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The pathogenic role of dendritic cells in non-infectious anterior uveitis

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ABSTRACT

Background: Anterior uveitis (AU) is characterised by infiltration of immune cells into the anterior chamber of the eye. Dendritic cells (DC) are professional antigen presenting cells that initiate and promote inflammation. This study aims to characterise DC in AU and to examine the effects of aqueous humor (AqH) on DC maturation and function.

Methods: The frequency and phenotype of AU and healthy control (HC) circulating DC was examined. AU and HC AqH was immunostained and assessed by flow cytometry. The effect of AU and HC AqH on DC activation and maturation was examined and subsequent effects on $CD4^+$ T cell proliferation assessed.

Results: AU peripheral blood demonstrated decreased circulating myeloid and plasmacytoid DC. Within AU AqH, three populations of $CD45^+$ cells were significantly enriched compared to HC; DCs ($CD11c^+$ $HLA\text{-}DR^+$), neutrophils ($CD15^+$ $CD11c^+$) and T cells ($CD4^+$ and $CD8^+$). A significant increase in IFNγ, IL8 and IL6 was observed in the AU AqH, which was also significantly higher than that of paired serum. AU AqH induced expression of CD40 and CD80 on DC, which resulted in increased T cell proliferation and the production of GM-CSF, IFNγ and TNFα.

Conclusion: DC are enriched at the site of inflammation in AU. Our data demonstrate an increase in inflammatory mediators in the AU inflamed microenvironment. AU AqH can activate DC, leading to subsequent proliferation and activation of effector T cells. Thus, the AU microenvironment contributes to immune cell responses and intraocular inflammation.

1. Introduction

Uveitis refers to inflammation of the uveal tract, the middle vascular layer of the eye. It accounts for up to 10% of blindness in the western world and is defined by anatomical location as anterior, intermediate, posterior or panuveitis (Miserocchi et al., 2013; Jabs et al., 2005). Anterior uveitis (AU) accounts for up to 92% of cases and is characterised by an influx of inflammatory cells into the aqueous humor (AqH) which is appreciable on slit lamp examination (Chang et al., 2005). Histological evidence of animal models of AU have identified the nature of these cells but human studies are rare (Smith et al., 1998).

Dendritic cells (DC) are a heterogeneous population of professional antigen presenting cells (APC) that provide the initial signals determining the magnitude and quality of the T cell response. DC play an important role in defence against pathogens but are also crucial in maintenance of immune tolerance to self-antigens (Manicassamy and Pulendran, 2011). In the immature, resting state they constantly probe their environment. Upon contact with antigen they become activated and develop veiled projections with upregulation of cell surface molecules and production of cytokine. They migrate to secondary lymphoid organs and present their antigen to naïve or memory T cells leading to proliferation and differentiation towards effector T cells. Myeloid DC (mDC) express CD11c and are effective stimulators of T cell activation which is enhanced in the presence of GM-CSF (O'Keeffe et al., 2015). Plasmacytoid DC (pDC) express CD123 and are associated with viral immunity (O'Keeffe et al., 2015). In addition, in times of acute inflammation there is potential recruitment of monocytes from blood to tissues being differentiated to 'inflammatory' DC (Nakano et al., 2009).

Resident DC are found in an immature state in iris tissue (Steptoe et al., 1995). These DC express TLR4 and its associated LPS receptor complex where it is thought they are strategically placed along blood vessels to capture disease causing antigens (Chang et al., 2004). DC

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exhibit plasticity in developing either pro-inflammatory or immune-regulatory characteristics depending on their environment. Evidence exists for the involvement of antigen presentation and DC in the pathogenesis of uveitis. Experimental autoimmune uveitis (EAU) is induced by immunization of animals with retinal antigen (eg. interphotoreceptor retinoid binding protein (IRBP)) which has greatly contributed to our current understanding of genetic and cellular mechanisms in human uveitis. An alternative model of EAU, based on inoculation of antigen-pulsed DC has been developed (Tang et al., 2007). As well as a pro-inflammatory role, DC have also been shown to suppress intraocular inflammation in the EAU model whereby pre-treatment of the animal model with fixed immature DC inhibits uveitogenic CD4 T cell activat (Oh et al., 2011). mDC have been identified in AqH of patients with AU and were characterised by elevated major histocompatibility complex classes I and II (Denniston et al., 2012). The levels of circulating mDC subpopulations have been found to correlate with disease activity in uveitis (Chen et al., 2015a).

We hypothesise that DC contribute to the pathogenesis of AU by migrating from the circulation to the anterior chamber of the eye under chemo-attractive influence whereby their subsequent activation and maturation leads to propagation of the inflammatory responses through effector T cell responses. To test this, we profiled the circulating DC and AqH microenvironment in AU and compared to HCs. We investigated the effect of AU and HC AqH on a DC model and assessed subsequent functional responses on T cells.

2. Methods

2.1. Patient recruitment

Patients with active, non-infections AU over the age of 18 were recruited following the tenets of the Declaration of Helsinki. Whole blood, peripheral blood mononuclear cells (PBMC), serum and aqueous humor (AqH) samples were analysed with samples obtained within 24 h of presentation – all patients were naïve to treatment at time of sampling. AqH was sampled via paracentesis under topical anaesthesia. HC AqH was obtained from patients undergoing cataract surgery for senile cataract after consent. The uveitis cohort comprised 27 patients of mean age 39 years (± 7) of which 11 were HLA-B27 related and 16 idiopathic with the ratio of B27 positive to negative comparable for experiments. Disease was classified as acute first episode (n = 14) or recurrent (n = 13). No patient had a concurrent associated systemic diagnosis at presentation.

