# REGULATION OF DENDRITIC CELL FUNCTION BY THE OCULAR MICROENVIRONMENT

By

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# Abstract

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The ocular microenvironment is immunosuppressive in animal models of antigen presenting cell function. My hypothesis was that in humans the normal ocular microenvironment maintains an immature dendritic cell (DC) phenotype, whereas in intraocular inflammation (uveitis) this regulation fails, permitting full DC maturation leading to the production and recruitment of pathogenic effector T cells to the eye.

Using an *in vitro* model of DC function, I observed that non-inflammatory aqueous humour (AqH) inhibited DC maturation, with reduced MHC and CD86 expression, and reduced capacity to induce proliferation of allogeneic T cells, an effect which was cortisol and TGFβ2 dependent. In contrast, exposure to uveitis AqH generated a distinct DC profile with IFNγ dependent elevation of MHC class I, but reduced MHC class II and CD86 expression and impaired induction of T cell proliferation. Exposure to uveitis AqH from patients on topical glucocorticoid treatment caused additional suppression of CD86.

Characterisation of *ex vivo* myeloid DC from patients with uveitis supported the findings of the in vitro model, with AqH-derived myeloid DC showing elevated MHC, but reduced CD86 expression.

In summary human AqH is shown to be a powerful inhibitor of DC maturation, retaining this regulatory role during uveitis.

*To Anna, and the many like her*

Two weeks ago Anna could see ...

But today, Anna is in trouble. Her own immune system has turned against her. White blood cells known as leucocytes have infiltrated most of the major structures of the eye. There are so many that I cannot see in much more than Anna can see out. At the back of the eye, the critical light-sensitive retina resembles the victim of a microscopic paint-ball competition. Clusters of leucocytes choke the retinal vessels and spill over into the surrounding tissues. The immune system is running riot.

Most of the time our eyes live in their own protected little world. They travel in the "quiet coach" of the human body in which the most dramatic thing that should ever happen is a change in the view. It is a civilised place, in which even the immune system is on low volume. Most of our research underlines the ways that the immune system is kept in check within the eye. Inflammation inside the eye should not happen. But sometimes, as with Anna, the eye's immune system ramps up the volume, releasing the destructive inflammatory process of uveitis.

Most uveitis is unexplained. We are good at describing it, reasonable at classifying it, moderate at treating it and, as yet, terrible at understanding it. In our research we probably get closest to what is going on through taking precious fluid samples from the front of the inflamed eye.

Detailed study has allowed us to start building an accurate picture of the types of white blood cells involved during an attack of uveitis. We only have snapshots, but from these images we are steadily working backwards towards the key events that kick this whole process off. Uveitis should not happen. But it does, and it can blind people.

I want to know why uveitis happens. I want to know because Anna is sitting in front of me in the clinic, and we've been filling in the forms that will officially register her blind. I want to know because she can't go back to her job. I want to know because the best treatments we had weren't good enough. And I want to know so that, next time, I have something more to offer.

*From* 'Blind Ignorance' *by Alastair Denniston*, *MRC Max Perutz Science Writing Competition*

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And finally, to my family. Thank you for your love and encouragement as I explore the 'fearful and wonderful' mysteries of the eye. To my darling wife, Sarah; my two remarkable boys, Arran and Ewan; my ever-encouraging parents, James and Inger; and my amazing brother, Richard...thank you.

'Ut Omnes Videant'

# Abbreviations

| | |
|---|---|
| AC | Anterior Chamber |
| ACAID | Anterior Chamber Associated Immune Deviation |
| ALK | Activin receptor Like Kinase |
| αMSH | α Melanocyte Stimulating Hormone |
| AP-1 | Activator Protein - 1 |
| APC | Antigen Presenting Cell |
| APC-Cy7 | Allophycocyanin-Cyanine 7 |
| AqH | Aqueous Humour |
| BSA | Bovine Serum Albumin |
| BΦ | Basophil |
| CD | Cluster of Differentiation |
| CFSE | Carboxyfluorescein diacetate, succinimidyl ester |
| CGRP | Calcitonin Gene Related Peptide |
| CNS | Central Nervous System |
| $CO_2$ | Carbon dioxide |
| CPM | Counts Per Minute |
| Cy | Cyanine |
| DC | Dendritic Cell |
| $dH_20$ | Distilled Water |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylaenediamine-Tetraacetic Acid |
| ELISA | Enzyme-linked Immunosorbant Assay |
| EΦ | Eosinophil |
| FACS | Fluorescence Activated Cell Sorting |
| FCS | Fetal Calf Serum |
| FITC | Fluorescein Isothiocyanate |
| FS | Forward Scatter |
| GI | Gastrointestinal |
| GM-CSF | Granulocyte/Macrophage-Colony Stimulating Factor |
| GTP | Guanosine Triphosphate |
| HEPES | (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) |
| HI | Heat Inactivated |
| HLA | Human Leukocyte Antigen |
| $H_2O$ | Hydrogen oxide (water) |
| HS | Human Serum |
| ICAM | Intercellular Cell Adhesion Molecule |
| iDC | Immature Dendritic Cell |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IV | Intravenous |
| L | Ligand |
| LFA | Lymphocyte Functional Antigen |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| MACS | Magnetic Assisted Cell Sorting |
| MADCAM | Mucosal Addressin-Cell Adhesion Molecule |
| MCP | Monocyte Chemotactic Protein |
| mDC | Mature Dendritic Cell |
| MHC | Major Histocompatibility Complex |
| MICA | MHC class I-Chain related gene A |
| MIP | Macrophage Inflammatory Protein |
| MLR | Mixed Lymphocyte Reaction |
| Mo | Monocyte |
| MΦ | Macrophage |
| NF-AT | Nuclear Factor of Activated T cells |
| NF-κB | Nuclear Factor Kappa-light-chain-enhancer of activated B cells |
| NK | Natural Killer |
| NS | Not significant |
| PB | Pacific Blue |
| PBMC | Peripheral Blood Mononuclear Cell |
| PCR | Polymerase Chain Reaction |
| PBS | Phosphate Buffered Saline |
| PE | Phycoerythrin |
| $PGE_2$ | Prostaglandin $E_2$ |
| PI | Propidium Iodide |
| PK | Protein Kinase |
| PPD | Purified Protein Derivative |
| R | Receptor |
| RA | Receptor Antagonist |
| RANTES | Regulated upon Activation, Normal T cell Expressed and Secreted |
| rpm | Revolutions Per Minute |
| RPMI | Roswell Park Memorial Institute |
| RNA | Ribonucleic Acid |
| SD | Standard Deviation |
| SEM | Standard Error of the Mean |
| SS | Side scatter |
| STAT | Signal Transducer and Activators of Transcription |
| TCR | T cell receptor |
| TGFβ | Transforming Growth Factor β |
| Th cells | Helper T cells |
| TLR | Toll-Like Receptor |
| TNF | Tumour Necrosis Factor |
| TRAIL | TNF Related Apoptosis Inducing Ligand |
| Tween-20 | Polyoxyethylyenessorbitan Monolautate |
| UV | Ultraviolet |
| VIP | Vasoactive Intestinal peptide |

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# 1 GENERAL INTRODUCTION

## 1.1 The hypothesis and its context

The primary role of the eye is to facilitate vision. Integral to this function is a transparent visual axis permitting light to pass through cornea, aqueous humour, lens and vitreous to reach the retinal photoreceptor layer which performs the remarkable feat of transducing light to nerve impulses.

In order to maintain this clear visual axis, the eye is one of a number of structures that has a relatively protected relationship with the immune system, known as immune privilege (Medawar, 1948; Kaplan and Niederkorn, 2007). Thus by a number of different strategies it achieves the balance of permitting the immune responses required for protective immunity, and at the same time restricting these responses to prevent significant pathological damage which might obstruct vision (Streilein, 2003b; Streilein, 2003a).

However potentially blinding intraocular inflammation (known as uveitis) does still occur. This can be due to intraocular infection, but most uveitis is non-infective and is thought to be an autoimmune (or at least an autoinflammatory) phenomenon. There is clear evidence from both human and animal work that activated T cells are involved in the pathogenesis of uveitis (Muhaya et al., 1999). It is proposed that, as in other organ systems, these T cells have been activated by mature dendritic cells (DC), typically within the draining lymphoid tissue(Guermonprez et al., 2002; Steinman, 1991). The site of DC maturation is not known, but is most likely to occur in the eye with trafficking of mature dendritic cells (mDC) to the draining lymphoid tissue

(Streilein, 2003a; Streilein, 2003b). Alternative and quite possibly coexisting mechanisms include the drainage of soluble antigen (accompanied by inflammatory cytokines and other molecules from the ocular microenvironment) to the lymphoid tissue with processing, maturation and presentation by a resident lymphoid DC or local antigen presentation within the eye by resident ocular DC to incoming CD4+ T cells (Camelo et al., 2006; Camelo et al., 2004b; Dullforce et al., 2004).

**My central hypothesis is that the normal resting ocular microenvironment maintains a relatively immature DC phenotype which generates anergic or regulatory T cells, whereas in intraocular inflammation (uveitis) this regulation breaks down permitting full DC maturation resulting in production and recruitment of pathogenic effector T cells to the eye.**

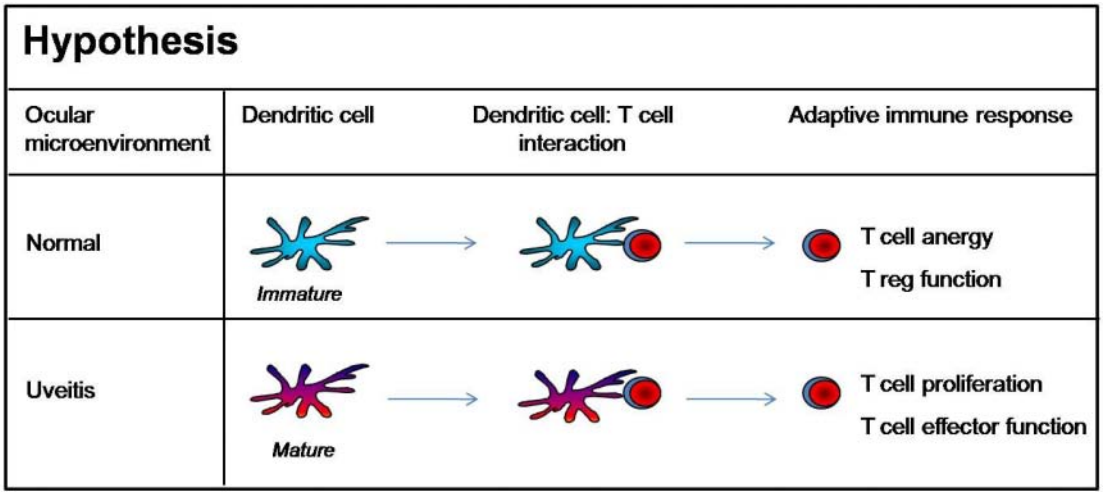


**Figure 1.1 Schematic representation of the hypothesis.**
My hypothesis is that the normal resting ocular microenvironment maintains a relatively immature DC phenotype which generates anergic or regulatory T cells. In contrast in uveitis I propose that this regulation breaks down resulting in full DC maturation and inducing an effective adaptive immune response with T cell proliferation and recruitment to the eye.

In the following introduction, I outline the immunological context of this hypothesis in more detail. I will consider the dendritic cell first within the framework of the normal immune system, second within the unique microenvironment of the normal eye and finally within the dysfunctional microenvironment of the uveitic eye.

## 1.2 The dendritic cell and its role in immunity

The immune system is a remarkable series of inter-related defence mechanisms which protect the host from danger, whether external (invasion by pathogens) or internal (neoplasia). The immune system can be broadly divided into innate and acquired processes. Innate immunity provides an immediate line of defence, and is of sufficient efficacy to deal with the majority of potential pathogens encountered in peripheral tissue. The target requirements of innate immunity are either non-specific or of broad specificity to ensure defence against a wide-range of pathogens. Key components of the innate system include the barrier itself (surface epithelium), specialised leucocytes (macrophages, neutrophils, natural killer cells, mast cells, eosinophils, basophils), and secreted molecules (acute phase response proteins, complement, and interferons).

In contrast, the processes of acquired immunity are highly specific, enabling a highly potent but customized response to almost any pathogen encountered. The acquired immune system also has ‘memory’, whereby any further encounter with the same pathogen will generate a faster and amplified response. The key players of the acquired immune system are lymphocytes, either B cells (which secrete antigen-specific antibody) or T cells (which may have a ‘cytotoxic’ role to kill infected cells or a cytokine-producing/‘helper’ role).

DC are bone-marrow derived leucocytes that provide a crucial link between the innate and adaptive immune responses. They are highly specialised antigen-presenting cells (APC) that respond to the presence of pathogens and inflammation at peripheral tissue sites, migrating to secondary lymphoid organs where they present antigen to either naïve or memory T cells, leading to T cell proliferation and differentiation towards effector cells (Steinman, 1991; Reis e Sousa, 2004a; Reis e Sousa, 2006; Rossi and Young, 2005). It is the DC that provides the initial signals that determine both the magnitude and quality of the T cell response (and via T helper cells, the B cell response). The DC is thus central to the potent and pathogen-specific defence characteristic of the acquired immune response (Reis e Sousa, 2004a; Reis e Sousa, 2006; Rossi and Young, 2005; Moser, 2003).

## 1.3 The paradigm of DC function

Our current understanding of antigen presentation is still largely based on studies on the Langerhans cell, a type of dendritic cell found in the epidermis.

In 1985 Steinman and colleagues showed that freshly isolated Langerhans cells expressed high levels of MHC class II but were poor at stimulating T cell proliferation in a mixed lymphocyte reaction. In contrast after two days of culture they became much more potent at simulating T cell proliferation (Schuler and Steinman, 1985; Inaba et al., 1986), but lost the capacity to process antigen (Romani et al., 1989). Parallel behaviour was observed *in vivo* by Austyn and colleagues, who noted that Langerhans cells from skin transplants and explants emigrate to lymphoid tissues,

undergoing a phenotypic ‘maturation’ in the process and losing the ability to process antigen (Larsen et al., 1990; Reis e Sousa et al., 1993). It was subsequently shown that ‘maturation’ could be induced both *in vitro* by the addition of inflammatory cytokines (notably TNFα), LPS or CD40 ligation (Sallusto and Lanzavecchia, 1994; Winzler et al., 1997), and *in vivo* by the addition of LPS (Roake et al., 1995; De Smedt T. et al., 1996).

In the simplest form of the paradigm, DC resident in the tissues encounter and process antigen in the context of a ‘danger signal’ such as LPS or TNFα, and ‘mature’ in terms of their chemokine receptors (to enable migration to the draining lymph node) and costimulatory molecules (to induce efficient T cell proliferation). In recent years this paradigm has had to evolve. First, the simple linear concept of maturation has struggled to encompass the range of DC behaviour observed *in vitro* and *in vivo*, particularly with regard to DC-induction of tolerance. Second, more recent studies in the skin show that a subcutaneous injection of antigen actually gives rise to three waves of antigen arriving in the draining lymph node: (1) soluble antigen which is then internalized by interdigitating DC (at around 90mins); (2) antigen borne by dermal DC (at around 24h); and (3) antigen borne by Langerhans cells (at around 48h) (Sixt et al., 2005; Kissenpfennig et al., 2005).Third, it has become clear that in addition to the ‘migratory’ DC of the paradigm, there are significant populations of non-migrating lymph node resident DC. Even within the Langerhans cell model, a number of studies have failed to show that the migrated cells directly induce T cell proliferation (Allan et al., 2003; Itano et al., 2003), and it now seems increasingly likely that there is transfer of antigen from migratory DC to lymph node resident DC

(such as the interdigitating DC) with subsequent antigen presentation by the resident DC (Carbone et al., 2004).

## 1.4 Classification of DC

### 1.4.1 Subsets of human DC

There are two major subsets of DC that have been identified from human blood: conventional myeloid DC and plasmacytoid DC. Conventional myeloid DC have a classic dendritic morphology and can be further subdivided on the basis of their surface molecules The most common (0.6% of all PBMCs) are $CD1a^{+}$ BDCA-$1(CD1c)^{+}CD11c^{high}$ BDCA-$3^{low}$ and are designated type-1 MyDC (Dzionek et al., 2000). The lesser population (0.04% PBMC) are $CD1a^{-}$ BDCA-$1(CD1c)^{-}CD11c^{low}$ BDCA-$3^{high}$ and are designated type-2 MyDC (Narbutt et al., 2004). Both populations are effective stimulators of T cells which may be enhanced by coculture with GM-CSF. In contrast plasmacytoid DC (0.4% PBMC) have a rounder morphology, and are poor stimulators of T cells unless stimulated with IL-3 and CD40L. They are functionally distinguished by the ability to produce high levels of IFNα in response to virus. They are BDCA-2+BDCA-4+ but are $CD1a^{-}$BDCA-$1^{-}CD11c^{-}$BDCA-$3^{-}$ (Kadowaki and Liu, 2002).

Although most studies of human DC depend on blood DC, conventional myeloid DC have also been isolated from tonsil, thymus and spleen; plasmacytoid DC have also been isolated from the tonsil and bone marrow.

| | Myeloid type-1 | Myeloid type-2 | Plasmacytoid |
|---|---|---|---|
| CD1a | ++ | - | - |
| CD4 | ++ | ++ | ++ |
| CD11b | + | - | - |
| CD11c | +++ | ++ | - |
| CD40 | + | + | - |
| CD80 | + | + | + |
| CD86 | + | + | - |
| BDCA-1 | ++ | - | - |
| BDCA-2 | - | - | ++ |
| BDCA-3 | - | ++ | - |
| BDCA-4 | - | - | ++ |
| MHCII | +++ | +++ | ++ |

**Table 1.1 Circulating human DC and their expression of key surface molecules**

### 1.4.2 Subsets of murine DC

Studies on murine DC have also shown a clear division between conventional DC and plasmacytoid DC (reviewed (Sato and Fujita, 2007)). In the mouse, conventional DC can be subdivided primarily on the basis of CD4 and CD8 expression into the following subtypes:

CD4-CD8αhighCD205+CD11b-, CD4-CD8α-CD205-CD11b+, CD4+CD8α-CD205-CD11b+, CD4-CD8αlowCD205highCD11b+ and CD4-CD8α-CD205+CD11b+ (Sato and Fujita, 2007). These subtypes show some degree of anatomical localisation: CD4-CD8αhighCD205+CD11b- are found in the T cell areas of spleen and lymph nodes, CD4-CD8α-CD205-CD11b+ and CD4+CD8α-CD205-CD11b+ are found in the marginal zone of spleen and lymph nodes, CD4-CD8αlowCD205highCD11b+ are

found in skin draining lymph nodes and CD4-CD8α-CD205+CD11b+ in all lymph nodes. The DC found in the spleen have been demonstrated to be capable of inducing Th1, Th2 and regulatory T cell responses. The other two subtypes are generally only found in the lymph nodes and are mature forms of DC which have migrated from the peripheral tissues (Sato and Fujita, 2007). Thus CD4-CD8αlow appear to be the mature form of Langerhans cells, whereas CD4-CD8α- represent the mature form of interdigitating tissue DC. Murine plasmacytoid DC are functionally similar to their human equivalents being distinguished by the ability to produce high levels of type-1 IFN in response to virus (Asselin-Paturel et al., 2001).

## 1.5 Anatomical localization

Dendritic cells are found in both blood and tissue. In the blood, dendritic cells and dendritic cell precursor cells comprise around 1% of peripheral blood mononuclear cells. In the non-lymphoid tissues dendritic cells are found in the skin (epidermal Langerhans cells and dermal cells), most organs (including liver, kidney, heart), connective tissues and mucosa (notably gastrointestinal tract). In the lymphoid tissues interdigitating dendritic cells are found in both T cell areas of the paracortex and in the germinal centres(Sato and Fujita, 2007). The germinal centre also contains follicular dendritic cells which are stromal cells with a dendritic morphology which trap whole antigen in the form of immune complexes for extended periods, promoting B cell activation and selection (Batista and Harwood, 2009). They are not “true” dendritic cells and will not be discussed further in this thesis.

## 1.6 DC maturation and cell surface molecules

It is clear that DC may exist in a number of states that are more or less immunogenic or tolerogenic. As outlined earlier, the paradigm of DC maturation is one of a unidirectional pathway with DC moving from an antigen sampling immature state to an antigen presenting mature state. Despite important caveats to this model, the concepts of "immature" and "mature" DC continue to be useful to describe states of DC phenotype and function.

A number of stimuli have been shown to induce maturation of DC, notably inflammatory cytokines and/or ligands for pattern recognition receptors (PRR) (De Smedt T. et al., 1996; Roake et al., 1995; Sallusto and Lanzavecchia, 1994; Winzler et al., 1997), although CD40 ligation has also been shown to be effective *in vitro* (Sallusto and Lanzavecchia, 1994). In response the DC undergoes a number of changes in terms of its surface molecules, cytokine production, migratory capacity and ability to act on other cells which are discussed below (Lutz and Schuler, 2002; Reis e Sousa, 2006)(Figures 1.2 and 1.3).

### 1.6.1 DC surface molecules

The immature DC in peripheral tissue express low levels of MHC class I and II and lack costimulatory molecules such as CD80/86. They do however express pattern-recognition receptors (PRR) such as Toll-like receptors (TLR)(Reis e Sousa, 2004b) which can recognise conserved pathogen associated molecular patterns (PAMPs), molecules such as DEC 205 which can facilitate phagocytosis (Bonifaz et al., 2002), and chemokine receptors such as CCR5 which serve to retain the iDC in the

peripheral tissue (Sallusto and Lanzavecchia, 1999). They also ingest large amounts of antigen indiscriminately by macropinocytosis (Sallusto et al., 1995).

