from a non-atopic donor outside of our group of experimental subjects and T cells from the same donor as for the experiments described in this study, adequate proliferative responses were observed (data not shown). This effect on stimulation is therefore not limited to the patients in our control group.

DISCUSSION

Phenotypic alterations in MoDCs derived from VKC patients

The MoDC derived from VKC patients are a more mature phenotype than those from control subjects as indicated in the statistically significant upregulation of MHC class II and the

co-stimulatory molecules CD86 and CD83. This finding is comparable to those of McCarthy *et al.* [11] who found that DCs in atopic asthma patients expressed higher levels of CD80 and CD86. The upregulation of MHC class II and co-stimulatory molecules is likely to increase the antigen presenting capacity of DCs in VKC patients. CD83 expression on the cell surface is a marker of DC maturation.[12] Zhou and Tedder have demonstrated that most CD83⁺ cells express high levels of CD86;[13] the VKC MoDCs in this study displayed upregulation of both molecules. As CD86 is upregulated in respiratory tract DCs and in circulating blood B cells in allergic asthma [11, 14] and on LC's in the conjunctiva of patients with VKC,[15] it is clear that this co-stimulatory molecule may contribute to the T-cell driven pathology of VKC.

Adhesion proteins upregulated on VKC MoDCs included CD58, CD11c and CD11b. These molecules facilitate the interaction of DCs with T cells and influence the ability of DCs to migrate to regional lymph nodes upon maturation.

Expression of FcεRI on MoDCs

DCs can express the high-affinity IgE receptor FcεRI, which, in the presence of specific IgE, facilitates the uptake of allergen, leading to increased activation of allergen-specific T cells. We found that MoDCs from VKC patients and normal subjects displayed similar levels of FcεRI expression. These results are consistent with those of Holloway *et al.*,[16] who found that the total expression of FcεRI on the surface of DCs isolated from the peripheral blood of healthy and asthmatic subjects were not significantly different. Other studies have associated elevated levels of DC FcεRI with atopic conditions.[17, 18] There are at least three possible explanations for the results in our study. First, the mature state of the VKC MoDCs may have led to downregulation of the FcεRI receptor: upon *in vitro* maturation, LCs completely and irreversibly downregulate their transcripts for FcεRI subunits, leading to a rapid loss of intracellular and surface receptor expression.[19] Second, although a direct correlation between serum IgE levels and expression of FcεRI on human basophils from allergic subjects has been shown,[20]

this control of FcεRI expression may not apply to DCs, since FcεRI on these cells exists as a trimeric complex lacking the β subunit, in contrast to the tetrameric form expressed by mast cells and basophils . Finally, DCs in different tissues may have different phenotypes which may be influenced by the microenvironment. Indeed we have found increased FcεRI expression in conjunctiva in allergic conjunctivitis.[9]

Mechanisms underlying reduced T cell proliferation in response to VKC MoDCs

Mature DCs are better able to prime T cells than immature DCs, an important early step in cell-mediated immunopathology. We found the expected potent T cell proliferation in response to MoDCs derived from control subjects, but it was surprising to find that our mature VKC MoDCs were *less* effective at stimulating T cell proliferation. This, however, is not the first report of a reduction in T cell function in atopic disease—the literature in support of this finding is well summarized in the review article by Strannegård and Strannegård.[21] In the setting of allergic disorders, this reduced capacity to cause T cell proliferation might be one reason why those with allergic disease are so prone to viral and fungal infections, in particular herpes simplex virus infection. Upon presentation of viral antigen from virus-infected cells it may be that the ineffective ability of DCs to stimulate T cell proliferation leads to impaired clearance of viral infection in atopic patients.