2.2. Cell isolation

PBMC were isolated from HC blood using a density gradient separation (Lymphoprep, Axis-Shield poC, Norway). $CD14^+$ monocytes were subsequently purified using CD14 microbeads (Miltenyi Biotec) as per manufacturer's instructions. $CD14^+$ monocytes were differentiated to monocyte derived dendritic cells (moDC) over 7 days in the presence of recombinant GM-CSF (70 ng/ml) and IL4 (50 ng/ml) (Peprotech). MoDC which were greater than 95% positive for the DC marker CD11c were considered pure and used for subsequent experiments.

2.3. Flow cytometry

Circulating DC subsets were identified in whole blood using the Cytofix/Cytoperm™ kit (BD) according to manufacturer's instructions. The following antibodies were used to distinguish mDC and pDC in the circulating blood; Lin 1: FITC (Lineage cocktail 1 contains antibodies against CD3, CD14, CD16, CD19, CD20, CD56), HLA-DR: V450, CD11c: APC/Cy7, CD123: PerCP/Cy5.5. The APC population was identified as being Lin^- $HLA\text{-}DR^+$ with mDC expressing CD11c and pDC expressing CD123. Cell surface DC activation and maturation markers (CD40: PE/Cy7, CD80: PE, CD83: APC, CD86: FITC, CD11c: APC/Cy7) were quantified.

A panel of antibodies was used to characterise infiltrating immune cells in the inflamed AqH, (CD45: FITC, CD14: PE, CD4: PE/CF594, CD64: PE/Cy5.5, CD8: PE/Cy7, HLA-DR: V450, CD15: BV510, CD3: APC, CD11c: APC/Cy7). Maturation and activation markers of stimulated moDC were assessed as per whole blood samples. Intracellular T cell cytokines were assessed using the following antibodies; IL17A: FITC, GM-CSF: PE, TNFα: PerCP/Cy5.5, IFNγ: APC, fixable viability dye eFluor: A780 (eBiosciences) in conjunction with FIX & PERM™ cell fixation and cell permeabilisation kit (Invivogen).

2.4. T cell assay

MoDC were differentiated as described above and stimulated with 50% AqH with supernatants harvested after 48 h. $CD4^+$ T cells isolated from a single healthy donor using CD4 microbeads (Miltenyi Biotec) were labelled with CellTrace™ Violet (CTV) (Invitrogen, 1 μM) and seeded in triplicate at 200,000 cells per well in a 96-well plate. Supernatants from AqH stimulated moDC (diluted 1:4) were added to $CD4^+$ T cells, which were stimulated with anti-CD3 and anti-CD28 (both 1 mg/ml, Immunotools). After 5 days, the T cells were re-suspended in fresh media and transferred to FACS tubes. $CD4^+$ T cell proliferation was measured by CTV dilution. T cells were then re-stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of Brefeldin A (5 μg/ml; all Sigma). Cells were harvested and $CD4^+$ T cells were stained for the intracellular cytokines IL17A, IFNγ, GM-CSF and TNFα. Samples were analysed using the LSRFortessa (BD Biosciences) and Flowjo software (Treestar Inc.).

2.5. Cytokine quantification

Protein concentration of IFNγ, IL10, IL12p70, IL1β, TNFα, IL6 and IL8 were measured by multiplex cytokine panel assay according to the manufacturer's instructions on a V-PLEX custom human cytokine assay kit (Meso Scale Discovery). Electrochemiluminiscence was measured using the MSD Sector Imager 2400. Cytokine standards ranges were as follows: IFNγ: 0.20–938 pg/mL; IL10: 0.03–233 pg/mL; IL12p70: 0.11–315 pg/mL; IL1β: 0.04–375 pg/mL; TNFα: 0.04–248 pg/mL; IL6: 0.06–488 pg/mL; IL8: 0.04–375 pg/mL.

2.6. Statistics

Graphpad Prism Version 6.0d was used for statistical analysis. For non -parametric data Wilcoxon signed rank test for related samples and Mann Whitney U for non-paired samples were used. Student's t-tests were used to analyse parametric data. $P < 0.05$ was deemed statistically significant. Results within text/figure legends are represented as mean (± SD).

3. Results

3.1. AU patients have decreased frequencies of circulating mDC and pDC

To examine differences in circulating DC, flow cytometry was used to phenotypically characterise these cells in the peripheral blood. We identified a significant decrease in the percentage of both mDC and pDC in AU patients compared to age matched HCs ($p < 0.01$ and $p < 0.05$, respectively; Fig. 1). AU patients have a mean of 25.7% (± 9.7) less circulating mDC and 13.3% (± 6.1) less pDC. These circulating DC were present in an immature state evidenced by undetectable levels of CD83 and CD86 and low levels of CD40 and CD80 for both AU and HC (data not shown). These data highlight that AU patients have fewer circulating DC than HC, although they appear to reside in a similar immature maturation state in both HC and disease.