Maturation signals (cytokine or TLR ligand) induce the upregulation of key molecules on the DC. These molecules are involved in presenting antigen to T cells as part of "signal 1" via MHC class I and II (Janeway, Jr., 1989); in contributing to T cell activation as part of "signal 2" via the costimulatory molecules CD80/CD86 (Greenwald et al., 2005), 4-1BBL(Bertram et al., 2002), ICOSL (Greenwald et al., 2005) and OX40L (Chen et al., 1999) and adhesion molecules (eg ICAM-2, LFA-1, CD58); or in directing the migration of the DC to the draining lymph node (CCR7) (Sallusto and Lanzavecchia, 1999; Sallusto et al., 1998). Selected molecules are considered further in table 1.2 (reviewed (Jeras et al., 2005b)).

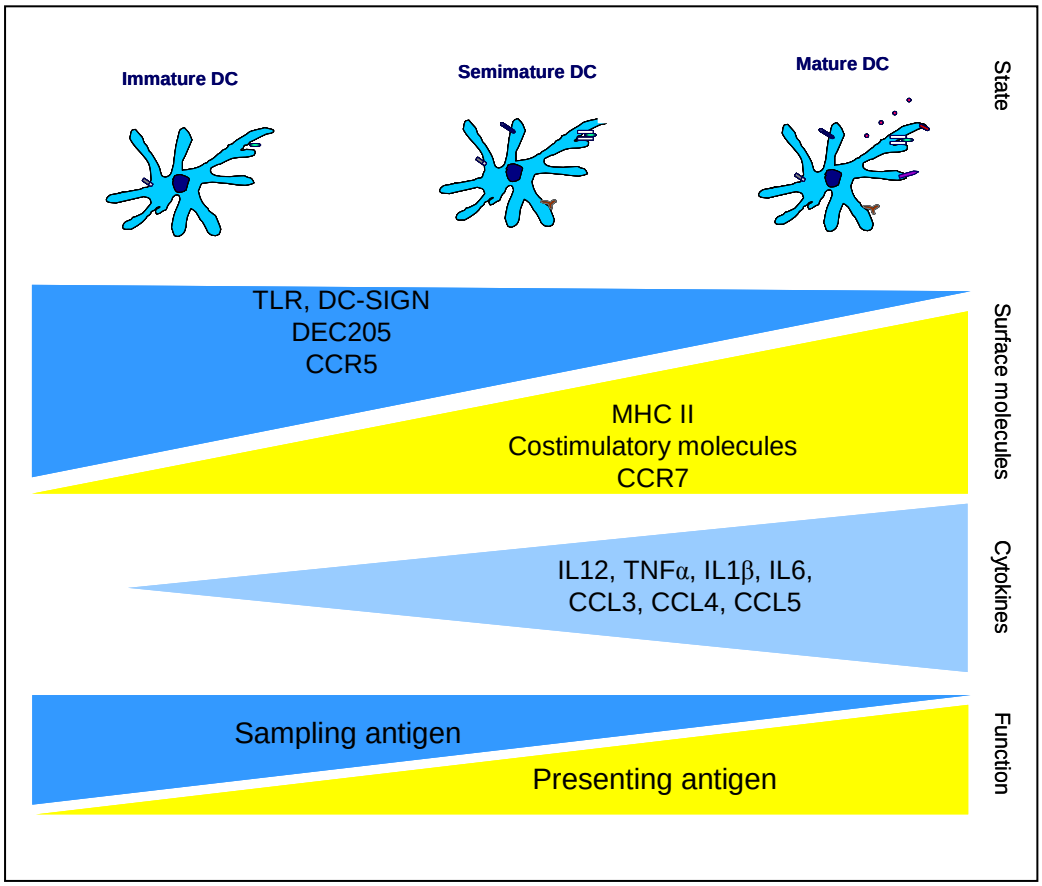


**Figure 1.2 Outline of dendritic cell development**
Outline includes key surface molecules, cytokine production and function.

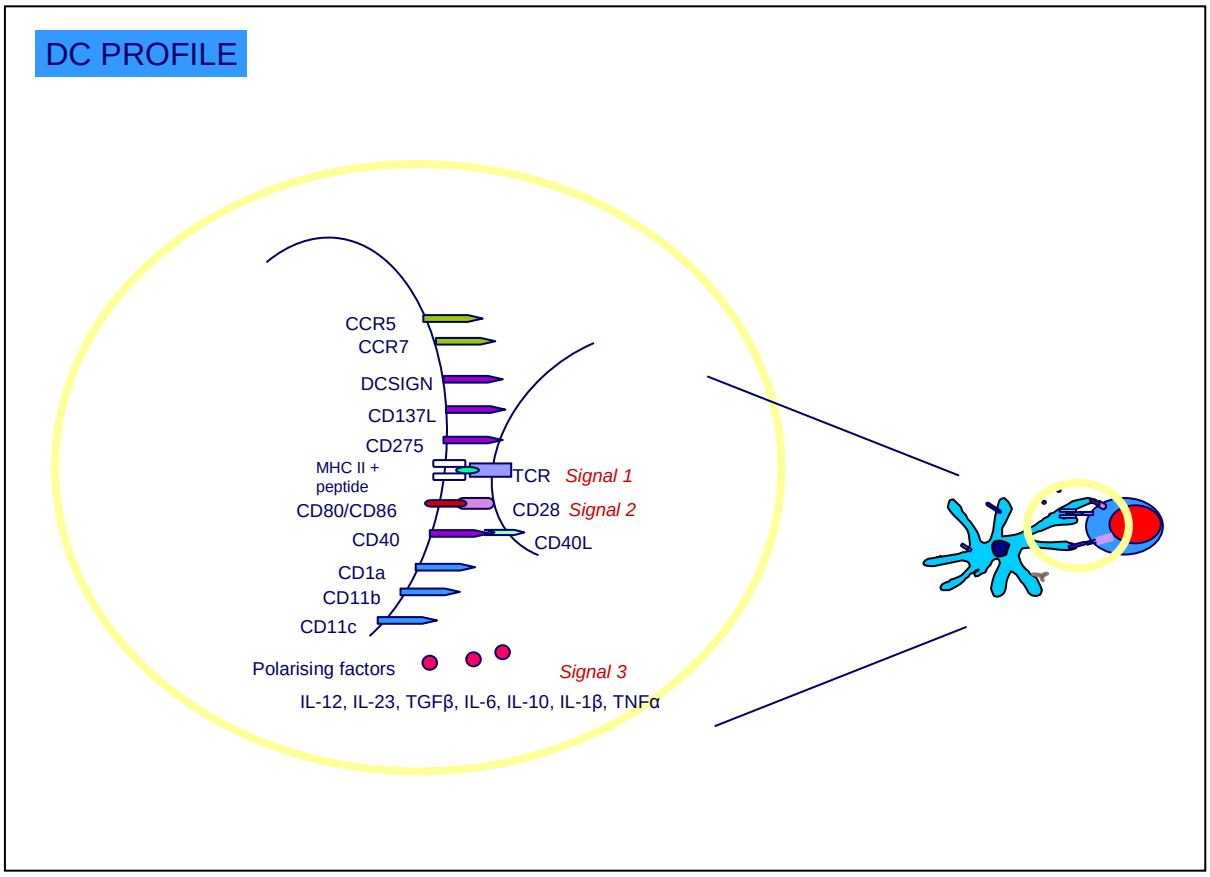


**Figure 1.3 Summary diagram of significant molecules related to DC function which are considered further in this project.**
These include molecules involved in the interaction between DC and T cells, chemokine receptors and soluble factors.

| | Mo | Mϕ | iDC | mDC | Function |
|---|---|---|---|---|---|
| CD1a | +/- | - | + | + | Presentation of non-peptide Ag to CD1 restricted T cells |
| CD1b | +/- | | + | + | Presentation of non-peptide Ag to CD1 restricted T cells |
| CD11b | + | +/++ | ++ | ++ | Assoc with CD18integrin β2; adhesion via ICAM1,2,3 |
| CD11c | + | ++ | + | ++ | Assoc with CD18integrin β2; adhesion and CTL |
| CD14 | ++ | ++ | +/- | +/- | LPS/LPS Binding Protein receptor |
| CD80 | - | + | - | ++ | Costimulation via CD28 |
| CD83 | - | | - | ++ | Unknown but marker of mature DC |
| CD86 | + | + | + | ++ | Costimulation via CD28 |
| CDw137L (4-1BBL) | - | + | +/- | + | Costimulation; 4-1BB on activated T/B |
| CD209 (DC-SIGN) | - | - | + | + | Capture of Ag |
| CD275 (ICOSL) | + | + | +/- | + | Costimulation; ICOS on T cells (especially antigen experienced T cells vs CD28 ) |
| PD-L1 | - | -/+ | + | ++ | Upregulated by IFNγ; regulates T-cells via PD-1 |
| PD-L2 | - | -/+ | - | + | Upregulated by IL-4; regulates T-cells via PD-1 |
| B7-H3 | - | - | + | + | Upregulated by IFNγ; regulates T-cells via unknown receptor |
| B7-H4 | - | - | - | + | Regulates T cells via unknown receptor |

**Table 1.2 Selected surface molecules relevant to the function of monocytes, macrophages and dendritic cells.**
Abbreviations: monocytes (Mo), macrophages (Mϕ), immature DC (iDC) and mature DC (mDC)**.**

### 1.6.2 Pattern Recognition Receptors

Pattern recognition receptors (PRR) are cell-surface molecules which recognise conserved pathogen-associated molecular patterns (PAMP). Toll like receptors are the archetypal PRR, recognising a range of PAMPs from viruses, bacteria, protozoa and fungi, and widely expressed by cells of the innate immune system. Of particular importance to monocyte derived DC are TLR3, TLR4 and TLR8 although they do also express TLR1, TLR2, TLR5, TLR6, and possibly TLR7 and TLR 10 (Mazzoni and Segal, 2004; Reis e Sousa, 2004b). Unlike monocyte-derived and myeloid DC, plasmacytoid DC do not express TLR4 (ligand LPS), but do express TLR9 (ligand CpG oligonucleotide), activation of which generates large amounts of IFNα (Krug et al., 2001).

DC-SIGN is a C-type lectin found on DC which can also act as a pattern recognition receptor. It has been shown to recognise human herpes virus 8 and Mycobacterium tuberculosis. DC-SIGN expression is IL-4 dependent and inhibited by IFNα, IFNγ and TGFβ(Granelli-Piperno et al., 2005; Relloso et al., 2002). Other C-type lectins which can act as PRR are dectin-1 (which can signal in response to β-glucan) and BDCA-2 (exact role still uncertain)(Reis e Sousa, 2004a).

### 1.6.3 Molecules involved in 'signal 1'

#### 1.6.3.1 TCR/CD3 complex and CD4/8

The T cell receptor (TCR) is a transmembrane disulphide-linked heterodimer containing immunoglobulin-like domains. The TCR recognises antigen in the form of

processed peptides bound to MHC molecules on the surface of the antigen presenting cell. Endogenously produced peptides (whether host or foreign from intracellular infection) are presented on class I MHC. Exogenous peptides are taken up by phagocytosis or macropinocytosis (Sallusto et al., 1995), and are processed and presented on either class II MHC, the usual pathway for exogenous peptides, or class I MHC; class I MHC presentation in this context is known as 'cross presentation' and is important in enabling DC to induce naive CD8+ T cells to respond to exogenous antigen eg viral infections of parenchymal cells (Rock and Shen, 2005).

TCR are antigen specific. When the TCR recognises their specific MHC-peptide complex on the APC, the TCR signals via the CD3 complex of tyrosine kinases (known as 'signal 1')(Janeway, Jr., 1989). T cells also express the coreceptor molecules CD4 or CD8 (or rarely both) which bind at different sites on invariant parts of the MHC molecule; CD4 binds to class II MHC and CD8 to class I MHC. Binding of CD4 or CD8 leads to the activation of tyrosine kinases associated with their cytoplasmic domains that, via phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 molecule, lower the threshold for activation of the TCR CD3 complex by about 100-fold.

### 1.6.4 Molecules involved in 'signal 2'

#### 1.6.4.1 CD40L and CD40

The engagement of the TCR with the MHC-peptide complex ('signal 1') leads to upregulation of CD40L on the T cell which interacts with CD40 on the DC. This interaction, known as licensing (Fujii et al., 2004), increases the capacity of the DC to

activate the T cell by increasing formation of MHC–peptide complexes (‘signal 1’), by increasing expression of costimulatory molecules such as CD80/86 (‘signal 2’) and by the production of cytokines including IL-12 (‘signal 3’). CD40 also influences DC survival, migration, and avoidance of suppression (Fujii et al., 2004).

#### 1.6.4.2 CD28 and CD80/86

TCR engagement with the MHC-peptide complex is not sufficient on its own to induce an effector T cell response, and indeed may lead to a refractory state known as anergy unless accompanied by an appropriate costimulatory signal (Schwartz, 2003). The most important costimulatory signal is the CD28 interaction with CD80/86 which promotes the activation of T cell responses, with a predominant role during initial T cell activation. CD80 is not expressed at significant levels on immature DC but is upregulated in response to maturation stimuli. CD86 is already present at low levels on immature DC and is also upregulated on mature DC(Jeras et al., 2005b). Signalling via CD28 upregulates T cell production of IL-2 both by increasing transcription of the gene and by stabilising IL-2 mRNA leading to autocrine stimulation and T-cell expansion(Lindstein et al., 1989). Signalling through CD28 can also upregulate distinct effector T cell cytokine responses characteristic of the distinct T cell subtypes, Th1 and Th2 (discussed later)(Fujii et al., 2004; Greenwald et al., 2005)

#### 1.6.4.3 Inducible costimulator (ICOS; CD278) and ICOSL (CD275)

ICOS interaction with ICOSL synergizes with the CD28 interaction with CD80/86 to promote T cell activation but predominantly affects antigen-experienced (rather than naïve) T cells. ICOS does not upregulate IL-2 production (so does not lead to T cell

proliferation) but can upregulate Th1 and Th2 cytokines and via IL-10 production may have a role in generating regulatory T cells. ICOS also provides important T cell help to B cells for their differentiation and effector functions (Greenwald et al., 2005).

#### 1.6.4.4 4-1BB (CDw137) and 4-1BBL

4-1BB is a costimulatory member of the TNFR family, expressed on activated CD4 and CD8 T cells(Vinay et al., 2006). Its ligand, 4-1BBL, is expressed on activated APC, including dendritic cells. 4-1BB is upregulated on the T cell in response to TCR stimulation, but after CD28-mediated costimulation (Bertram et al., 2002). It is thought to augment T cell proliferation, cytokine production, and prevent activation-induced cell death. Although it can induce IL-2 production in resting T cells, it is much less effective than CD28 (Wen et al., 2002).

#### 1.6.4.5 Inhibitory molecules

CTLA-4 and PD-1 are CD28 family members which provide inhibitory signals that can attenuate T cell responses. Although CTLA4 predominates they have synergistic roles (Greenwald et al., 2005). Levels of the ligand for PD-1 (PD-1L) increase in primary culture murine RPE cells on exposure to IFNγ, and this is associated with inhibition of bystander T cell responses although this appeared to be Th1 (as opposed to Th17) selective (Sugita et al., 2009).

### 1.6.5 Chemokine receptors

Immature DC are usually resident in the peripheral tissue and so are appropriately placed to deal with pathogen entry. However they, like monocytes, may be recruited

from blood to sites of inflammation under the influence of the chemokine receptors CCR1, CCR2, CCR5 and CXCR1 responding to the chemokines CCL2, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CXCL8 (IL-8) (Sallusto et al., 1999).

During maturation these chemoreceptors are downregulated or desensitised, whereas CCR7 and CXCR4 are upregulated. This leads to DC responsiveness to chemokines, CCL19 and CCL21 (for CCR7), and CCL12 (for CXCR4), which are constitutively expressed in the lymph nodes and T cell areas of the spleen. DC (from the afferent lymphatic vessels) and CCR7/CXCR4 expressing T cells (from the high endothelial venules) are thus directed to the appropriate compartments of the lymphoid tissue to facilitate efficient antigen presentation (Scandella et al., 2002).

### 1.6.6 Cytokines and chemokines produced by DC

In addition to cell-surface signals between DC and T cell, mature DC produce secreted molecules (cytokines and chemokines) with wide-ranging effects on the immune system. DC secretion of IL-1β (Gardella et al., 2001), TNFα (Telusma et al., 2006) and IL-6 (Dodge et al., 2003) have broad pro-inflammatory functions including fever and endothelial activation. Most fundamentally, DC effectively launch the adaptive immune system by the activation of CD4+ and CD8+ T cells and the direction of their differentiation (discussed further below). Additionally secretion by mature DC of the chemokines CCL3, CCL4 and CCL5 recruit further monocytes and iDC (Sallusto et al., 1999; Scandella et al., 2002). The cytokines and chemokines considered further in this project are listed in tables 1.3 and 1.4.

| Cytokine | Producer | Receptor | Target | Action |
|---|---|---|---|---|
| IL-1β | MΦ, epithelial cells | CD121a, CD121b | T cell, MΦ, | Activation of T cells and MΦ; Fever |
| IL-1RA | Mo, MΦ, N Φ, Hepatocytes | CD121a | | Competitive antagonist of IL-1 |
| IL-2 | T cell | IL-2Rαβγ | T cell | Proliferation |
| IL-4 | T cells, Mast cells | CD124 | B cell<br>T cell | B activation, IgE switch; T → Th2 |
| IL-6 | T cells, MΦ, DC, Endothelial cells | CD126/CD130 | T cell<br>B cell | T cell growth + differentiation<br>B cell growth + differentiation<br>Fever |
| IL-8 (CXCL8) | MΦ | CXCR1,2 | NΦ, BΦ, T cell | Chemotaxis |
| IL-10 | T cells (TH2), MΦ, | | T cells, MΦ, DC, B cells | TH1 inhibition<br>Inhibits MΦ activation<br>Inhibits DC response to chemokines<br>Increase MHCII on B cells |
| IL-12 | MΦ, DC | IL-12R | NK<br>CD4+ T cell | NK activation<br>T → Th1 |
| IL-13 | T cells | IL-13R | B cell<br>T cell<br>MΦ | B growth and differentiation<br>Inhibits MΦ inflammatory cytokine production<br>Inhibits Th1 |
| IL-15 | Many | IL-15R | T cell<br>NK | T Proliferation and CD8 memory T cell survival<br>NK Proliferation |
| IL-17A,F | TH17 | IL-17R | Fibroblasts, endothelium, MΦ | Induce pro-inflammatory cytokine production by other cells |
| TNFα | MΦ, NK, T cells | TNFR-I, and –II | MΦ, endothelial cells | Endothelial activation |
| GM-CSF | MΦ, T cells | CD116 | Mo | Mo → DC, MΦ, |

**Table 1.3 Selected cytokines relevant to the function of DC and T cells.**
Abbreviations: macrophages (Mϕ), monocytes (Mo), neutrophils (Nϕ), dendritic cells (DC).

| Chemokine | Producer | Receptor | Target | Action |
|---|---|---|---|---|
| CCL2 (MCP-1) | Mo, MΦ, Fibroblasts, Keratinocytes | CCR2 | Mo, BΦ, T, | Chemotaxis |
| CCL3 (MIP-1α) | Mo, T cells Fibroblasts, Mast cells | CCR1,5 | Mo, MΦ, iDC, NK, BΦ, T (Th1 >Th2), | Chemotaxis |
| CCL4 (MIP-1β) | Mo, MΦ, Neutrophils, endothelium | CCR5 | Mo, MΦ, iDC, NK, BΦ, T (Th1 >Th2), | Chemotaxis |
| CCL5 (RANTES) | T cells, endothelium, platelets | CCR1,3,5 | Mo, MΦ, DC, NK, BΦ, EΦ, T (memory > other; Th1 >Th2), | Chemotaxis |
| CXCL8 (IL-8) | Mo, MΦ, fibroblasts, endothelium, keratinocytes | CXCR1,2 | NΦ, BΦ, T cell | Chemotaxis |
| CXCL9 (Mig) | Mo, DC, fibroblasts | CXCR3 | Activated T (Th1 > Th2) | Chemotaxis |
| CXCL10 (IP-10) | Keratinocytes, Monocytes, T cells, fibroblasts, endothelium | CXCR3 | Activated T (Th1 > Th2) | Chemotaxis |

**Table 1.4. Selected chemokines relevant to DC and T cells**.
Abbreviations: macrophages (Mϕ), monocytes (Mo), neutrophils (Nϕ), basophils (Bϕ), dendritic cells (DC), eosinophils (Eϕ), natural killer cells (NK).