This is the first study to show a functional difference between the MoDCs derived from VKC patients and normal control subjects who had no known history of allergy. There are several possible explanations for reduced T cell-stimulating activity. The proliferative signal presented by the MoDCs to the T cells might be defective or insufficient. Indeed, the ‘strength of stimulation’ model of T cell activation and differentiation is based on the fact that naïve T cells interacting with DCs receive stimulatory signals of differing strengths, depending on the degree of DC maturation, the level of peptide-MHC and co-stimulatory molecule interaction, and the duration of T cell-DC interaction.[22] According to this model, T cells receiving a signal of

excessive strength undergo activation-induced cell death. The MoDCs from VKC patients, being in a more mature state of differentiation, may deliver a signal of excessive strength to the T cells, thereby causing cell death and a reduced proliferative response. Alternatively, inhibitory signals (DC-derived immunoglobulin receptor 2 (DigR2), cytotoxic T lymphocyte-associated receptor 4 (CTLA-4), programmed death-1 (PD-1), and DC-associated heparan sulfate proteoglycan-dependent integrin (DC-HIL)) presented or secreted by the VKC MoDCs hinder an adequate proliferative response from T cells. Finally, the difference in MoDC stimulatory capacity might be due to the differences in age and blood volumes between the donor groups, unavoidable limitations of this study. The normal control patients were older, on average, than the VKC patients, and the blood volumes withdrawn from the normal patients were higher than for the VKC patients.

In conclusion, we have shown that MoDCs derived from circulating monocytes in VKC individuals display a more mature phenotype than their normal counterparts, as evidenced by enhanced expression of MHC II, CD86, CD83, CD58, CD11c and CD11b. Despite this phenotypic maturity, the VKC MoDCs are less-potent T cell stimulators - and potentially less-effective APCs - than their normal counterparts. The impaired T cell proliferation may contribute to the abnormal inflammatory response, not least to viral or fungal infection, seen in patients with VKC and other allergic disorders.

ACKNOWLEDGEMENT

This work was supported by a grant from the Medical Research Council (grant number G78/7987) and supported in part by the NIHR Biomedical Research Centre in Ophthalmology at Moorfields Eye Hospital and the UCL Institute of Ophthalmology.

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291 Competing interests: None.

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Figure & Table Legends

Figure 1: Flow cytometric analysis of **(a)** MoDCs cultured for analysis of CD11c surface marker. The FACS pattern was generated based on cells that were gated ('A'), and confirms their phenotype as CD11c+. The same methodology was used for characterization of normal MoDCs and VKC MoDCs. **(b)** and **(c)** Staining for surface markers labelled with FITC and PE respectively. Expression of each of these markers was determined by flow cytometry and is represented in the form of histograms. A shift of the curve to the right in comparison with the isotype control indicates surface expression of this marker. In **(b)** MoDCs derived from normal control and VKC patients were labelled with FITC-labelled antibodies against MHC class II, CD83 and CD86. The histograms show significant upregulation of the DC surface markers MHC class II, CD86 and CD83 in VKC subjects compared to normal control subjects; in **(c)** MoDCs derived from normal control and VKC patients were labelled with PE-labelled antibodies against CD11b, CD11c, CD23, CD58 and FcεRI. Significantly higher expression of CD11c, CD11b and CD58 was found on VKC-derived cells with significantly lower expression of the CD23 receptor but not of FcεRI. (* denotes $p < 0.05$.)

Figure 2: Flow cytometric analysis of MoDCs and T cells for MLRs. CD11c labelling **(a)** of cells derived from normal control and VKC patients that were gated ('A') indicated DC phenotype. CD3 labelling **(b)** of isolated lymphocytes that were gated ('A') confirmed T cell phenotype. **(c)** Allogeneic T cell proliferation stimulated by MoDCs derived from normal control and VKC patients. Donor T cells tested were identical for the MLRs involving control MoDCs and VKC MoDCs. T cells were co-cultured with MoDC for 5 days. Triplicate assays were performed at each MoDC:T cell ratio. Results shown indicate mean of triplicate assays for each patient and then mean of all patients at that ratio. Error bars indicate standard deviation. At all cell ratios

382 tested, higher T cell proliferation, as determined by thymidine incorporation, was found in

383 response to incubation with MoDCs from normal control subjects than from VKC patients.

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385 **Table 1:** Background patient information.

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387 **Table 2:** The mean fluorescent intensity (MFI) recorded for each marker measured during flow

388 cytometric analysis. * Denotes a statistically significant result.

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