## 1.7 DC effects on T cell phenotype

The DC interaction with the naive CD4+ T cell is central to most adaptive immune responses (Figure 1.4). The phenotype of the DC in terms of the surface molecules expressed and cytokines produced are the most important influence on the differentiation of the CD4+ T cell into Th1, Th2, Th17, and Treg T cell subtypes. Thus IL-12 and IL-18 production by mature DC, and DC stimulation of NK cells to produce IFNγ biases T cell differentiation to Th1 (Trinchieri, 2003). Similarly IL-6

and IL-23 production by DC may bias T cell differentiation down a Th17 pathway (Boniface et al., 2008). DC regulation of Th2 responses is less clear. It has been suggested that the Th2 may be the default pathway with predominantly negative regulation by DC via IL-12 inhibition of GATA-3 expression. These mature DC responses contrast with immature DC antigen presentation to T cells where there is minimal proliferative response and the resulting T cells are frequently anergic (as reviewed earlier) , or may develop regulatory function (discussed below). DC therefore have the ability to control the balance between tolerance, immunity and autoimmunity (Moser, 2003; Lutz and Schuler, 2002; Moser, 2003; Reis e Sousa, 2006).

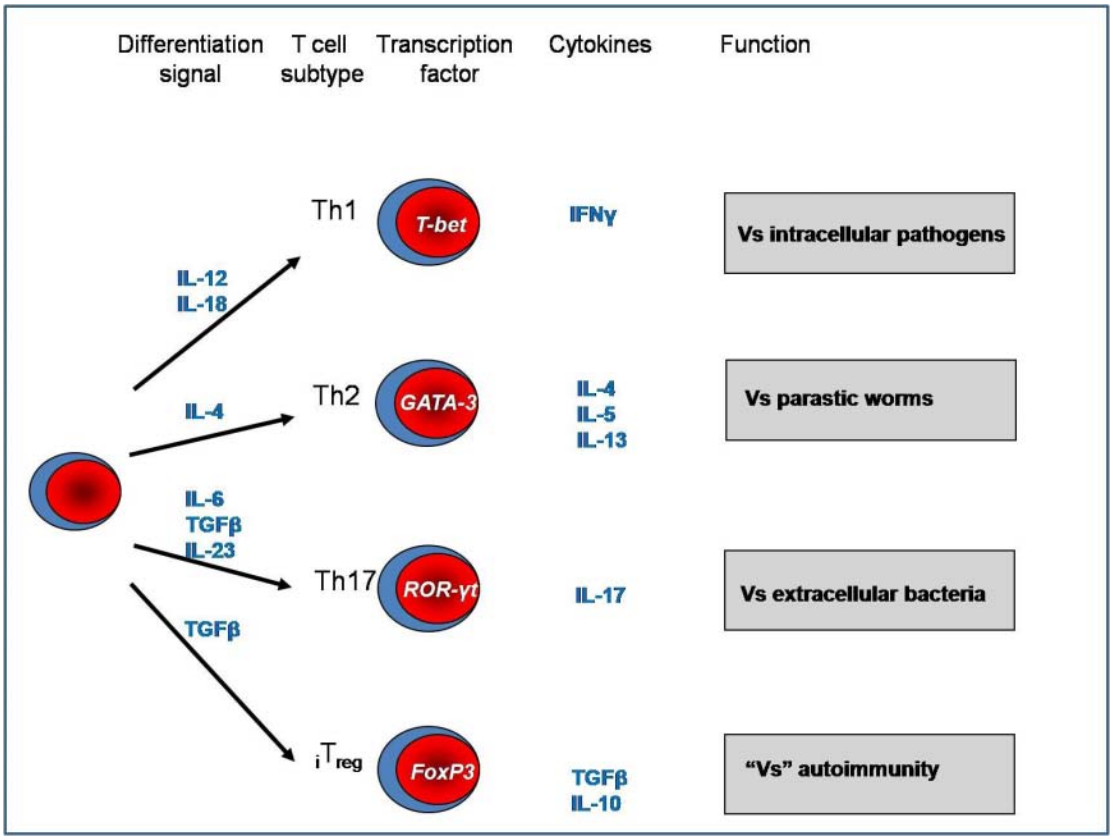


**Figure 1.4 Summary of CD4+ T cell lineages indicating the primary signals of differentiation, their transcription factors, cytokine profile and function.**

## 1.8 DC and regulatory T cells

In addition to the long-established mechanisms of anergy and deletion, it has become apparent that T cell function may be regulated by specific populations of T cells known as regulatory T cells. These appear to have an important role in the maintenance of peripheral tolerance and the prevention of autoimmune disease. Several types of "regulatory" T cell have been identified (Tarbell et al., 2004; Sakaguchi, 2005). Natural T regs develop in the thymus and are CD4+CD25+Foxp3+; CD25 being the high affinity IL-2 receptor, Foxp3 being a transcription factor(Sakaguchi, 2005). Their development may depend on DC. DC conditioned with thymic stromal lymphoprotein can stimulate the development of CD4+CD25+ T regs from CD4+CD25- thymocytes under human thymus culture conditions (Watanabe et al., 2005). To proliferate and to enhance their suppressive function the T regs require an antigen-specific signal from a DC, however their suppressive function is then relatively widespread, being capable in experimental models of blocking autoimmunity due to multiple antigens(Tarbell et al., 2006). Induced T regs are produced in the periphery in response to peripheral stimuli. These include IL-10-secretors (sometimes called Tr1) and TGFβ-secretors (sometimes called Th3)(Tarbell et al., 2006; Kojo et al., 2005). As with CD4+ T cell differentiation in general, it is the nature of the DC: T cell signal (and therefore the phenotype of the DC itself) that is primarily responsible for T cell differentiation into a regulatory or effector T cell (Lutz and Schuler, 2002; Reis e Sousa, 2006).

## 1.9 Immune privilege and the eye

The immune system has evolved to protect the individual from a wide variety of pathogens, but alongside this protective immunity there is frequently significant non-specific damage to the surrounding tissues. Although this can be tolerated in the majority of tissues, certain sites are more sensitive to these effects due to their limited capacity for self-renewal or the delicate physiology of the tissues. Immune responses in these sensitive tissues are highly specialised, with a reduction in the pathological damage caused by the immune response, conferring immune privilege to these sites(Streilein, 2003b; Streilein, 2003a). The eye (Figure 1.5) is the archetypal site of immune privilege and will be considered first, before reviewing other sites of immune privilege such as the brain, the pregnant uterus, the hamster cheek pouch, the hair follicle, and the testes.

In order to maintain a clear visual axis, the eye must allow immune responses required for protective immunity, and at the same time restrict these responses to prevent significant pathological damage. Many mechanisms have been suggested to contribute to the maintenance of immune privilege in the eye, both immunological and anatomical. Immunological mechanisms include: a complex network of immunosuppressive molecules in the ocular microenvironment (aqueous humour – AqH) (Taylor et al., 1992; Taylor, 2007; Cousins et al., 1991b), expression of FasL on ocular surfaces (pro-apoptotic signal notably against activated T cells) (Ferguson and Griffith, 2007), expression of atypical rather than typical class I MHC (inhibit NK cells, and not recognised by CD8+ cytotoxic T cells), and the phenomenon of anterior chamber associated immune deviation (ACAID)(Streilein, 2003b; Streilein, 2003a).

Anatomical mechanisms comprise a tightly regulated blood:ocular barrier and limited lymphatic drainage.

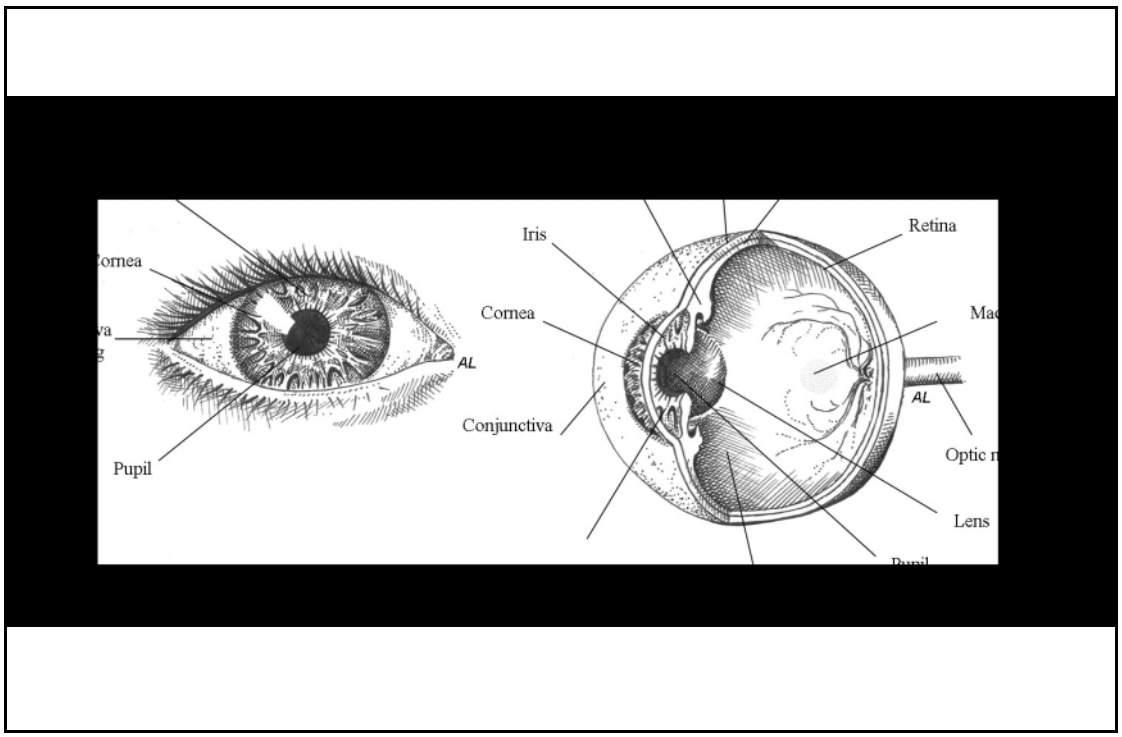


**Figure 1.5 Key structures of the eye**.
Extraocular and intraocular views showing key anatomical structures, including the uveal tract comprising iris, ciliary body and choroid. Illustration by Angela Luck; adapted with permission.

### 1.9.1 Ocular immune privilege

Evidence for the unusually protected status of the anterior segment of the eye stretches back to the observation by Van Dooremaal in 1873 that murine-to-canine skin grafts were tolerated when inserted into the anterior chamber of the dog eye.

Medawar, recognising that immunity was responsible for allograft rejection, regarded allograft tolerance by the eye and the brain as evidence that they were 'immune privileged' (Medawar, 1948)(recently reviewed (Kaplan and Niederkorn, 2007)). Further investigation has highlighted the contribution of aqueous humour to ocular immune privilege, and its regulation of both afferent and efferent arms of the immune response. Whilst other immunological and anatomical mechanisms are undoubtedly also important in maintaining immune privilege, within the context of this thesis I will be primarily considering the role of aqueous humour and its influence on the ocular antigen presenting cell with specific regard to the dendritic cell.

### 1.9.2 Aqueous humour

Aqueous humour is a clear colourless plasma-like fluid produced by the ciliary body at around 2.5µl/min (in human). Its functions include structure (providing support to the anterior segment of the eye), nutrition (to the avascular lens and cornea), light transmission and immunoregulation. It differs biochemically from plasma by being high in ascorbate but lower in glucose and protein, unless there has been breakdown of the blood-ocular barrier.

The earliest direct demonstration of the immunomodulatory effects of AqH was Kaplan's observation that injection of alloantigenic cells into the anterior chamber of the eye resulted in immune deviation in which there was antigen-specific suppression of classical Th1 immune responses (Kaplan et al., 1975). Subsequent studies by Streilein and colleagues refined this concept of Anterior Chamber Associated Immune Deviation (Kaplan and Streilein, 1977; Kaplan and Streilein, 2007; Streilein and

Niederkorn, 2007; Wilbanks et al., 1991; Wilbanks and Streilein, 1991; Wilbanks et al., 1992), one of the fundamental tenets of which was that the differentiation of ocular antigen presenting cells and their processing of antigen under the influence of aqueous humour results in a unique immunoregulatory phenotype (recently reviewed by Niederkorn (Niederkorn, 2007)). Several investigators also observed that when APC and T cells are co-cultured in the presence of AqH or iris/ciliary body supernatants, proliferation is inhibited (Cousins et al., 1991a; Streilein and Bradley, 1991). Whilst interesting, it should be noted that these experiments do not identify whether it is the afferent arm (ie the APC) or the efferent arm (the T cell) or both which are affected by AqH. More recently, however, Shen has shown that murine bone-marrow derived dendritic cells when cultured *in vitro* with rabbit AqH, are inhibited in their capacity to induce a mixed lymphocyte reaction (Shen et al., 2007).

AqH was also found to modulate the efferent arm of the DTH response. Benson and Niederkorn demonstrated local suppression of DTH responses to a highly immunogenic tumour (UV5C25) within the eye of BALB-c mice despite persistence of systemic DTH responses (Benson and Niederkorn, 1991). As noted above, most studies on the effects of AqH on T cell proliferation are hard to interpret due to the exposure of both APC and responder T cell to the AqH. Important exceptions include AqH inhibition of T cell proliferation in the IL-2 dependent CTLL assay (Cousins et al., 1991a), and AqH inhibition of lymphocyte proliferation to polyclonal mitogens and to growth hormones (Kaiser et al., 1989).

Investigation into the mechanism by which AqH achieves its effects have identified the presence of a number of potential immunomodulatory molecules including TGFβ,

the neuropeptides αMSH, VIP, CGRP, somatostatin and the endogenous glucocorticoid, cortisol.

### 1.9.3 Transforming Growth Factor β (TGFβ)

TGFβ was the first significant immunomodulatory molecule to be identified in AqH (Cousins et al., 1991a; Wilbanks et al., 1992). TGFβ exists in three isoforms, TGFβ1-3. Although mRNA for all three isoforms may be found in ocular cells, TGFβ2 is the only isoform to actually be expressed at sufficient levels to be recovered from healthy aqueous humour. (Cousins et al., 1991a; Pasquale et al., 1993) In healthy aqueous humour TGFβ2 levels are 1-10ng/ml, the vast majority being in its latent form (Knisely et al., 1991b).

TGFβ2 has been shown to have a number of immunomodulatory properties on APC. In some of the early experiments into the phenomenon of ACAID, Wilbanks showed that conferral by AqH of ACAID-inducing properties on non-ocular macrophages was TGFβ-dependent (Wilbanks et al., 1992). TGFβ2-treated APC lose the ability to activate Th1 cells and induce delayed type hypersensitivity (DTH). These APC produce less IL-12 and have lower expression of various accessory molecules (Takeuchi et al., 1998; Tsunawaki et al., 1988). Recently Shen has shown that recombinant TGFβ2 recapitulated the inhibitory effects of rabbit AqH on murine bone-marrow derived DC, and that the effects of AqH were blocked by AqH-pre-treatment with anti-TGFβ2 (Shen et al., 2007). TGFβ2 treatment of macrophages also

reduces their production of inflammatory cytokines and their ability to generate reactive oxygen species(Tsunawaki et al., 1988).

TGFβ2 present in AqH has also been shown to be capable of modulating T cell proliferation directly. In an IL-2 dependent CTLL assay, Cousins *et al* showed that AqH inhibition of proliferation was completely reversed by the addition of anti-TGFβ2 (Cousins et al., 1991a). TGFβ has been shown to inhibit proliferation of naive T cells by inhibiting IL-2 production via Smad3 (in contrast activated T cells are relatively insensitive (Cottrez and Groux, 2001)), and to inhibit the differentiation of Th1 and Th2 subtypes via T-bet/Stat4 and GATA-3/NF-AT respectively (recently reviewed by Li *et al* (Li et al., 2006)). Significantly TGFβ also induces FoxP3 expression and the induction of CD4+CD25+ T regs from CD4+ CD25- naive T cells (Chen et al., 2004).

### 1.9.4 α -Melanocyte Stimulating Hormone (αMSH)

αMSH is a 1.6 kDa, 13-amino acid neuropeptide which is cleaved from the protein, pro-opiomelanocortin hormone (POMC). It is produced by a number of cell types including neurons, macrophages and keratinocytes. αMSH is constitutively expressed in the healthy eye with aqueous humour levels of around 20pM (Taylor et al., 1992). DC respond to αMSH via the melanocortin receptor MC-1R, with downregulation of CD86 and CD40, inhibition of the production of IL-1, IL-2, IL-4,IL-6, IL-13, IFNγ and TNFα and increased secretion of IL-10 (reviewed (Luger et al., 2003)). Similarly αMSH has been noted to suppress activated macrophages, reducing their expression of pro-inflammatory cytokines, reactive oxygen species and nitric oxide, whilst

increasing their production of IL-10, αMSH and their expression of αMSH receptors (Star et al., 1995). It has also been found to suppress the pro-inflammatory effects of LPS (Chiao et al., 1996), IL-1 (Watanabe et al., 1993) and TNFα (Martin et al., 1991).

In addition to its effects on APC, αMSH has a number of important effects on T cells. AqH and αMSH (at its ocular concentration) inhibit IFNγ production by antigen-stimulated T cells; neutralization of the αMSH component of AqH reverses this inhibition. αMSH can also induce a regulatory phenotype in CD4+ T cells which may be augmented by TGFβ2. This has been demonstrated both *in vitro* and in the mouse model of experimental autoimmune uveitis (Taylor et al., 1992). In addition in a model of local adoptive transfer of delayed type hypersensitivity (DTH) in which lymph node cells from immunized C57BL/6 are reintroduced after incubation with Mycobacterium tuberculosis antigen, the suppressive effects of AqH were found to be partially reversed by removal of the αMSH fraction (Taylor et al., 1994a).

### 1.9.5 Vasoactive Intestinal Peptide (VIP)

VIP is a 3.3 kDa, 28 amino acid neuropeptide produced by neurons, with variant forms also being produced by neutrophils and mast cells. It is present in the anterior chamber at 12 $\pm$ 1 nM. VIP has been shown to inhibit mitogen-driven lymphocyte proliferation and function (Ottaway, 1988; Stanisz et al., 1986). Its effects on APC are not well defined. At its ocular concentration it has been shown to inhibit Ag-stimulated lymph node cell proliferation and IFNγ production *in vitro*. Interestingly removal of VIP from the low molecular weight fraction of AqH did not neutralize the

effect of AqH on proliferation but did neutralize the suppression of IFNγ-production. In addition removal of VIP neutralized the suppression of local adoptive transfer of delayed type hypersensitivity (DTH) (Taylor et al., 1994b). Ferguson subsequently showed that VIP was essential for the induction of ACAID, that it was antagonised by substance P and that the presence of VIP was dependent on a normal diurnal rhythm of light exposure.

### 1.9.6 Cortisol

Cortisol (hydrocortisone) is one of a class of hormones known as 'glucocorticoids', a group of steroid hormones produced in the cortex of the adrenal gland, and the functions of which include glucose metabolism. It has wide-ranging effects on many homeostatic processes including metabolism, fluid balance, the cardiovascular system, and the immune system. Cortisol is secreted according to a diurnal rhythm , peaking in the morning, but is secreted at increased levels in response to stress.

Cortisol has been observed at biologically significant levels in human and rodent AqH with levels of 18.0 $\pm$ 1.0 ng/ml (mean $\pm$ SD) for human; cortisone (corticosterone) levels were significantly lower at 0.7 $\pm$ 1.0ng/ml. These levels equate to 49.7nmol/l and 1.9nmol/l respectively (362.5Mol mass). Our own group has measured levels of cortisol of 4.8 $\pm$ 0.38 nmol/l and cortisone of 1.9 $\pm$ 0.13 nmol/l (mean $\pm$ SEM) by RIA in patients attending for cataract surgery(Rauz et al., 2003). Other groups observed human hydrocortisone levels of between 2.5 and 17ng/ml (Weinstein et al., 1991; Rozsival et al., 1981). Interestingly the binding protein Cortisol Binding Globulin (CBG) was not present at detectable levels (by radioimmunoassay) (Knisely et al.,

1994). Thus it would appear that the vast majority of cortisol in AqH exists in free form, in contrast to the situation in serum where 80-90% is bound to CBG, 4-10% is bound to albumin and the remaining 6-10% is free cortisol.

The effects of cortisol on the immune system are primarily mediated via the glucocorticoid receptor (GR). The availability of cortisol for a specific tissue or cell may also be modified by the actions of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) which catalyzes the cortisone-cortisol shuttle. 11β-HSD1 catalyzes the generation of active cortisol from cortisone, whereas the isoform 11β-HSD2 catalyzes the reverse process.

Binding of glucocorticoid to the glucocorticoid receptor results in downstream inhibition of the transcription factors NF-κB and AP-1, and, via binding to negative glucocorticoid response elements (GRE) in inhibition of the POMC gene (reviewed (Webster et al., 2002)). Cortisol has wide-ranging effects including the apoptosis of monocytes, inhibiting IL-1 (Lee et al., 1988), IL-6 and TNFα production (Steer et al., 2000), inhibiting IL-12 production by DC and inducing a shift from Th1 to Th2 (Freeman et al., 2005). The addition of cortisol at the levels found in AqH inhibited PHA/IL-1-stimulate murine thymocyte proliferation and CCL64 cell proliferation (Knisely et al., 1994).

### 1.9.7 Extraocular sites of immune privilege

Other sites of immune privilege include the brain, the pregnant uterus, the hamster cheek pouch, the hair follicle, and the testes. The advantage of immune privilege

varies from organ to organ. For the brain (like the eye) it reduces the chance of immune-mediated collateral damage in a structure with limited regenerative capacity and where minor anatomical damage can cause survival-compromising loss of function. The pregnant uterus, effectively a semiallogeneic graft, requires immune privilege to prevent rejection of the fetus. Finally, without the immune privilege of the cheek pouch, the hamster would not be able to store food for the prolonged periods necessary for its survival.

Some of the mechanisms of immune privilege familiar to the ocular immunologist, have also been observed in other immune privileged structures. Mechanisms of immune privilege shared by the eye, brain and feto-placental unit include the presence of immunomodulatory molecules such as TGFβ, αMSH, VIP, CGRP and somatostatin, expression of FasL, absence of typical MHC class I molecules, and, more speculatively, brain and fetal equivalents to ACAID (recently reviewed by Niederkorn (Niederkorn, 2006)).

## 1.10 Antigen presentation in the ocular context

In contrast to the role of the Langerhans cell within the skin (reviewed earlier), the nature and role of the antigen presenting cells of the eye are much less well-defined. Some of the most fundamental questions of ocular immunology centre on the ocular APC: its nature (dendritic cell, macrophage or other), its function (stimulatory or regulatory) and its location/migratory behaviour (resident or migratory). I will first summarise the background to each of these questions, before reviewing our central hypothesis within this context.

### 1.10.1 The identification of the ocular APC

The nature of the ocular APC-like cell has been a matter of some debate. In human cadaveric studies of 12 eyes Chang observed that a population of dendritiform stromal cells of the iris, ciliary body and choroid were HLADR+ TLR4+MD2+ but CD68-, and were therefore most typical of dendritic cells.(Chang et al., 2004) However the same cells appeared to express CD14, which whilst expressed at high levels in macrophages is also expressed on iDC. In an earlier cadaveric study of 34 human eyes Flugel observed similar CD45+HLADR+ dendritiform cells in the trabecular meshwork, around the intra- and episcleral vessels, the stroma of the ciliary body and the iris, and, with increasing age of the patient, in the "uveoscleral" pathway. In this paper co-authored with Streilein, Flugel argued that the CD45+HLADR+ cells in the trabecular meshwork were migrating APC "in transit" which could go on to mediate ACAID(Flugel et al., 1992).

In mouse and rat anterior uveal tissues both Knisely and McMenamin observed networks of Class II MHC (Ia) + dendritiform cells which they identified as dendritic cells (Knisely et al., 1991a) (McMenamin et al., 1994). Further work in the rat showed that the pleomorphic or dendritiform DC express MHC II and/or OX62 antigen (α2 E integrin) and have a half-life of around 3 days whereas the macrophage population are positive for CD163, are usually MHCII- and have a much longer half-life (Steptoe et al., 1995).

Meanwhile in the mouse Streilein and colleagues observed F4/80+ cells in the anterior uveal tissues which they identified as monocyte/macrophages. This was of

particular interest because they also showed that these F4/80+Class II MHC (1a)-cells when derived from either iris/ciliary body or from the peritoneal cavity could be loaded with antigen (*in vivo* or *in vitro*) and transferred intravenously or intracamerally (ie into the anterior chamber) to induce ACAID in a second animal.(Wilbanks et al., 1992; Wilbanks et al., 1991) (Wilbanks and Streilein, 1991). Although F4/80 is often considered as a marker of murine macrophages it may also label groups of dendritic cells such as the Langerhans cell group of DC(McMenamin et al., 1994; Camelo et al., 2003). Immunolabelling of murine iris and ciliary body by McMenamin suggested that 80% of the F4/80+ cells were also positive for the macrophage marker SER4 (and apparently similarly for CD11b). However 33% of the F4/80+ cells also expressed MHCII (Ia) which has been primarily described on the DC population. Two thirds of the class II(Ia) + cells also expressed specific DC markers such as CD11c (McMenamin, 1999).

### 1.10.2 The function of the ocular APC

Although there is general agreement over the existence of both a DC-like population and a macrophage-like population, there is considerable argument over two issues: first, their function – do they act as immunogenic APCs or do they have an immunoregulatory role?; second, their migration – do they act purely locally or do they migrate, and if so where to? Camelo and colleagues have hypothesised that the anterior uveal dendritic cells are the sentinel APC which migrate to the draining lymph node to induce naïve T cell proliferation and so generate the primary ocular immune response, whereas the macrophage may be involved in the secondary immune response by restimulating activated T cells (Camelo et al., 2003).

Previous studies by Williamson and Streilein found that single-cell suspensions and whole preparations of the iris/ciliary body not only failed to function as allogeneic APCs but actually suppressed the mixed lymphocyte reaction. Intraocular injection of IFNγ led to upregulation of MHCII on resident bone-marrow derived cells of the iris-ciliary body and could abrogate these immunosuppressive properties, although the exact mechanism of this altered function is unclear (Streilein et al., 1992). In contrast Steptoe found that purified MHCII (Ia)+ED2- iris DC, but not MHCII-ED2+ macrophages, did have moderate allostimulatory capacity in the mixed leucocyte reaction assay and that this could be further enhanced by the addition of GM-CSF (Steptoe et al., 2000). These authors suggested that the failure to observe allostimulation in other studies might be due to contamination with iris/ciliary body epithelial or stromal cells which are known to have a strong immunoregulatory effect, rather than being a feature of the iris DC themselves.

### 1.10.3 The traffic of soluble antigen and of ocular antigen presenting cells

In the absence of a well-developed system of draining lymphatics it is unclear where antigen presentation actually takes place. A number of explanations have been proposed which are not mutually exclusive and may indeed coexist. The three dominant models are discussed below.

### 1.10.3.1 Model 1: Antigen presentation occurs in the eye only

The simplest solution, proposed by Chang and colleagues, is that the traffic of antigen or APC out of the eye is unnecessary. They propose that naïve T cells may be recruited directly to the uvea and that priming and activation could occur in the uvea itself (Chang et al., 2004). Although there is no direct evidence for this occurring within the eye, direct antigen presentation to naive and memory CD4+ T cells can occur in peripheral tissues. Although under normal circumstances, only primed CD4+ T cells would be recruited to the site of inflammation, it is possible that in the presence of generalised endothelial activation (such as in the presence of severe blood ocular barrier breakdown) naïve CD4+ T cells may also traffic through.

### 1.10.3.2 Model 2: Ocular APC migrate from the eye and present antigen in the spleen

In contrast the Anterior Chamber Associated Immune Deviation (ACAID) model postulates that antigen introduced to the anterior chamber is picked up by APC which follow the conventional outflow pathway of aqueous humour via the venous system to the spleen(Streilein, 2003b). In the context of ACAID antigen presentation occurs in the spleen triggering an immunoregulatory (rather than an effector) response (Streilein, 2003b) . This has similarities to venous induction of tolerance where antigen is directly injected into the vein resulting in the traffic of large quantities of antigen directly to the spleen, although most advocates of ACAID argue that it is a distinct tolerogenic mechanism (Niederkorn, 2007) .

### 1.10.3.3 Model 3: Soluble antigen migrates to the draining lymph node and is processed by resident DC

Other investigators have noted that almost all antigen is carried in mainly soluble form and not by an ocular APC. They propose that soluble antigen leaves the eye by either the conventional route or the unconventional uveoscleral route, and may then be transported in the venous system or in the afferent lymphatics to the secondary lymphoid tissues (Camelo et al., 2006; Dullforce et al., 2004). Wilbanks and Streilein compared the subsequent distribution of intracameral, subconjunctival and intravenous injections of 125I labelled BSA. In contrast to intravenous administration, both intracameral and subconjunctival sites acted as antigen depots, with detection of antigen even 4 weeks later. However they noted that 95% of the injected antigen left the eye in soluble form ie without being processed by ocular antigen presenting cells (Wilbanks and Streilein, 1989).

Egan *et al* found that both the peptide OVA 323-339 and the whole OVA protein when inoculated into the posterior chamber of the eye led to the expansion of OVA-specific T cells in the ipsilateral submandibular lymph node by day 3. This effect is abrogated by enucleation of the eye at 30mins, 1h or 5h, but persists with enucleation at 24h suggesting that the antigen (in whatever form) exits the eye between 5 and 24h (Egan et al., 1996).

Becker and colleagues subsequently used intravital microscopy in the mouse to identify which ocular APC ingested ovalbumuin after intracameral administration. Fluorescent ovalbumin and/or fluorescent antibodies to MHC II, CD11b, CD11c and F4/80 were injected into the anterior chamber of healthy mice. They found that

ovalbumin was taken up by some cells with morphologic and surface molecules typical of macrophages and some more typical of dendritic cells. At 6h after injection most (95%) ovalbumin+ cells were CD11c+, 90% were F4/80+, around 60% were MHCII+, and around 15% were CD11b+. On colabelling only 10% of the CD11b+ cells were MHCII+, and only 10-20% of the MHCII+ cells were CD11b+. At 24h after injection only 24% of ovalbumin+ cells were CD11c+, and around 30% were MHCII+, whereas the proportion of CD11b+ cells had increased to 60% and F4/80+ cells had remained at 90%. They also found that at 24h most of the MHCII+ cells were positive for an antibody mix to CD80 and CD86. The authors suggest that this apparent shift from a typical dendritic cell phenotype (CD11c+ and/or MHCII+) to a more macrophage-like phenotype (CD11b+ and/or MHCII-) may reflect actual differentiation of the DC, emigration of the DC or death of the DC with ingestion by macrophages (Becker et al., 2003).

The same group then compared the uptake of texas red labelled ovalbumin (TR-OVA) with the uptake of fluorescent 2μm latex beads after injection into the murine anterior chamber. Cells positive for TR-OVA but not for fluorescent beads were observed in the submandibular lymph nodes from 6h onwards. The majority of the beads remained clumped in the angle of the eye, although a few could be found in the spleen at 24h post injection(Dullforce et al., 2004). Video microscopy showed that no bead-containing APC from the iris showed directionally significant movement or left the iris at any time point (4h, 5h, 6h, 12h, 24h, 48h) even under the additional inflammatory stimulus of LPS (Dullforce et al., 2004). This contrasted with their parallel experiments in which intradermal injection of identical beads resulted in

bead-containing APC in the draining lymph nodes, so recapitulating the work of Randolph (Randolph et al., 1999).

Using *in vivo* video microscopy in the rat Camelo found that 24h after intracameral injection of fluorescent-labelled dextran, antigen had been taken up by numerous cells of the iris, ciliary body, anterior suprachoroidal space and episcleral connective tissue. Confocal microscopy revealed that the majority of these cells were MHCII (Ia) -, and bore typical macrophage markers ED1 (*syn* macrosialin; stains most rat tissue macrophages, may also be on DC; homologue of CD68), and ED3 (CD169, stains marginal zone and subcapsular sinus subsets of lymphoid macrophages); however there was also a population of cells which were positive for both ED2 (CD163;*syn* scavenger receptor B) usually found on monocytes/macrophages (particularly alternatively activated (M2) macrophages(Tiemessen et al., 2007)) as well as OX62+ (ie rat $\alpha_{E2}$ integrin) which is a typical DC marker (Camelo et al., 2003).

The same group then attempted to identify which extraocular sites the fluorescent-labelled dextran drained to. They observed dextran+ cells in the marginal zone of the spleen, in the subcapsular sinus of the ipsilateral submandibular, superficial cervical and deep cervical lymph nodes, and unexpectedly in the mesenteric lymph nodes. In some cases dextran+ cells were also found in the contralateral submandibular and superficial cervical lymph nodes (Camelo et al., 2004a). In the spleen these dextran+ cells were CD1+, CD11b+, CD11c-, CD68+ (clone ED1), CD86low, CD163low (clone ED2), CD169+ (clone ED3),MHCII (Ia)+ (clone OX6), ED5- (marker of FDC), and in the lymph nodes were CD4+, CD8+, CD80+ and CD172a+ (OX41).

The authors argue that these phenotypes suggest that they are resident lymphoid macrophages, and do not represent migrated APC from the eye (Camelo et al., 2006).

The work by Camelo (Camelo et al., 2003) suggesting that in the rat eye intracameral dextran is mostly taken up by macrophages contrasts with the work of Dullforce(Dullforce et al., 2004) (discussed earlier) suggesting that in the mouse eye intracameral ovalbumin was first taken up by DC and only later appeared in cells of a macrophage phenotype. These differences could reflect differences in the nature of the antigen, interspecies variation or issues of experimental design and technique. Camelo and colleagues attempted to address some of these issues by repeating their earlier work with dextran (a polysaccharide) using ovalbumin and BSA (both proteins). With all three antigens, antigen positive cells were observed within the iris, iridocorneal angle, pre-equatorial choroid and around limbal/episcleral vessels. Interestingly whereas with BSA and dextran (40-70kDa), antigen positive cells were found lining both the conventional and non-conventional pathways, with albumin antigen positive cells were found mainly along the conventional outflow pathway. At 24h most of the antigen positive cells were of a similar phenotype regardless of the antigen used; specifically they were CD68+ (ED1), CD163+ (ED2), mostly MHCII- and occasionally CD169+ (ED3) suggestive of a macrophage phenotype. A few antigen positive MHCI++ dendriform cells were identified in the iris, trabecular meshwork, choroid and episclera, and these were proposed as possible DC.(Camelo et al., 2004b) It should be noted that the studies by Camelo did not involve examining the eye earlier than 24h post injection, and thus direct comparison with the Dullforce (Dullforce et al., 2004) study is not possible.

In a subsequent series of experiments with parallels to the Dullforce paper, Camelo and colleagues injected cascade-blue-dextran into the AC of the right eye and FITC-dextran into the AC of the left eye: significantly individual APC bearing both fluorochromes were identified. Similarly when CB-Dx was injected into the AC of the right eye and FITC-DX was injected into the tail vein, APC bearing both fluorescent antigens were identified in the marginal zone of the spleen. They also showed that the distribution to lymph nodes following intracameral injection was almost identical to that seen after subconjunctival injection; they argued that this suggests that the intracameral antigen that ends up in these sites also travels via the conjunctival lymphatics (Camelo et al., 2006).

### 1.10.4 Summary of antigen presentation in the eye

My central hypothesis is that the normal resting ocular microenvironment maintains a relatively immature DC phenotype which generates anergic or regulatory T cells, whereas in intraocular inflammation (uveitis) this regulation breaks down permitting full DC maturation resulting in production and recruitment of pathogenic effector T cells to the eye.

In terms of the identity of the ocular APC, MHC class II+ cells have been identified in the mouse, rat and cadaveric human eye, mainly around the iris (Becker et al., 2003; McMenamin et al., 1994; Forrester et al., 1994). These can be divided into two groups according to whether they express typical dendritic cell or macrophage markers. The iris dendritic cells express low CD11c, CD80 and CD86 suggesting that they are relatively immature (McMenamin et al., 1994) . Although less abundant than

macrophage-like populations, there is now good evidence that DC are present within the anterior (and posterior) segment of the eye (Chang et al., 2004; Forrester et al., 1994; Forrester et al., 1993; Forrester et al., 2005; McMenamin et al., 1994; McMenamin, 1999; McMenamin, 1997; McMenamin et al., 1992).

In terms of function, indirect evidence for the role of dendritic cells in uveitis (or at least a form of ocular antigen presenting cell) comes from the presence during inflammation of recently primed, activated T cells (CD45RO+, CD69+) suggesting recent activation by professional APC(Muhaya et al., 1999) . Antigen injected into the AC is captured within 6-24h by macrophage and DC-like cells in the iris and subconjunctival tissue(Dullforce et al., 2004; Camelo et al., 2003), however it remains unclear whether antigen presentation is by these ocular DC themselves, or by resident DC of the lymphoid tissues which have imbibed soluble antigen or transferred antigen (from migratory DC). It is also uncertain whether antigen presentation takes place in the draining lymph nodes, the spleen (as per ACAID model) or indeed in the ocular tissue itself (as proposed by Chang).

In the light of my hypothesis and the limits of our current knowledge (summarised above), one of the challenges of this thesis was therefore to develop an *in vitro* model of DC function that would reflect DC behaviour in both the resting ocular microenvironment and under inflammatory conditions and would enable us to assay its capacity to induce T cell responses both of naive cells, and of memory cells.

## 1.11 Uveitis

### 1.11.1 The clinical problem of uveitis

Uveitis comprises a group of diseases characterised by intraocular inflammation, which by its very existence challenges the paradigm of ocular immune privilege. Many cases resolve rapidly, but a significant number of patients develop persistent disease, with damage to ocular structures resulting in severe visual impairment. It is currently unclear whether the development of uveitis reflects a failure of immune privilege or whether these mechanisms are overwhelmed and conversely whether disease resolution represents a re-establishment of the normal mechanisms of immune privilege function.

Although uveitis *per se* is a term correctly used to describe inflammation of the uveal tract (iris, ciliary body and choroid; Figure 1.5) alone, the term has come to include a large group of diverse diseases affecting also the retina, optic nerve and vitreous(Bora and Kaplan, 2007; Forrester, 2007). It predominantly affects people of working age, peaking in the 20 – 50 year age group, with an annual incidence of 14 – 50 per 100,000(Durrani et al., 2004). Whilst there is some variation in prevalence across the globe, in the developed world it is the 5$^{th}$ commonest cause of visual loss accounting for 10 – 15% of total blindness and up to 20% of legal blindness(Durrani et al., 2004).

Uveitis can be a devastating sight-threatening condition. The main causes of reduced vision are cystoid macular oedema and secondary cataract. Other vision threatening complications include secondary glaucoma, band keratopathy, vitreous opacities, optic neuropathy, retinal scars and phthisis (involution of the eye). Depending on the

primary site and extent of inflammation it may be described as anterior, intermediate, posterior or panuveitis (Table 1.5), each of which have their own constellation of typical clinical features (Table 1.6)((Jabs et al., 2005; Bora and Kaplan, 2007; Forrester, 2007).

| **Type** | **Primary site of inflammation** | **Includes** |
|---|---|---|
| Anterior uveitis | Anterior chamber | Iritis<br>Iridocyclitis<br>Anterior cyclitis |
| Intermediate uveitis | Vitreous | Pars planitis<br>Posterior cyclitis<br>Hyalitis |
| Posterior uveitis | Retina or choroid | Focal, multifocal or diffuse choroiditis<br>Chorioretinitis<br>Retinochoroiditis<br>Retinitis<br>Neuroretinitis |
| Pan uveitis | Anterior chamber, vitreous and retina or choroid | |

**Table 1.5 Anatomical classification of uveitis**
The Standardization of Uveitis Nomenclature (SUN) anatomical classification of uveitis (2005)(Jabs et al., 2005)

| Type | Common symptoms | Common signs |
|---|---|---|
| Anterior uveitis | If acute: pain, redness<br>If chronic: usually asymptomatic | Ciliary injection<br>AC cells<br>AC flare<br>Keratic precipitates<br>Posterior synechiae |
| Intermediate uveitis | 'Floaters'<br>Reduced vision<br>May be asymptomatic | Vitreous cells<br>Exudation at the ora serrata |
| Posterior uveitis | Reduced vision<br>'Floaters' | Macular oedema<br>Retinal or choroidal infiltrates/oedema<br>Retinal haemorrhages<br>Periphlebitis or arteritis |
| Panuveitis | Any of the above | Any of the above |

**Table 1.6 Common clinical features observed in anterior, intermediate, posterior and panuveitis**
Clinical presentation varies according to the primary site of intraocular inflammation (Bora and Kaplan, 2007; Forrester, 2007).

One of the challenges of inflammatory disease is to score activity and so permit monitoring of disease and assessment of any intervention. Anterior segment involvement is scored using the SUN grading score for anterior chamber cells and flare(Jabs et al., 2005). For AC cells this involves scoring the number of AC cells in a 1.0 x 1.0mm beam as follows: <1 cells, score 0; 1-5 cells, score 0.5+; 6-15 cells, score 1+, 16-25 cells, score 2+, 26-50 cells, score 3+, >50 cells, score 4+. For AC flare the grading is similarly from 0 to 4, and is based on the increasing visibility of the beam within the normally transparent AqH, the loss of clarity of the intraocular structures and the visibility of fibrin (Jabs et al., 2005).

In the majority of patients the cause of the intraocular inflammation is unknown (Table 1.7). Infectious agents, such as Toxoplasma gondii, and the herpes viruses are

well-recognised causes, but, particularly amongst industrialized nations, only account for a minority of disease. Of the non-infectious cases, some are part of a systemic disease process, such as sarcoidosis, Behçet's disease, multiple sclerosis and the HLA-B27 related group of diseases, but in most cases the pathogenesis is not understood. It is thought that many of these 'idiopathic' cases have an autoimmune aetiology(Bora and Kaplan, 2007; Forrester, 2007). They share similarities (and indeed are often associated) with established autoimmune conditions; they also respond to immunosuppresives drugs, such as glucocorticoids (Curnow et al., 2004; Curnow et al., 2005; Muhaya et al., 1999; Becker et al., 2002).

| **Group** | **Subgroup** |
|---|---|
| Infectious | Bacterial<br>Viral<br>Fungal<br>Parasitic<br>Others |
| Non-infectious | Known systemic association<br>No known systemic association |
| Masquerade | Neoplastic<br>Non-neoplastic |

**Table 1.7 Clinical classification of uveitis**
The clinical classification of uveitis (infectious/non-infectious/masquerade) has been described by the International Uveitis Study Group, 2008)(Deschenes et al., 2008)

### 1.11.2 Treatment of uveitis

In common with most other inflammatory diseases, uveitis responds well to treatment with glucocorticoids (Oksala, 1960; Dick et al., 1997; Gaudio, 2004). In most anterior uveitis topical synthetic glucocorticoids are sufficient: typically dexamethasone 0.1% or prednisolone 1%. In more severe episodes of uveitis or where uveitis is not confined to the anterior part of the eye systemic glucocorticoids are usually given(Oksala, 1960; Dick et al., 1997; Gaudio, 2004). Where prolonged immunosuppresssion is required (or where systemic glucocorticoids at an acceptable dose are insufficient to control disease) alternative agents are used in addition or instead of glucocorticoids. Such agents include cyclosporine (Dick et al., 1997; Murphy et al., 2005), tacrolimus (Murphy et al., 2005), methotrexate (Gangaputra et al., 2009), azathioprine(Pasadhika et al., 2009), mycophenolate mofetil (Kilmartin and Dick, 1999; Teoh et al., 2008), cyclophosphamide (Rosenbaum, 1994), anti-TNF therapies (Murphy et al., 2004; Mushtaq et al., 2007; Suhler et al., 2009), IFNα (Deuter et al., 2009) and other 'biological' therapies(Sen et al., 2009). All of these agents, like glucocorticoids, have significant potential side-effects (Okada, 2005; Gaudio, 2004; Dick et al., 1997) (Table 1.7, 1.8). Within the field of uveitis there is a dearth of large scale high quality randomised controlled trials to direct the appropriate use of these agents. Although there is a general reliance on glucocorticoids first-line, the selection of supplementary or alternative immunosuppressants (and their dosing regimen) still tends to be led more by personal experience, retrospective studies or by extrapolation from other diseases than from randomised controlled trials specific to uveitis. Fortunately this situation is beginning to improve with the development of

consensus guidelines (Jabs et al., 2000) and a move to prospective studies (Sen et al., 2009; Suhler et al., 2009).

Experimental therapies currently under investigation for use in uveitis include T reg infusion and tolerogenic DC vaccination, both of which have shown to be effective in EAU (Jiang et al., 2003). Other targets which have shown promise in animal models include IL-17, CD44(Xu et al., 2002; Reis e Sousa et al., 1999), chemokines (such as MIP-1α)(Crane et al., 2003), and adhesion molecules (such as ICAM-1) (Uchio et al., 1994; Xu et al., 2003).

Part of this project was to investigate how glucocorticoid treatment may alter the effect of the ocular microenvironment on DC, by studying the effect on DC both of AqH from patients with uveitis pre or post-commencement of treatment and of the therapy itself .

| Drug | Mechanism | Side-effects (selected) |
|---|---|---|
| *Glucocorticoid* | | |
| Prednisolone<br>Methylprednisolone<br>Dexamethasone | Inhibits NF-κB and AP-1 | Glucose intolerance/diabetes mellitus<br>Weight gain<br>Osteoporosis<br>Adrenal suppression<br>Cataracts |
| *Antimetabolites* | | |
| Azathioprine | Inhibits purine metabolism | Bone marrow suppression<br>Gastro-intestinal (GI) upset<br>Secondary malignancies<br>Alopecia |
| Methotrexate | Inhibits dihydrofolate reductase | Hepatotoxicity<br>Bone marrow suppression<br>GI upset |
| Mycophenolic acid | Inhibits purine metabolism | Bone marrow suppression<br>GI upset<br>Secondary malignancies |
| Leflunomide | Inhibits pyrimidine synthesis | Bone marrow suppression<br>Hepatotoxicity |
| *Calcineurin inhibitors* | | |
| Ciclosporin | Inhibits calcineurin/NF-AT transcription factor → reduce IL-2 + other cytokines | Nephrotoxicity<br>Hypertension<br>Hepatotoxicity<br>Gingival hyperplasia<br>Hypertrichosis |
| Tacrolimus | Inhibits calcineurin/NF-AT transcription factor → reduce IL-2 + other cytokines | Nephrotoxicity<br>Hypertension<br>Neurotoxicity<br>Hepatotoxicity |
| Voclosporin | Inhibits calcineurin/NF-AT transcription factor → reduce IL-2 + other cytokines | Nephrotoxicity<br>Hypertension |
| *Cytotoxics* | | |
| Cyclophosphamide | Alkylating agent: DNA cross-linking blocks cell replication | Bone marrow suppression<br>Haemorrhagic cystitis<br>GI upset<br>Sterility |

**Table 1.8 Conventional anti-immunosuppressive drugs used in uveitis, their chief mode of action and selected important side-effects**

| Drug | Mechanism | Side-effects (selected) |
|---|---|---|
| *TNFα inhibitors* | | |
| Adalimumab | Anti-TNFα: fully human monoclonal antibody against TNFα | TB and Hep B reactivation<br>Severe infections |
| Etanercept | Anti-TNFα: Fc fusion protein which binds extracellular TNF-α | Hypersensitivity reactions<br>TB and Hep B reactivation<br>Severe infections |
| Inflixamab | Anti-TNFα: chimeric monoclonal antibody against TNF-α | Human antichimeric antibodies serum sickness<br>TB and Hep B reactivation<br>Anaphylaxis<br>Severe infections |
| *Interleukin receptor antagonists* | | |
| Anakinra | Anti-IL1R: recombinant version of IL1 receptor antagonist (IL1RA) | Injection-site reaction<br>Neutropenia<br>Severe infections (especially in asthma) |
| Daclizumab | Anti-IL2R: humanized monoclonal antibody against the IL2 receptor | Hypersensitivity reactions<br>Hypertension<br>Severe infections |
| *Anti- B cell* | | |
| Rituximab | Anti-CD20: chimeric monoclonal antibody against CD20 (B-cells) | Severe infusion reactions(inc dyspnoea, hypoxia, bronchospasm)<br>Cytokine release syndrome<br>Cardiac dysfunction (inc arrest, hypotension, angina, arrhythmias)<br>TB reactivation<br>PML |
| *Interferons* | | |
| Interferon-$\alpha^2$ | Antiviral and anti-tumour: decreases NK cell activity | Leukopenia<br>Depression<br>TB reactivation<br>Flu-like symptoms<br>Nephrotoxicity<br>Hepatotoxicity |

**Table 1.9 Biological therapies currently used in uveitis, their chief mode of action and selected important side-effects.**
A number of emerging biological therapies (such as anti-IL-17 therapies) are also currently under consideration.

### 1.11.3 The initiation of uveitis

The processes leading to the initiation of non-infective uveitis are still unclear. With regard to the most common group, acute anterior uveitis (AAU), there is good evidence for both a genetic and environmental component. Genetic factors include a strong association with HLA-B27. In the West the prevalence of HLA-B27 is $\leq$ 10% in the general population but around 50% in those presenting with AAU(Brewerton et al., 1973a; Ehlers et al., 1974). Conversely being HLA-B27+ is estimated to carry around a twenty five-fold relative risk of AAU (vs non-carriers) (Brewerton et al., 1973a; Ehlers et al., 1974). It is not clear whether the HLA-B27 association for AAU (and spondylarthropathies such as ankylosing spondylitis) is directly due to the molecule itself (eg via inappropriate recognition of self antigen on the HLA-B27 molecule) or whether it is due to the presence of neighbouring genes existing in linkage disequilibrium with HLA-B27. Candidates include MHC I chain-related gene A (MICA; MICA A4 is associated with AAU (Goto et al., 1998); MICA*009 with Behcet's disease (Mizuki et al., 2007)) and TNF(Kaijzel et al., 1999).

Despite its high relative risk of AAU, being positive for HLA-B27 only carries a lifetime incidence of around 1% (Linssen et al., 1991) raising the possibility of a significant environmental contribution. Most attention has focused on the presence in a proportion of AAU of recent gram negative bacterial infection, particularly Yersinia and Salmonella(Saari and Kauranen, 1980; Saari et al., 1980b; Saari et al., 1980a). The role of Gram negative bacteria is reasonably well established in other HLA-B27 diseases (notably in reactive arthritis)(Liu et al., 2001) and thus a natural target in AAU. Interestingly both mice and rats transgenic for HLA-B27 only develop an

Ankylosing Spondylitis-like inflammatory disease when exposed to non-sterile conditions.

In the eye the expression of TLR-4 and its associated LPS receptor complex is limited to DC-like APC found in perivascular and sub-epithelial locations where they can potentially respond to either intraocular or blood-borne LPS (from Gram negative bacteria); indeed this is used to induce a hyperacute anterior uveitis in the model endotoxin-induced uveitis (EIU)(Rosenbaum et al., 1980). It is proposed that the precipitating event in acute anterior uveitis may be an inappropriate TLR-4-mediated response within the eye, driven by either LPS or endogenous TLR-4 ligands. The self perpetuating autoimmune cycle may become established once LPS acts as an adjuvant for hypothetical self-antigens leading to full APC maturation and breakdown of peripheral tolerance. Additionally in the presence of inflammation and tissue damage endogenous TLR-4 ligands are generated which may reinforce the TLR-4 response (Chang et al., 2004). Alternative animal models of anterior uveitis include collagen-induced anterior uveitis (CAU) induced by a type I collagen fragment (Bora et al., 2004)and experimental melanin induced uveitis induced by choroid derived melanin-associated antigen (Chan et al., 1994)(Figure 1.10).

The situation with regard to intermediate and posterior uveitis is even more complex. First it is a matter of debate as to whether the various clinical entities are different diseases or part of the spectrum of a single pathological process. Evidence for the former view comes from the clinical, prognostic, and genetic differences between some types of uveitis, for example, pars planitis (a form of intermediate uveitis with good prognosis) and the strongly HLA-A29 associated bird-shot retinochoroidopathy

(a form of posterior uveitis with poor prognosis). The interesting possibility that these are artificial groupings to describe a spectrum of inflammatory response has been recently reviewed by Forrester (Forrester, 2007) who points out that in animal models (summarised in table 1.10) the induction of autoimmune intraocular inflammation by ocular antigens can lead to a spectrum of disease with strong similarities to the range of human uveitis.

Animal models such as experimental autoimmune uveitis (the most widely used model of posterior uveitis; recently reviewed (Kerr et al., 2008)) have demonstrated the capacity of ocular antigens (notably retinal-S antigen and interphotoreceptor retinoid binding protein) to induce uveitis when immunised with an adjuvant (usually a Mycobacteria tuberculosis extract such as Complete Freund's Adjuvant)(Table 1.10). Modifications of this model (species, genetic background, immune status) result in variations in clinical phenotype which can be used to mimic acute monophasic, acute recurrent, and chronic disease. Traditionally regarded as a Th1 disease, it has become increasingly apparent that Th17 cells may also play a critical role(Peng et al., 2007). This switch in paradigm (similar to that which has occurred with Experimental Autoimmune Encephalomyelitis (EAE)(Chen et al., 2006)) explains why switching off IFNγ alone does not cure EAU(Raveney et al., 2008), and why the presence of IL-6 (in the constitutively TGFβ2 rich ocular microenvironment) may be so central to the development of EAU and possibly human uveitis (Peng et al., 2007; Amadi-Obi et al., 2007). Despite this recent change in focus it should be noted that EAU can also be induced in IL-17 knock-out mice, and thus it seems that both pathways have a pathogenic role to play (Luger et al., 2008).

More recently spontaneous models have been developed which, it is argued, better model the spontaneous nature of human disease. These models include spontaneous uveoretinitis in nu/nu mice after thymic reconstitution at 4wks of age (Ichikawa et al., 1991), bird-shot retinopathy-like uveitis in mice made transgenic for HLA-A29 (Szpak et al., 2001), or the introduction of a foreign antigen (eg Hen egg lysozyme (HEL)) under an ocular promoter (eg the αcrystallin promoter) with either the adoptive transfer of specific T cells or crossing onto a mouse transgenic for the specific TCR (eg anti-HEL)(Zhang et al., 2003; Lambe et al., 2007).

| **Model** | **Antigen** | **Adjuvant** | **Animal** |
|---|---|---|---|
| Experimental autoimmune uveitis (EAU) | Retinal S-Ag or IRBP (bovine) | CFA | Mouse or rat |
| Endotoxin-induced uveitis (EIU) | LPS (systemic or intravitreal) | As for Antigen | Mouse or rat |
| Mycobacterium tuberculosis adjuvant-induced uveitis (MTU) | Mycobacterium tuberculosis adjuvant (intravitreal) | As for Antigen | Mouse |
| Experimental melanin induced uveitis (EMIU) | Melanin (bovine) | Hunter's adjuvant | Rat |
| Experimental Autoimmune Encephalomyelitis (EAE) | Myelin basic protein | CFA | Rat |
| Collagen-induced autoimmune uveitis (CAU) | Type I Collagen (bovine) | CFA<br>Pertussis toxin | Rat |

**Table 1.10 Outline of commonly used animal models of uveitis, their antigens and adjuvants.**
Abbreviations: IRBP = interphotoreceptor retinoid binding protein; CFA = complete Freund's adjuvant

In human uveitis evidence for the role of autoantigens is less complete. De Smet noted lymphocyte stimulation responses to peptide determinants of retinal S antigen in a range of uveitis conditions, being most frequent in uveitis associated with Behcet's disease or sarcoidosis (de Smet et al., 1990; de Smet et al., 2001). Attempts to identify responding T cells in the peripheral blood of uveitis patients are extremely challenging but have suggested a higher rate of responding cells in uveitis patients vs controls (0-400/$10^7$ cells vs 0-4/$10^7$ cells)(de Smet et al., 1998), and, at least in Behcet's patients a thirty-fold expansion of the retinal S-Ag specific T cell pool after an episode of intraocular inflammation (de Smet and Dayan, 2000). No difference in serum levels of anti-retinal S Ag or anti-IRBP have been observed between patients and controls (Chan et al., 1985; Doekes et al., 1987).

### 1.11.4 Immune privilege and inflammation

Does AqH still have immunosuppressive properties in the presence of inflammation? In the murine model of experimental autoimmune uveitis, Ohta observed that blood ocular barrier breakdown is associated with increased levels of IL-6 and loss of the capacity to suppress anti-CD3 driven T cell proliferation at the onset of disease (day 11 after inoculation with IRBP). However at day 17 when disease was progressively severe AqH recovered the ability to suppress anti-CD3 driven T cell proliferation associated with a fall in IL-6 and in the presence of TGFβ-1 (from blood) and TGFβ-2 (from the eye)(Ohta et al., 2000a). Subsequently using the model of endotoxin induced uveitis the same group found that 6 to 24 hours after LPS injection AqH lost its capacity to suppress T-cell activation associated with an increase in IL-6 measured in the AqH and high levels of IL-6 mRNA in the iris/ciliary body tissue.

Neutralization by the addition of IL-6 specific antibodies to inflamed AqH led to recovery of its capacity to suppress T-cell activation. Similarly CD3-induced T cell proliferation suppressed by exogenous TGFβ2 (at 0.5 or 5 ng/ml) could be overcome by the addition of IL-6 (at 1 or 10ng/ml). Additionally they noted that the induction of antigen-specific anterior chamber–associated immune deviation (ACAID) in normal mice was prevented by the addition of IL-6 into the anterior chamber (Ohta et al., 2000b). Conversely using a different model based on the injection of LPS into the vitreous cavity of BALB/c mice, Mo and colleagues found that even at the peak of intraocular inflammation (9h) these eyes would both permit the proliferation of allogeneic tumour cells and support ACAID, and their AqH would still strongly inhibit T cell activation.(Mo and Streilein, 2007)

## 1.12 Objectives

In this thesis, I set out to achieve the following:

- To develop an *in vitro* model of DC differentiation, maturation and function that would best reflect the microenvironment of the human eye.
- To study the effects of non-inflammatory AqH (or its significant components) on DC phenotype and function.
- To compare the effects of non-inflammatory AqH with AqH from patients with uveitis.
- To consider the effects of treatment on the ocular microenvironment as reflected by the effects of AqH from patients on or off treatment at the time of sampling.
- To establish whether DC can be identified in aqueous humour from uveitis patients, and to characterize their phenotype.
- To identify the mechanisms within the eye that regulate DC maturation under both non-inflammatory conditions and during uveitis.

# 2 MATERIALS AND METHODS

## 2.1 List of reagents

### 2.1.1 Media and solutions

All Sigma-Aldrich, Irvine, UK unless otherwise specified.

| | |
|---|---|
| RPMI medium | RPMI (Roswell Park Memorial Institute) 1640 L-glutamine (1.64mM), benzylpenicillin (40U/ml), streptomycin (0.4mg/ml) and HEPES buffer (10mM) |
| RPMI/ITS+3 | RPMI medium with 1% ITS+3 liquid media supplement; ITS+3 liquid media supplement contains 1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin (substantially iron-free), 0.5 μg/ml sodium selenite, 470 μg/ml linoleic acid, 470 μg/ml oleic acid and 50 mg/ml bovine serum albumin (equivalent to 2 moles each of linoleic acid and oleic acid per mole of albumin). |
| RPMI/ITS+3/NEAA/Na pyruvate | RPMI medium with 1% ITS+3 liquid media supplement, 1% Nonessential amino acids, 1% Sodium pyruvate<br>Non-essential amino acid solution (100x) stock solution contains 0.89g/l L-alanine, 1.5g/l L-asparagine, 1.33g/l L-aspartic acid, 1.47 g/l glutamic acid, 0.75 g/l glycine, 1.15 g/l L-proline and 1.05 g/l L-serine.<br>Sodium pyruvate stock solution contains 100mM sodium pyruvate solution. |
| RPMI/1% BSA | RPMI medium with 1% bovine serum albumin (BSA) |

| | |
|---|---|
| RPMI/10% HS | RPMI medium with 10% pooled human AB+male serum (HS; HD Supplies, Aylesbury,UK) |
| RPMI/10% FCS | RPMI medium with 10% pooled fetal calf serum (Biosera, Ringmer, UK) |
| PBS | Phosphate buffered saline contains 8g/l NaCl, 0.2g/l KCl, 1.15 g/l $Na_2HPO4$, 0.2g/l $KH_2PO4$ in distilled $H_2O$; prepared as 1 PBS tablet per 100ml distilled $H_2O$ (tablets supplied by Oxoid, Basingstoke, UK) |
| MACS buffer | Phosphate buffered saline (PBS) with 0.5%BSA, 2mM ethylenediamine tetra-acetic acid (EDTA) |

### 2.1.2 Cytokines and prostaglandins

Recombinant human IL-4 (Immunotools, Friesoythe, Germany)

Recombinant human GM-CSF (Immunotools)

Recombinant human IL-1β (Peprotech, London, UK)

Recombinant human IL-6 (Immunotools)

Recombinant human TNFα (Peprotech)

Recombinant human PGE2 (Sigma-Aldrich).

Recombinant human TGFβ1 (R & D Systems, Abingdon, UK)

Recombinant human TGFβ2 (Peprotech)

Recombinant human TGFβ3 (R & D Systems)

Recombinant human αMSH (Bachem, Weil am Rhein, Germany)

Recombinant human VIP (Bachem)

### 2.1.3 Antibodies

| Target | Conjugate | Species | Isotype | Clone | Company | Cat No | Dilution |
|---|---|---|---|---|---|---|---|
| CD1a | PB | Mouse | IgG1 | HI149 | Biolegend | 300117/8 | 1in 50 |
| CD1c (BDCA-1) | APC | Mouse | IgG2a | AD5-8E7 | Miltenyi | 130-090-903 | 1in 10 |
| CD4 | PECy5 | Mouse | IgG2a | 53.5 | Caltag | MHCD0406 | 1 in 50 |
| CD11b | APCCy7 | Mouse | IgG1 | ICRF44 (44) | BD Pharmingen | 557754 | 1 in 40 |
| CD11c | PECy7 | Mouse | IgG1 | 3.9 | Biolegend | 301607 | 1 in 10 |
| CD14 | FITC | Mouse | IgG1 | MEM-18 | Immunotools | 21270143 | 1 in 20 |
| CD14 | APCCy7 | Mouse | IgG2b | MφP9 | BD Pharmingen | 557831 | 1 in 20 |
| CD16 | FITC | Mouse | IgG1 | LNK16 | Immunotools | 21279163 | 1 in 10 |
| CD19 | FITC | Mouse | IgG1 | LT19 | Immunotools | 21270193 | 1 in 10 |
| CD40 | PECy5 | Mouse | IgG1 | 5C3 | BD Pharmingen | 555590 | 1 in 5 |
| CD45RA | PETR | Mouse | IgG1 | 2H4LDH 11LDB9 | Beckman Coulter | PN IM2711 | 1 in 80 |
| CD45RO | FITC | Mouse | IgG2a | UCHL1 | Dako | F0800 | 1 in 20 |
| CD56 | FITC | Mouse | IgG2a | MEM-188 | Immunotools | 21270563 | 1 in 10 |
| CD80 | FITC | Mouse | IgG1 | 2D10 | Biolegend | 305206 | 1 in 10 |
| CD80 | PE | Mouse | IgG1 | L307.4 | BD Pharmingen | 557227 | 1 in 20 |
| CD80 | Biotin | Mouse | IgM | BB1 | BD Pharmingen | 555682 | 1 in 50 |
| CD83 | PE | Mouse | IgG1 | HB15 | Serotec | MCA1582PE | 1 in 10 |
| CD86 | PECy5 | Mouse | IgG1 | 2331 FUN-1 | BD Pharmingen | 555659 | 1 in 20 |
| CDw137L (4-1BBL) | PE | Mouse | IgG1 | 5F4 | Biolegend | 311503/4 | 1 in 10 |
| CD209 (DCSIGN) | FITC | Mouse | IgG2b | DCN46 | BD Pharmingen | 551264 | 1 in 5 |
| CD275 (ICOSL) | Biotin | Mouse | IgG2b | 2D3 | Biolegend | 309405/6 | 1 in 50 |
| CD303 (BDCA-2) | FITC | Mouse | IgG1 | AC144 | Miltenyi | 130-090-510 | 1in 10 |
| CCR5 (CD195) | PECy7 | Mouse | IgG2a | 2D7 / CCR5 | BD Pharmingen | 557752 | 1 in 10 |
| CCR7 | FITC | Mouse | IgG2a | 150503 | R+D | FAB197F | 1 in10 |

**Table 2.1 List of primary antibodies used for flow cytometry (against surface molecules other than MHC).** Abbreviation used Allophycocyanin (APC), Cyanine 5 (Cy5), Cyanine 7 (Cy7) Fluorescein isothiocyanate (FITC), Pacific blue (PB), Phycoerythrin (PE), Phycoerythrin Texas Red (PETR)

| Target | Conjugate | Species | Isotype | Clone | Company | Cat No | Dilution |
|---|---|---|---|---|---|---|---|
| HLA-A,B,C | PB | Mouse | IgG2a | W6/32 | Biolegend | 311417/8 | 1 in 50 |
| HLA-DR | FITC | Mouse | IgG2a | L243 | BD Pharmingen | 347400 | 1 in 20 |
| HLA-DR | PE | Mouse | IgG2a | L243 | BD Pharmingen | 347401 | 1 in 50 |
| HLA-DR | PETR | Mouse | IgG1 | Immu-357 | Beckman Coulter | PN IM3636 | 1 in 20 |

**Table 2.2 List of primary antibodies used for flow cytometry (against MHC)**

| Target | Conjugate | Species | Isotype | Clone | Company | Cat No | Dilution |
|---|---|---|---|---|---|---|---|
| Biotin | Qdot 525 | Mouse | N/A | N/A | Invitrogen | Q10141MP | 1 in 50 |
| Biotin | Qdot 605 | Mouse | N/A | N/A | Invitrogen | Q10101MP | 1 in 50 |

**Table 2.3 List of streptavidin-conjugated secondary antibodies used for flow cytometry**

| Target | Conjugate | Species | Isotype | Clone | Company | Cat No | Dilution |
|---|---|---|---|---|---|---|---|
| Irrelevant | FITC | Mouse | IgG1 | 203 | Immunotools | 21335023 | 1 in 20 |
| Irrelevant | PE | Mouse | IgG1 | 203 | Immunotools | 21335014 | 1 in 10 |
| Irrelevant | PETR | Mouse | IgG2b | MOPC-195 | Caltag | MG2b17 S | 1 in 40 |
| Irrelevant | PECy5 | Mouse | IgG2a | UPC-10 | Immunotools | 21335025 | 1 in 20 |
| Irrelevant | PECy7 | Mouse | IgG2a | 5.205 | Caltag | MG2a12 S | 1 in 10 |
| Irrelevant | PB | Mouse | IgG2a | 5.205 | Caltag | Mg2a 28 S | 1 in 10 |
| Irrelevant | APC Cy7 | Mouse | IgG1 | MOPC-21 | BD Pharmingen | 557873 | 1 in 20 |
| Irrelevant | Biotin | Mouse | IgG1 | DAK-GO1 | Dako | X0945 | 1 in 10 |

**Table 2.4 List of isotype control antibodies used for flow cytometry**

| Target | Conjugate | Species | Isotype | Clone | Company | Cat No | Dilution |
|---|---|---|---|---|---|---|---|
| FoxP3 | PE | Rat | IgG1 | eBR2a | eBioscience | 12-4321-73 | 1 in 5 |
| Irrelevant | PE | Rat | IgG1 | PCH101 | eBioscience | 12-4776-73 | 1 in 5 |

**Table 2.5 List of antibodies used for flow cytometry against intracellular targets**

### 2.1.4 Other reagents

Ficoll-Paque Plus (GE Healthcare Biosciences, Amersham, UK)

MACS CD14-microbeads (Miltenyi Biotec, Surrey, UK)

MACS Naïve CD4+ T cell isolation microbeads (Miltenyi Biotec)

MACS Memory CD4+ T cell isolation microbeads (Miltenyi Biotec)

MACS CD8+ T cell isolation microbeads (Miltenyi Biotec)

MACS Myeloid Dendritic Cell isolation microbeads (Miltenyi Biotec)

Carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Invitrogen)

Cortisol ($\geq$ 98% HPLC Hydrocortisone; Sigma-Aldrich)

RU486 (Mifepristone; Sigma-Aldrich)

SB431542 hydrate (Sigma-Aldrich)

Flow Check Fluorospheres (Beckman Coulter Inc)

Counting beads (CALTAG/Invitrogen, Paisley, UK)

Propidium iodide (Sigma-Aldrich)

### 2.1.5 Other consumables

96-well suspension culture plates sterile U bottom with lid (Greiner Bio-one Ltd, Stonehouse, UK)

96-well adherent culture plates sterile flat bottom with lid (Sarstedt, Leicester, UK)

24-well adherent culture plates sterile flat bottom with lid

Tissue culture flasks for adherent cells (25cm$^2$ surface area size) (Sarstedt)

96-well multiscreen-MIC transmigration plates (Millipore, Watford, UK)

## 2.2 Aqueous humour samples

Ethical approval for the collection of AqH and matched peripheral blood from patients either with uveitis (cases) or cataract (controls) had previously been obtained in 1996 from West Birmingham Local Research Ethics Committee. I oversaw the successful renewal of both our ethical application (Dudley Local Research Ethics Committee) and our research and development applications, including writing the new patient information sheets and consent forms (see Appendix).

A team of ophthalmologists at the Birmingham and Midland Eye Centre led by Professor P.I. Murray regularly undertake AqH sampling both from patients with uveitis (for clinical and research purposes) and from patients attending for cataract surgery (for research purposes). Under the previous and current ethical application we have been able to collect between 20-80 uveitis AqH samples each year. As part of this team, and as the named contact for reporting accrual of patients to the United Kingdom Clinical Research Network (UKCRN) I oversaw the recruitment of 188 uveitis patients (of whom 182 had successful sampling of peripheral blood and 87 of aqueous humour).

Clinical data recorded comprised anatomical type of uveitis (Table 1.5), aetiology (where known), whether unilateral or bilateral, duration of current episode, duration of disease, current medical treatment (if any) and anterior chamber cellular activity (as per Standardization of Uveitis Nomenclature 2005 classification (Jabs et al., 2005)). Anterior chamber flare was not recorded at the outset of the study, but has since been added to the minimum data set. AqH sampling from patients with active

uveitis was performed at the slit lamp following a previously published protocol(Cheung et al., 2004) and with typical sampling volumes of 40-100µl. The procedure was only carried out by the following trained personnel: Prof P.I.Murray, Mr I.J.Khan, Mr J.Abbott, Mr P.Tomlins, Miss K.Oswal and myself.

Patients attending for routine cataract surgery provided the group of non-inflammatory control AqH samples. AqH sampling from the control group was performed in theatre at the start of their cataract surgery, using a 1ml syringe with a 27 gauge needle via either the main incision or a paracentesis port prior to any other manipulation of the anterior chamber, obtaining samples of 50-100µl. Working with Professor P.I. Murray and Miss S. Rauz we recruited 311 cataract patients (of whom all 311 had uncomplicated sampling of aqueous humour) (Figure 2.1). There were no significant adverse events following peripheral blood or aqueous humour sampling in either the uveitis or the control group.

Each sample was centrifuged (300g, 5mins, 20˚C), before aspirating the supernatant for freezing at -80ºC in aliquots. Detailed demographic data including classification of uveitis (anatomical and clinical, and specific uveitis syndromes), and therapy (where relevant) was documented. The cells were resuspended in 100µl of RPMI/10% heat inactivated fetal calf serum (HIFCS) prior to staining for analysis by flow cytometry.

During the period 1/11/06 to 1/9/09 (the period during which we were specifically recruiting for this project) we recruited 188 patients with uveitis with a mean $\pm$ SD age of 52yrs 9mths $\pm$ 17yrs 4mths. Of these 107 had anterior uveitis, two intermediate uveitis, four posterior uveitis and 54 panuveitis. Of this group of 188 patients, 87

underwent sampling of AqH (Figure 2.1). The clinical features of the group who underwent AqH sampling are set out in Table 2.6.

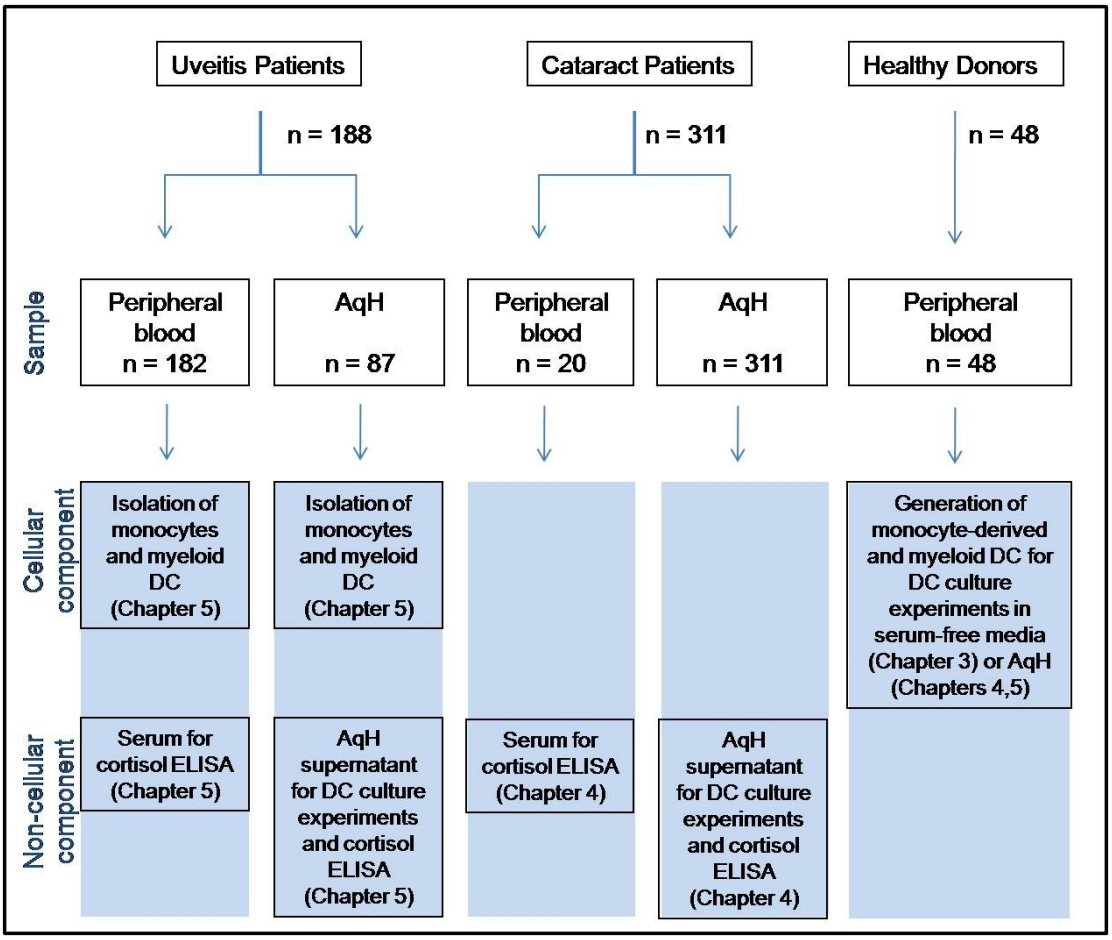


**Figure 2.1 Flow-chart highlighting the recruitment of uveitis patients, cataract patients and healthy donors, and the utilization of samples as part of this study (UKCRN4654).**
Note that all DC culture experiments were based on monocyte-derived DC or myeloid DC from healthy controls. Non-inflammatory 'control' AqH was taken at the time of surgery from patients without uveitis attending for routine cataract extraction.

| Parameter | Aqueous humour samples | |
|---|---|---|
| **Number recruited** | 87 | |
| **Age** (Mean ± SD) | 49.3 ±16 yrs | |
| **Anatomical classification** | Anterior<br>Intermediate<br>Posterior<br>Panuveitis | 72 /82<br>0<br>0<br>10 /82 |
| **Eyes affected** | Unilateral<br>Bilateral | 68 /78<br>10 /78 |
| **Course** | Acute (first episode)<br>Recurrent<br>Chronic | 17 /82<br>64 /82<br>1 /82 |
| **Aetiology** | Idiopathic<br>HLA-B27 or related disease<br>Fuchs Heterochromic Uveitis<br>Viral (Herpes family)<br>Vogt-Koyanagi-Harada disease | 52 /82<br>18 /82<br>7 / 82<br>2 / 82<br>1 / 82 |
| **Duration of episode** (Mean ± SD) | 5.9 ± 4.9 days | |
| **Treatment** | Nil<br>Topical corticosteroid<br>Oral corticosteroid<br>Intravenous corticosteroid | 49 /77<br>24 /77<br>2 /77<br>2 /77 |
| **Activity (cells)** | 0<br>1+<br>2+<br>3+<br>4+ | 6 /82<br>20 /82<br>24 /82<br>29 /82<br>3 /82 |

**Table 2.6 Demographic and clinical features of patients with uveitis undergoing AqH sampling during the course of this study.**
All data (including duration of episode and treatment) was recorded at the time of the sampling and therefore does not reflect subsequent disease course. Anterior chamber cellular activity was graded as per Standardization of Uveitis Nomenclature 2005 classification criteria(Jabs et al., 2005).

## 2.3 Purification of cells

### 2.3.1 Purification of peripheral blood mononuclear cell fraction from whole blood

Heparinised peripheral blood samples were obtained from healthy donors (recruited from amongst work colleagues). Informed consent was taken in accordance with the Human Tissue Act 2004. Peripheral blood was diluted 1:1 with "RPMI medium" comprising RPMI (Roswell Park Memorial Institute) 1640 supplemented with L-glutamine (1.64mM), benzylpenicillin (40U/ml), streptomycin (0.4mg/ml) and HEPES buffer (10mM)(all Sigma-Aldrich). Diluted blood was layered on 8ml Ficoll-Paque Plus (GE Healthcare Biosciences) in 25ml universal tubes and centrifuged at 300g, 20°C, 30 mins, without brake. The peripheral blood mononuclear cell layer was transferred to fresh universal tubes and washed four times in RPMI medium with centrifugation of 300g at 20°C for 8mins each time.

The yield of peripheral blood mononuclear cells (PBMC) was calculated by use of the Improved Neubauer haemocytometer (Weber Scientific) as described by the manufacturer. The range of yield was $5 \times 10^5 - 1.5 \times 10^6$ per ml of peripheral blood.

### 2.3.2 Purification of monocytes (CD14+)

Monocytes were then isolated using MACS CD14-microbeads (Miltenyi Biotec) as described by the manufacturer. MACS Microbeads are superparamagnetic particles precoated with a specific antibody (here monoclonal anti-CD14) to enable positive or negative selection of cell populations when passed over a MACS column placed within a strong magnetic field. PBMC were incubated for 15mins at 4°C with 20µl

beads/$10^7$ cells and 80µl MACS buffer /$10^7$ cells. MACS buffer was filter-sterilized and consisted of phosphate buffered saline (PBS; Oxoid limited), 0.5% bovine serum albumin of 98% purity (BSA; Sigma-Aldrich) and 2mM ethylenediamine tetra-acetic acid, (EDTA; Sigma-Aldrich). The cells were then washed once with MACS buffer (centrifugation of 300g, 4°C , 8 mins), the supernatant pipetted off to a dry pellet, and cells resuspended in 500µl MACS buffer/$10^8$ cells. An LS MACS separation column was prepared by a single rinse with 3ml MACS buffer and placed on a QuadroMACS separator magnet. The resuspended cells were passed over the column, and the negative fraction eluted with a series of three x 3ml MACS buffer washes. The column was then removed from the magnet and the CD14-microbead positive fraction eluted with 5ml MACS buffer and firm column pressure from the plunger.

The yield of monocytes was calculated by use of the haemocytometer. The range of yield was $5 \times 10^4 – 1.5 \times 10^5$ per ml of peripheral blood. These populations were highly enriched with purity of >95% as determined by percentage of cells expressing CD14 analysed by flow cytometry (Figure 2.2).

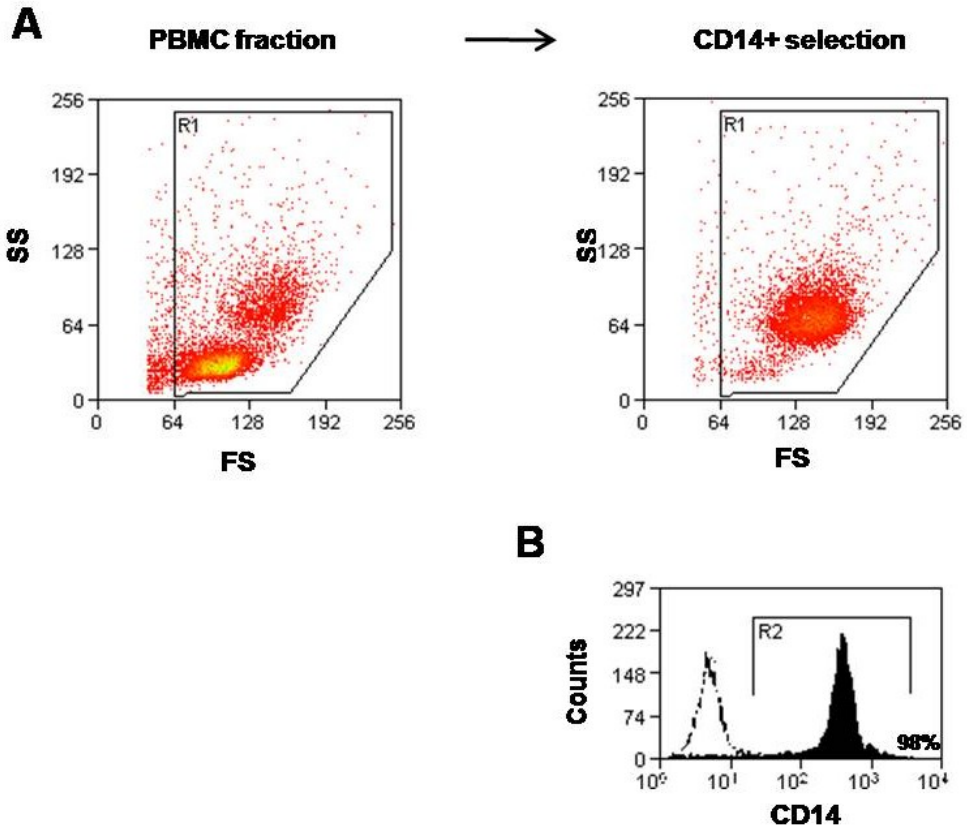


**Figure 2.2 Selection of CD14+ monocytes by CD14-microbeads**
CD14+ monocytes were isolated by MACS CD14-microbeads as described in the text, and purity checked by flow cytometry with staining for CD14. (A) Scatter plots show distinctive high forward scatter (FS) vs side scatter (SS) of monocytes compared to predominant lymphocyte population in peripheral blood mononuclear cell (PBMC) fraction. (B) Gating on region 1 (R1) for all cells (ie excluding debris) shows the purified fraction (filled-in area) to be 98% positive for CD14+ expression. Background staining was measured by an isotype control (broken line).

### 2.3.3 Purification of myeloid dendritic cells (BDCA-1+)

Myeloid dendritic cells were also isolated using MACS technology in a two-stage process involving the depletion of CD19+ B cells prior to the positive selection of BDCA-1+ cells; the depletion step is required due to BDCA-1 also being present on a subset of B cells. PBMC were incubated for 15mins at 4°C with 200µl MACS buffer /$10^8$ cells, 100µl CD19 beads/$10^8$ cells, 100µl FcR blocking reagent/$10^8$ cells, and 100µl BDCA-1-Biotin antibody /$10^8$ cells. The cells were then washed once with MACS buffer (centrifugation of 300g, 4°C, 8 mins ), before being resuspended in 500µl/$10^8$ cells MACS buffer and passed over a prepared LD MACS depletion column (single preparatory rinse with 2ml MACS buffer) placed on a QuadroMACS separator magnet. The B cell-depleted fraction was then eluted with a series of two x 1ml MACS buffer washes. The B-cell depleted fraction was then washed again with MACS buffer (centrifugation of 300g, 4°C ), before being resuspended in 400µl MACS buffer /$10^8$ cells and 100µl anti-biotin beads/$10^8$ cells and incubated for 15mins at 4°C. The cells were then washed once more with MACS buffer (centrifugation of 300g, 4°C , 8 mins), the supernatant pipetted off to a dry pellet, and cells resuspended in 500µl MACS buffer/$10^8$ cells before being passed over an MS MACS separation column placed on a MiniMACS separator magnet. Having prepared the MS column (by a single rinse with 500µl MACS buffer), the negative fraction was washed through with a series of three x 500µl MACS buffer. The column was then removed from the magnet and the BDCA-1-microbead positive fraction eluted with 1ml MACS buffer and firm column pressure from the plunger. To improve purity, elution was performed directly into a second prepared MS column, the negative

fraction was again eluted (three x 500µl MACS buffer) before final collection of the BDCA-1-microbead positive fraction by the addition of 1ml MACS buffer and firm column pressure from the plunger.

The yield of myeloid DC was calculated by use of the haemocytometer. The range of yield was $2 \times 10^3 - 5 \times 10^3$ per ml of peripheral blood. These populations were highly enriched with purity of $\geq$80% as determined by percentage of cells being BDCA-1+CD19- as analysed by flow cytometry (Figure 2.3).

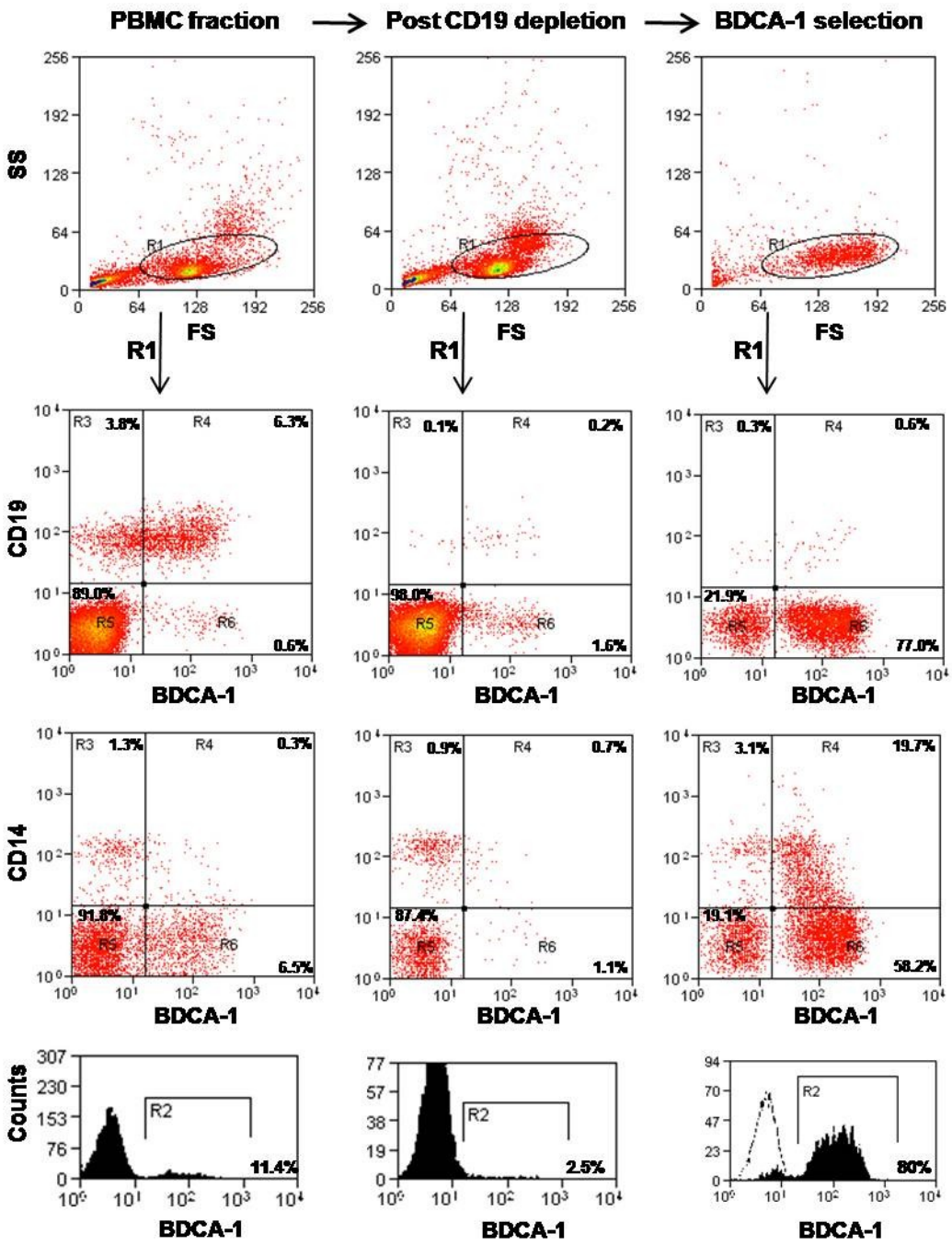


**Figure 2.3 Selection of BDCA-1+ myeloid DC by microbeads**
BDCA-1+ myeloid DC were isolated by MACS myeloid DC isolation kit as described in the text, and purity checked by flow cytometry with staining for BDCA-1, CD14 and CD19. Scatter plots show distinctive intermediate forward scatter (FS) and side scatter (SS) when compared to monocytes or lymphocytes. CD19+ B cells (including those expressing BDCA-1) are successfully depleted, enabling enrichment of the BDCA-1+ CD19- myeloid DC to 80% purity.

### 2.3.4 Purification of naive (CD45RA+) CD4+ T cells, memory (CD45RO+) CD4+ T cells and CD8+ T cells

Naive and memory CD4+ and CD8+ T cell subsets were isolated using MACS Isolations kits. This involved the depletion of non-CD4+ cells and memory T cells (for purification of naive CD4+ T cells), the depletion of non-CD4+ cells and naive T cells (for purification of memory CD4+ T cells) or the depletion of all non-CD8+ T cells (for purification of CD8+ T cells). In the CD4+ T cell isolation kits antibiotin-microbeads are used after first labelling the PBMC fraction with a cocktail of biotin-conjugated antibodies against CD8, CD14,CD16, CD36, CD56, CD123, TCRγδ, glycophorin A and either CD45RO (if isolating naive CD4+ T cells) or CD45RA (if isolating memory CD4+ T cells). The CD8+ T cell isolation kit is identical but comprises a cocktail of biotin-conjugated antibodies against CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCRγδ, and glycophorin A.

PBMC were incubated for 10mins at 4°C with 10µl biotin-antibody cocktail/$10^7$ cells and 40µl MACS buffer /$10^7$ cells. A further 30µl MACS buffer/$10^7$ cells and 20µl anti-biotin microbeads /$10^7$ cells were then added. The cells were then washed once with MACS buffer (centrifugation of 300g, 4°C , 8 mins), the supernatant pipetted off to a dry pellet, and cells resuspended in 500µl MACS buffer/$10^8$ cells before being passed over a prepared LS MACS separation column placed on a QuadroMACS separator magnet. The negative fraction (containing the population of interest) was eluted with a series of three x 3ml MACS buffer washes.

The yield of CD4+ or CD8+T cells was calculated by use of the haemocytometer. The range of yield was 2 x $10^5$ – 4 x $10^5$ per ml of peripheral blood for naive CD4+ T

cells, 5 x $10^4$ – 2 x $10^5$ per ml of peripheral blood for memory CD4+ T cells and 5 x $10^4$ – 5 x $10^5$ per ml of peripheral blood for CD8+ T cells. These populations were enriched with purity of >90% as determined on flow cytometry by percentage of cells being CD4+ CD45RA+ (for naive CD4+ T cells) and CD4+CD45RO+ (for memory CD4+ T cells). For both the naive and memory CD4+ populations, the contaminating population (comprising 3-6%) were CD4- cells, and were distributed evenly between positive and negative fractions with regard to the CD45 splice variant of interest. For CD8+ T cells purity was ≥80% (Figure 2.4).

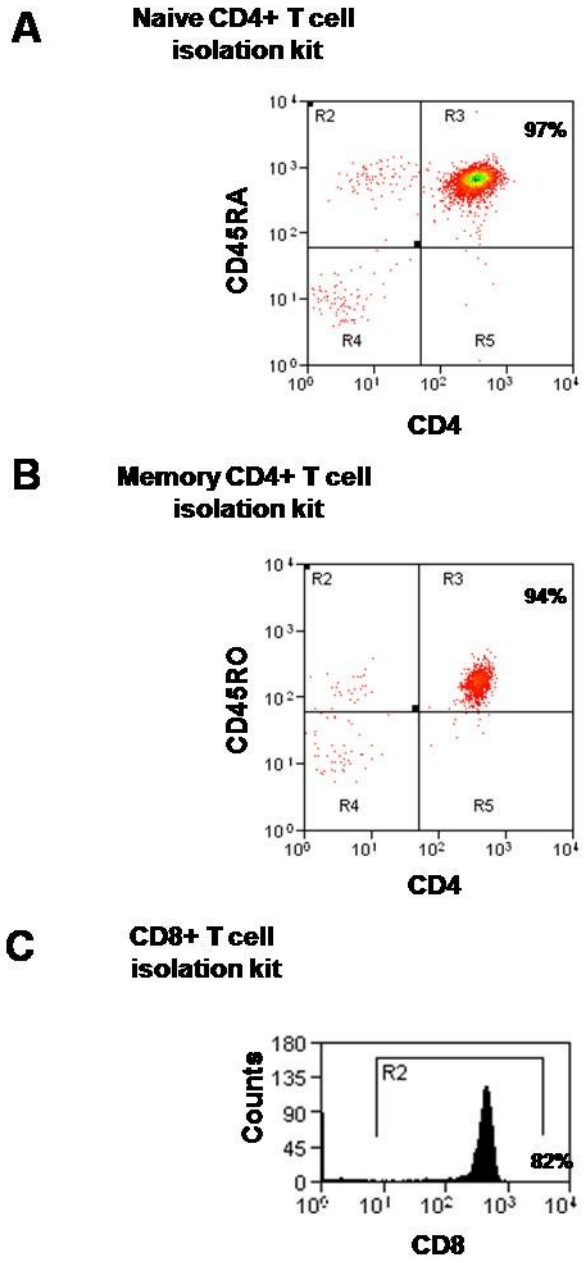


**Figure 2.4 Selection of T cell subsets by microbeads**
Naive CD4+, memory CD4+ and CD8+ T cell subsets were isolated by MACS isolation kits as described in the text. Purity was checked by flow cytometry with staining for (A) CD4 and CD45RA, (B) CD4 and CD45RO or (C) CD8 as appropriate. Untouched naive CD4+ and memory CD4+ T cells could be isolated to ≥94% purity; untouched CD8+ T cells could be isolated to >80% purity).

## 2.4 Cell culture

### 2.4.1 Standard culture conditions

Human monocyte-derived DC were generated using well-established protocols(Sallusto and Lanzavecchia, 1994) . The standard culture conditions are described below. Variations from this protocol necessitated by experimental design are indicated in the text. Monocytes were resuspended in RPMI/10% pooled human AB+male serum (10%HS; HD Supplies, Aylesbury, UK) at 2ml medium/$10^6$ cells supplemented with recombinant human IL-4 (500U/ml) and GM-CSF (1000U/ml)(both Immunotools, Friesoythe, Germany). These suspensions were placed in T25 flasks (Sarstedt, Leicester, UK) at 2.5 x $10^6$ cells/flask and cultured at 37°C with 5% $CO_2$. At day 3, 2ml medium was removed and 2.5ml fresh medium (ie RPMI/10%HS) with IL-4 (final concentration 500U/ml) and GM-CSF (final concentration 1000U/ml) was added. At day 6 immature DC (iDC) were harvested by shaking the flask gently and collecting (and counting) non-adherent cells.

### 2.4.2 Pooled human serum vs serum-free culture conditions

To test the effect of alternative serum-free media on the differentiation and activation of DC the following serum-free media were used: RPMI/ITS+3, RPMI/ITS+3/NEAA/Na pyruvate, RPMI/1%BSA. These were compared to standard RPMI/10% pooled human serum.

Cultures were carried out in 96 well flat-bottomed plates (Sarstedt) with initial counts of 50,000 cells in 100µl medium supplemented with IL-4 and GM-CSF (at standard concentration) per well at 37°C with 5% $CO_2$. At day 3, 25µl medium was removed and 50µl fresh medium with IL-4 and GM-CSF (standard concentrations) was added.

At day 6 immature DC (iDC) were harvested, and both number and surface expression of HLA-DR, CD80 and CD86 were measured by flow cytometry.

### 2.4.3 Pooled human serum vs alternative sera culture conditions

To test the effect of alternative serum-free media on the differentiation and activation of DC the following media were used: RPMI/10%HS, RPMI/10% heat inactivated pooled human serum (HIHS), RPMI/10% fetal calf serum (FCS) and RPMI/10% heat inactivated fetal calf serum (HIFCS). Heat inactivation comprised being heated to 56°C for 1 hour in a water bath.

Cultures were carried out in 96 well flat-bottomed plates with initial counts of 50,000 cells in 100µl medium supplemented with IL-4 and GM-CSF (at standard concentration) per well at 37°C with 5% $CO_2$. At day 3, 25µl medium was removed and 50µl fresh medium with IL-4 and GM-CSF (standard concentrations) was added. At day 6 immature DC (iDC) were harvested, and both number and surface expression of HLADR, CD80 and CD86 were measured by flow cytometry.

### 2.4.4 Pooled human serum vs autologous human serum culture conditions

Cultures were carried out under standard conditions but with the addition of RPMI supplemented with either 10% pooled human serum or autologous serum supplemented with IL-4 and GM-CSF (standard concentrations). Cultures were carried out in T25 flasks, fed at day 3 and harvested at day 6 as per standard. Autologous serum was prepared by placing 10ml heparinised blood on ice for 10mins, before centrifuging at 500g, 4°C, 10mins and keeping the supernatant. Surface

expression of HLADR, CD80 and CD86 was analysed as previously by flow cytometry.

## 2.5 DC maturation

Immature DC were cultured for a further 2d, under serum-free conditions, to induce maturation and/or modulation of their function. The standard culture conditions are described below. Any variations from this protocol necessitated by experimental design are indicated in the text.

### 2.5.1 Standard maturation conditions

At day 6 harvested DC were resuspended at $10^6$/ml in serum-free medium (RPMI/ITS+3/NEAA/Na pyruvate), and 500µl of DC suspension were added to each well of a 24 well plate (Sarstedt). To these wells were added either 500µl of the same serum-free medium alone or 500µl of a cocktail of the following inflammatory cytokines: IL-1β (final concentration 1µg/ml; Peprotech, London, UK), IL-6 (final concentration 200ng/ml; Immunotools), TNFα (final concentration 10ng/ml; Peprotech) and $PGE_2$ (final concentration 1µg/ml; Sigma-Aldrich).

### 2.5.2 Studies on the effect of non-inflammatory AqH

Since the volume of individual samples of AqH is a limiting factor, these experiments were carried out in smaller total volumes (80µl/well). Cultures of DC (monocyte-

derived or myeloid) were carried out in two matched 96 well round-bottomed plates (Greiner). After 2d plate 1 was harvested for analysis of surface molecule expression by flow cytometry, whilst plate 2 was used for the allogeneic proliferation assays. Experiments investigating the effects of non-inflammatory AqH were conducted using pooled samples of non-inflammatory AqH unless otherwise specified in the text. It was felt that under non-inflammatory conditions the properties of AqH from healthy individuals were likely to show sufficiently little variation, that pooling AqH would not mask its effects. In addition pooling of AqH was necessary to provide sufficient identical AqH within a single experiment to permit two plates (one for surface staining and one for allogeneic proliferation assay) of triplicate cultures of DC in the presence or absence of inflammatory cytokines with or without glucocorticoid blockade (or combined glucocorticoid and TGFβ blockade), an experimental design which requires 1920µl human AqH per experiment pooled from around 40 patients.

At day 6 DC cultured under standard conditions were harvested, counted (by haemocytometer) and resuspended at $5x10^5$ cells/ml in serum-free medium (RPMI/ITS+3/NEAA/Na pyruvate). 20µl of DC suspension (ie $10^4$ cells) was added to each well of a 96 well plate (Greiner) with 20µl of medium or 20µl cytokine cocktail described previously: IL-1β (final concentration 1µg/ml; Peprotech), IL-6 (final concentration 200ng/ml; Immunotools), TNFα (final concentration 10ng/ml; Peprotech) and $PGE_2$ (final concentration 1µg/ml; Sigma-Aldrich). AqH was added at 50% final concentration (ie 40µl per well) in all experiments. Plates were cultured for 2d at 37°C with 5% $CO_2$.

### 2.5.3 Studies on the effect of uveitis AqH

For experiments involving uveitis AqH, I opted not to pool specimens as I was concerned that the wide variation of clinical disease might be reflected in a diversity of properties of AqH which would be obscured if pooled. In these experiments comparison is therefore between the effects of medium alone, individual uveitis AqH samples and individual non-inflammatory AqH samples; each AqH group comprised at least six individual samples. Due to the volumes of AqH available this meant that each sample could only be tested once (ie replicates were not possible). For studies of surface molecule staining these cultures were carried out as described previously but using Terasaki plates (vWR International, Lutterworth, UK) in a total volume of 20µl. For allogeneic proliferation assays, DC were cultured in 96 well plates (Greiner) as described previously.

### 2.5.4 Studies on the effect of TGFβ and its inhibition

To compare the effects of TGFβ isoforms, 20µl of DC suspension (as above, ie $10^4$ cells) was added to each well of a 96 well plate (Greiner) with 20µl of medium or 20µl inflammatory cytokine cocktail as described previously. TGFβ isoforms were then added at a final concentration of 10ng/ml. TGFβ1 (R & D systems, UK), TGFβ2 (Peprotech) and TGFβ3 (R+D sytems) were compared. All experiments were carried out in a total volume of 80µl serum-free medium (RPMI/ITS+3/NEAA/Na pyruvate) and at 37°C with 5% $CO_2$.

To investigate the dose-response effect of TGFβ2, experiments were carried out as above but with TGFβ2 being added at a final concentration of 0.1, 1, 10 or 100ng/ml.

Inhibition of TGFβ was achieved with the synthetic molecule SB431542 which is a potent and selective inhibitor of TGFβ receptor type I kinases, specifically the Activin receptor-Like Kinases (ALK) -4, -5 and -7 which signal via phosphorylation of the transcription factors Smad-2 and Smad-3. In all experiments requiring TGFβ blockade, SB431542 was added at a final concentration of $10^{-7}$M.

### 2.5.5 Studies on the effect of cortisol and its inhibition

To study the effect of cortisol, 20µl of DC suspension (as above, ie $10^4$ cells) was added to each well of a 96 well plate (Greiner) with either 20µl of medium or 20µl inflammatory cytokine cocktail as described previously. Cortisol was then added at a final concentration $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$M. All experiments were carried out in a total volume of 80µl serum-free medium (RPMI/ITS+3/NEAA/Na pyruvate) at 37°C with 5% $CO_2$.

Inhibition of cortisol or other glucocorticoids (eg dexamethasone) was achieved with the synthetic molecule RU486 (also known as mifepristone) which is a potent inhibitor of both the glucocorticoid receptor and the progesterone receptor. In all experiments requiring glucocorticoid blockade RU486 was added at a final concentration of $10^{-7}$M.

## 2.6 Transmigration studies

Ninety-six well transmigration plates (Millipore (UK), Watford, UK) were used to determine DC migration to the chemokines CCL-19 (MIP-3β) and CCL5 (RANTES). Monocyte-derived DC were harvested at day 6 and placed into fresh serum-free medium culture for 48h under the conditions of interest (as described previously), but at a higher cell density of $8x10^4$cells/80µl medium in each well. After 48h DC were harvested, washed and resuspended in fresh serum-free medium at $8x10^4$ cells/200µl. 150µl of serum-free medium containing 0, 1, 10 or 100ng/ml of either CCL-19 (MIP-3β) and CCL5 (RANTES) was placed into the lower chambers of the 96 well transmigration plate. DC were placed in the top wells at $2x10^4$ cells in 50µl of serum-free medium., and the plate was incubated at 37ºC for 3h. The plate was then disassembled and the cells in each chamber harvested and added to FACS tubes containing 195µl of PBS/2%BSA and 5µl of counting beads, and counted by flow cytometry.

## 2.7 Carboxyfluorescein diacetate, sucinnimidyl ester (CFSE) labelling of T cells

The population of interest (either naive CD4+ T cells, memory CD4+ T cells or CD8+ T cells) was labelled with carboxyfluorescein diacetate, sucinnimidyl ester (CFSE; Invitrogen) as follows. The cells were kept in a universal tube and were washed twice with 10ml sterile PBS, before being resuspended in PBS at 50ul/$10^6$ cells. 2.5µl of 10M CFSE was added to 5ml of sterile PBS to give a 5uM solution. This was added at a 1:1 ratio to the cell suspension to give a final CFSE concentration of 2.5uM, and

incubated for 10min at room temperature with periodic shaking. At 10mins an equal volume of RPMI/10%HIFCS was added. After 1further minute the cells were washed once with PBS once and twice with RPMI/10%HIFCS. The cells were resuspended in RPMI/10%HIFCS at $10^6$/ml and incubated overnight in a 24 well plate at 2ml/well.

## 2.8 Allogeneic proliferation assay

At day 8 of DC culture the CFSE labelled naïve CD4+ T cells were harvested, and washed twice in RPMI/10%HIFCS. They were counted by haemocytometer, and resuspended in RPMI/10%HIFCS at $10^6$/ml.

One of the 96 well plates containing the DC cultures was spun down (300g, 4mins, 21°C) and washed with 100µl RPMI/10%HIFCS three times under tissue culture conditions. This was achieved by performing the washes in the hood including flicking off the supernatant onto paper towels, and lightly dabbing the plate on further IMS-soaked paper towels. The DC were then resuspended in 100µl of RPMI/10%HIFCS and 100µl of the naïve CD4+ T cell suspension was added ie $10^5$ CD4+ T cells/well. Three wells containing T cells alone ($10^5$ in 200µl RPMI/10%HIFCS) were also set up. At day 4 of proliferation assay the cells were harvested and proliferation was measured by flow cytometry.

## 2.9 Multiplex bead immunoassay

Culture supernatants which had been stored at -80ºC were analysed by multiplex bead immunoassay (Luminex), a technique which permits the analysis of up to 100 analytes in a single sample of 50µl. I used a Human Cytokine 25-plex Antibody Kit (Biosource, Nivelles, Belgium) which assays the following analytes: IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNFα, IFNα, IFNγ, GM-CSF, CCL2 (MCP-1), CCL3(MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL9 (MIG), CXCL10 (IP-10) and Eotaxin.

The following solutions were prepared: wash solution (PBS/0.05% Tween 20), assay buffer (PBS/0.05% Tween 20/1%BSA), standard solution (lyophilised concentrate rehydrated with assay buffer as per data sheet), bead solution (vortex and sonicate, dilute tenfold in assay buffer, protect from light), biotinylated detector antibody solution (vortex, tenfold dilution in assay buffer), streptavidin-RPE solution (vortex, tenfold dilution in assay buffer). The standard curve, blanks and samples were all initially prepared in a 96 well flexiplate to allow rapid transfer to the luminex plate. 100µl of standard solution was placed in the first well of the standard curve and 75µl of assay buffer in wells 2 – 10. Serial transfer of 25µl from wells 1 – 8 gave the 1 in 4 dilutions of the standard curve. Wells 9 and 10 were left with assay buffer only as "blanks". The samples were placed in wells 11 onwards.

The luminex filter plate was hydrated with wash solution 200ul/well and allowed to stand for a minimum of 30s, before aspirating via the vacuum manifold. 25µl bead solution was added to each well followed by 200µl wash solution. After 30s this was aspirated using the vacuum manifold. 25µl of assay buffer was then added to each

well prior to transferring 50µl from each well of the 96 well flexiplate to its corresponding well in the luminex plate. The plate was sealed and incubated for 2 hours on an orbital shaker.

The fluid was then aspirated via the vacuum manifold and washed twice by adding 200µl wash buffer and again aspirating via the vacuum manifold. 100µl of biotinylated detector antibody was added to each well, the plate sealed and incubated for a further 1 hour on the orbital shaker. The fluid was then aspirated via the vacuum manifold and washed twice by adding 200µl wash buffer and again aspirating via the vacuum manifold. 100µl of streptavidin-RPE solution was added to each well. The plate was sealed and incubated for 30mins on an orbital shaker. The fluid was then aspirated via the vacuum manifold and washed three times by adding 200µl wash buffer and again aspirating via the vacuum manifold. The wells were then resuspended in 100µl of assay buffer and run on the luminometer (Luminex).

Analysis was performed using StarStation 2 software (Applied Cytometry Systems, Sheffield, UK). The standard curve was adjusted to be five parameter weighted with the reciprocal of y fit. Upper and lower limits of sensitivity were defined by excluding any regions where the curve plateaued.

## 2.10 Cortisol Enzyme-linked Immunosorbant Assay

A high sensitivity cortisol acetylcholinesterase competitive EIA assay (Cayman, US) was used to determine the concentration of free cortisol in human AqH and serum. EIA buffer, wash buffer, standard, cortisol tracer and cortisol antiserum were prepared

as per manufacturer's instructions. Human AqH was diluted 25-fold prior to testing. 50µl aliquots of the following were added to the plate: the standard curve comprising standard and seven serial 1 in 2 dilutions, and the samples. Additional wells comprise a Blank well (Blk), Total Activity (TA) well, Non-Specific Binding (NSB) well, and Maximum Binding well (B0). 50µl of cortisol tracer was then added to all wells except the Total Activity and Blank wells, and 50µl of cortisol antiserum was added to all wells except Total Activity, Non-Specific Binding and Blank wells. The plate was the covered and incubated for 12-18h at 4ºC. The plate was then washed five times with wash buffer before adding 200µl of Ellman's reagent (reconstituted on the day as per manufacturer's instructions) to all wells and 5µl of cortisol tracer to the Total Activity well. The plate was again covered and put on an orbital shaker for 120-240mins before being read with a multiwall plate reader (Anthos HT III, Anthos labtec Instruments, Salzburg, Austria).

As the ELISA is a competitive assay, absorbance values are proportional to concentration of competitive bound cortisol tracer (and inversely proportional to concentration of cortisol). Standard curves were plotted using a 4-parameter logistic equation fitted to the logarithmic transformation of the standard concentrations vs the percentage cortisol bound.

## 2.11 Flow cytometry

All flow cytometry was performed using either a Beckman Coulter Epics XL bench-top flow cytometer (Beckman Coulter Inc, Fullerton, CA) or a Dako Cyan ADP High Performance flow cytometer (Dako, Colorado, US). Calibration was checked on a

daily basis with Flow Check Fluorospheres (Beckman Coulter Inc). Isotype controls were used to determine the level of background non-specific binding. For all multi-colour cytometry compensation was performed using cells stained with each fluorochrome conjugated-antibody singly. This adjusts for false positives arising from detection of other fluorochromes due to spectral overlap. The level of median fluorescence intensity (MFI) was used to distinguish between high and low levels of expression.

### 2.11.1 Analysis of DC differentiation

At day 6 of DC differentiation the plate was centrifuged (300g, 4mins, 4°C), supernatant discarded and the plate was lightly vortexed. The cells were then labelled with anti-HLA-DR FITC, -CD80 PE and –CD86 PECy5 (or the matched irrelevants) made up to optimal concentrations in PBS/2%BSA and to a total volume of 50µl/well (see table 2.1). They were then incubated for 20mins at 4°C.

The cells were then washed with 100µl PBS/2%BSA, centrifuged at 300g, 4mins, 4°C, the supernatant discarded and cells resuspended in 100µl PBS/2%BSA. The cell suspension was then transferred to flow cytometry tubes containing 180µl PBS/2%BSA and 20µl counting beads (CALTAG/Invitrogen, Paisley, UK). Flow cytometry was performed on the Beckman Coulter Epics XL bench-top flow cytometer.

### 2.11.2 Analysis of DC maturation

After 2d culture the 96 well plate containing the DC cultures for surface staining (ie plate 1) was spun down (300g, 4mins, 4°C) and the supernatants removed and kept (frozen down at -80°C). The plate was then spun down again, any remaining supernatant discarded and the cells labelled as described earlier. Unless otherwise specified in the text, the standard panel was anti-CCR7 FITC, HLA-DR PE, CD86 PECy5, CCR5 PECy7 and MHCI PB. After 20mins at 4º the cells were washed with 100µl PBS/2%BSA (300g, 4mins, 4°C), the supernatant discarded and cells resuspended in 100µl PBS/2%BSA. The cell suspension was then transferred to flow cytometry tubes containing 180µl PBS/2%BSA and 20µl counting beads and 100µl of 40µg/ml propidium iodide (Sigma-Aldrich) which acts as a dead cell exclusion dye. Flow cytometry was performed on the Dako Cyan ADP High Performance flow cytometer (Dako Colorado).

### 2.11.3 Analysis of T cell proliferation

After 4 days of culture the 96 well plate of DC: T cell cocultures was centrifuged (300g, 4mins, 21°C) and 100µl supernatant harvested and kept (frozen down at -80°C). The cells were then spun down again, any remaining supernatant discarded and the cells were resuspended in 100µl PBS/2%BSA before being transferred to flow cytometry tubes containing 180µl PBS/2%BSA, 20µl counting beads and 100µl propidium iodide (Sigma-Aldrich) which acts as a dead cell exclusion dye. Flow cytometry was performed on the Dako Cyan ADP High Performance flow cytometer (Dako Colorado). When only part of the sample is run the actual numbers of cells in

the original sample can be calculated from the number of counting beads counted using the following equation:

Total live cells = total PI negative cells counted/(total beads counted/total beads added).

Undivided cells have the highest concentration of CFSE (and therefore highest signal) and this halves with successive generations of proliferated cells. The number of proliferated cells can therefore be calculated from the percentage of live cells to the left of the first peak (ie of highest signal).

### 2.11.4 Analysis of FoxP3 expression of CD4+ T cells

To assess FoxP3 expression in dividing cells, intracellular staining was performed as follows. At day 4 (or timepoint specified in the text) DC:T cell co-cultures were harvested, and each well divided in two to enable comparison of FoxP3 staining vs isotype for each well. Cells were washed twice in 100µl PBS/0.5%BSA (300g, 4ºC) before being resuspended in 100µl of fix buffer (eBioscience; made up as per manufacturer's instructions) and incubated for 30mins at 4ºC. The plate was then washed once in 150µl PBS/0.5%BSA and twice in 150µl permeabilisation buffer (eBioscience; made up as per manufacturer's instructions). 1µ rat serum and 30µl of permeabilisation buffer was then added to each well and the plate was incubated for 15mins at 4ºC. Anti-human FoxP3 antibody (or isotype control) was then added to each well and the plate was incubated for a further 30 mins at 4ºC. After two further washes in 150µl permeabilisation buffer, the cells were resuspended in 100µl PBS/0.5%BSA, and transferred to FACS tubes containing 140µl PBS/0.5%BSA and

10µl counting beads. Flow cytometry was performed as previously with the important exception that propidium iodide was not used concurrently.

## 2.12 Statistical tests

The Kolmogorov-Smirnov test was used to test for normality of distribution. For normally distributed data, parametric tests were used as follows: Student's t-test (paired or nonpaired as appropriate) to determine significant differences between two conditions and one-way ANOVA for comparison of three or more conditions with Bonferroni's post hoc test for selected conditions. For non-normally distributed data, nonparametric tests were used: Mann−Whitney U test to determine significant differences between nonpaired groups, Wilcoxon−matched pairs analysis for paired samples and Kruskal-Wallis for comparison of three or more conditions with Dunn's post-hoc test for selected conditions. Correlation was tested by use of the Pearson correlation coefficient (for normal data) or the Spearman rank test (for non-normal data). The minimal level of confidence at which the results were judged significant was $p < 0.05$.

# 3 THE DEVELOPMENT OF AN *IN VITRO* MODEL TO STUDY DENDRITIC CELL FUNCTION IN THE OCULAR MICROENVIRONMENT

## 3.1 Introduction

*In vivo*, the anterior segment of the eye is a unique physiological niche in which tightly- regulated homeostatic and immunological mechanisms minimise the perturbations in the ocular microenvironment. Under resting non-inflammatory conditions, the blood-ocular barrier ensures that the aqueous humour which bathes the tissues of the ocular anterior segment is a distinctive serum-free environment. During uveitis, blood-ocular barrier break-down and local generation of inflammatory mediators cause significant changes in this environment with elevations in a number of key inflammatory cytokines such as IL-6, IL-8, CCL2 (MCP-1), and IFNγ accompanied by a fall in the regulatory molecule, TGFβ2 (Curnow et al., 2005). Dendritic cells present in the anterior segment of the eye have been difficult to characterise but appear to have a distinct, immature phenotype, possibly reflecting their exposure to this unique immunosuppressive environment (Forrester et al., 1994; Camelo et al., 2003; Chang et al., 2004).

The aim of this body of work was to establish an *in vitro* model of ocular dendritic cell function in which human DC could be cultured and exposed to AqH or its constituents under serum-free conditions. Achieving this would require: (1) generating pure populations of homogeneous human dendritic cells either by differentiation from precursors, or by direct isolation; (2) establishing a serum-free environment in which the effects of AqH could be studied; (3) ensuring that the

dendritic cells would tolerate these serum-free conditions and function normally in terms of cytokine production, chemotaxis and induction of T cell responses.

Monocyte-derived dendritic cells are the most widely studied *in vitro* model of human DC function, having the advantages over direct isolation of myeloid DC of being (1) reasonably abundant (ie easily generated from circulating monocytes which comprise around 10% of peripheral blood mononuclear cells), (2) available at high purity and (3) of known experience (ie naive). *In vitro* generation of monocyte-derived dendritic cells is commonly carried out in serum-supplemented media (Jeras et al., 2005a) . Our group has previously used 10% pooled human AB+ male serum supplementation of RPMI as standard for all our DC cultures, but I wished to investigate whether I could generate DC under serum-free conditions (as reported elsewhere (Bender et al., 1996; Royer et al., 2006)) and whether supplementation with other types of sera, notably Fetal Calf Serum (FCS) – which is often used as standard by other investigators (Sallusto and Lanzavecchia, 1994; Jeras et al., 2005b) – would alter DC differentiation and activation. Serum-free differentiation of monocyte-derived DC has received increasing attention mainly due to the use of these DC in clinical trials (Cerundolo et al., 2004).

One of the conditions of my proposed *in vitro* model of ocular dendritic cell function, was that the effects of AqH on DC should be studied under serum-free conditions, primarily so as to better reflect the environment of the anterior segment and secondarily to avoid the effects of AqH being obscured by the effects of serum. It was therefore important to characterise the behaviour of DC on being transferred to a

serum-free environment and to ensure that they demonstrated normal DC function under these conditions.

The culture of cells in human AqH *in vitro* raises a number of challenges of miniaturisation. Even when used at 50% rather than 100% the volumes of AqH that can be safely obtained (usually 50 – 100µl) place severe constraints on the total volume (and therefore the number of cells) that can be used in each culture. It was therefore necessary to establish whether DC could still undergo normal maturation in total culture volumes of 100µl. It was also important to characterise the baseline phenotype of these DC in some detail (surface molecules, cytokine production, migration, induction of T cell responses) before moving on to consider the effects of aqueous humour.

More recently it has become possible to isolate and study *in vitro* significant numbers of circulating myeloid dendritic cells. Although there are still a number of disadvantages to their use, notably lower yields and lower purities than with monocyte-derived DC, myeloid DC are of interest as being a true *ex vivo* DC, which avoids the criticism levelled at the monocyte-derived DC that it is an *in vitro* generated cell type which happens to resemble the paradigm of DC behaviour. I therefore investigated the phenotype and function of myeloid DC as compared to monocyte-derived DC in my *in vitro* model of dendritic cell maturation.

## 3.2 The role of serum on the differentiation of monocyte derived dendritic cells *in vitro*

As outlined above monocyte-derived DC are most commonly differentiated in FCS (Sallusto and Lanzavecchia, 1994; Jeras et al., 2005b), but emerging DC-based immunotherapies have led to interest in the generation of DC under serum-free conditions. Additionally whilst FCS is reported to provide good yields of monocyte-derived DC, it is likely that it is a less good model than human serum (HS) of the environment in which *human* DC differentiate *in vivo*. I therefore investigated the role of serum-supplementation and its alternatives in the differentiation of monocyte-derived dendritic cells *in vitro*.

### 3.2.1 Differentiation in serum-supplemented rather than serum-free medium improves yield and dendritic cell profile

In order to determine whether DC differentiation could be supported in the absence of serum, CD14+ bead-purified monocytes from healthy volunteers were cultured in 96 well plates in either standard conditions (RPMI/10% HS) or a serum-free alternative: (1) RPMI with 1% ITS+3, (2) RPMI with 1% ITS+3, 1% NEAA and 1% Na pyruvate, or (3) RPMI with 1%BSA. After six days of culture, cells were harvested, and cell number and surface expression of class II MHC (specifically HLA-DR) and the key costimulatory molecules CD80 and CD86 were measured by flow cytometry.

The number of DC harvested per culture was lower in all serum-free conditions than standard RPMI/10% human serum (Figure 3.1A,B). Compared to the low yield with

RPMI/ITS+3 or RPMI/1%BSA, mean cell recovery was two-fold higher with RPMI/ITS+3/NEAA/Na pyruvate and four-fold higher with RPMI/10% human serum. DC cultured in serum-free conditions were more heterogeneous and had significantly lower expression of HLA-DR and CD86 than DC cultured under standard conditions (Figure 3.1A,C).

### 3.2.2 Differentiation of dendritic cells is affected by serum type (human or fetal calf serum) with reciprocal changes in CD80 and CD86

Having established that serum-supplementation during differentiation improved the yield and profile of the DC generated, I compared the effect of standard pooled human serum (HS) with fetal calf serum (FCS), heat-inactivated HS (HIHS), or heat-inactivated FCS (HIFCS) under the same culture conditions. Yield of DC at day 6 was comparable in all serum-supplemented conditions. There was a trend towards a higher cell recovery with FCS, but this was not statistically significant (Figure 3.2).

DC cultured in any of the four serum-supplemented media had comparable expression of HLADR, but differed significantly in terms of the level of costimulatory molecules (Figure 3.2B) . Supplementation with FCS or HIFCS resulted in significantly higher expression of CD80 with an MFI that was higher than with HS supplementation by a factor of 3.1 and 2.6 respectively. Conversely supplementation with FCS or HIFCS resulted in significantly lower expression of CD86 with an MFI that was four-fold lower than with HS supplementation. Heat inactivation of the serum, whether for HS or FCS, did not significantly alter HLA-DR, CD80 or CD86.

Fig 3.1

Fig 3.2

The reciprocal effects of HS and FCS on CD80 and CD86 were then investigated by culturing DC in varying concentrations of each serum individually and in combination (Figure 3.3). FCS induced CD80 to near maximal levels (twice baseline levels) from 2.5% FCS supplementation, but was completely suppressed by the presence of even low levels of human serum (2.5%). Conversely CD86 expression was near maximal from 2.5% HS supplementation upwards, but was only partially suppressed by increasing concentrations of FCS (cf titration curve of HS alone).

### 3.2.3 Dendritic cell differentiation in human serum is not affected by the serum being autologous or pooled

Subsequently it was considered whether the donor-to-donor variation in yield and differentiation which occurs even under standard conditions might be partly due to individual "compatibility" with the pooled human serum. I hypothesized that the use of autologous human serum might therefore lead to more consistent results. In these experiments DC were cultured under standard conditions in T25 flasks with media supplemented with either autologous or pooled human serum (Figure 3.4).

The use of autologous vs standard pooled HS did not affect DC cultures in terms of yield or levels of HLA-DR, CD80 or CD86 (Figure 3.4). DC yields were 26 $\pm$14 % for autologous and 23 $\pm$13% (mean $\pm$SD) for standard pooled serum conditions.

Fig 3.3

Fig 3.4

## 3.3 The effect of inflammatory cytokines on the maturation of monocyte-derived dendritic cells *in vitro*

The previous experiments established (1) the suitability of pooled human serum for the differentiation of DC from monocytes in my model system, and (2) the potential advantages over serum-free or FCS based media. The subsequent experiments were directed towards establishing a successful *in vitro* model of DC maturation. To establish a model in which the putative effects of AqH on DC maturation could be investigated, it was important to first assess whether normal DC maturation could occur under serum-free conditions. *In vivo*, dendritic cell maturation may be driven by the milieu of inflammatory cytokines and/or by TLR stimulation (Reis e Sousa 2004). I had previously observed that under standard serum-supplemented conditions cytokine-driven maturation of DC was more effective than TLR stimulation alone, as measured by upregulation of HLA-DR, CD80, CD83, CD86 and CCR7 (Curnow personal communication).

Monocyte-derived DC which had differentiated under standard serum-supplemented conditions until day 6, were washed and then cultured for a further 48h with or without inflammatory cytokines (IL-1β, IL-6, TNFα, PGE2) under either serum-supplemented (RPMI/10%HS) or serum-free (RPMI/1%ITS+3/1%NEAA/1%Na pyruvate) conditions (Figure 3.5). Serum supplementation was associated with higher yields of dendritic cells under non-inflammatory conditions with a 1.6 fold increase in cell recovery for iDC ($p < 0.01$). Under both serum-free and serum-supplemented conditions, the pro-inflammatory cytokines caused similar DC maturation in terms of upregulation of HLA-DR, CD80 and CD86. The absence of serum was not associated

Fig 3.5

with any significant difference in the levels of CD80 or CD86, but was associated with 20-30% higher levels of HLA-DR.

### 3.3.1 Inflammatory cytokines can induce a surface phenotype characteristic of dendritic cell maturation under serum-free conditions

Having established that under my defined serum-free conditions, monocyte-derived DC could respond appropriately to an inflammatory stimulus by upregulating HLADR, CD80 and CD86, these 'serum-free' immature DC (iDC) and mature DC (mDC) were analysed in more detail in terms of their surface phenotype and function. In addition to the upregulation of class II MHC, and the costimulatory molecules CD80 and CD86, the pro-inflammatory cytokine cocktail caused upregulation of CD40, and of the accessory costimulatory molecule ICOSL, but not of the accessory molecule 4-1BBL; there was also reciprocal downregulation of CCR5 and upregulation of CCR7 consistent with the published literature (Figure 3.6)(Greenwald et al., 2005; Scandella et al., 2002).

### 3.3.2 DC maturation under serum-free conditions alters DC chemotactic function

In order to test whether this characteristic change in surface phenotype towards a mature DC phenotype was accompanied by a change in DC behaviour, DC function was tested in three key areas: migration to chemokines, cytokine production and induction of T cell proliferation.

Fig 3.6

Ninety-six well plates with transwell inserts were used to assess DC migration to a range of concentrations of either CCL5 (RANTES; ligand for CCR5 expressed on iDC) or CCL19 (ligand for CCR7 expressed on mDC). Monocyte-derived dendritic cells which had differentiated under standard serum-supplemented conditions until day 6, were washed and then cultured for a further 48h with or without inflammatory cytokines (IL-1β, IL-6, TNFα, $PGE_2$) under serum-free conditions (RPMI/1%ITS+3/1%NEAA/1%Na pyruvate). They were then harvested and placed in fresh serum-free medium in the top chambers of the 96-well insert, with serum-free medium or 1 -100ng/ml chemokine placed in the bottom chamber. After three hours of culture both chambers were harvested and cell number was counted by flow cytometry (Figure 3.7). iDC showed directional responses to CCL5 (18% transmigration to 100ng/ml vs 1.5% baseline) but not to CCL19, consistent with their high expression of CCR5 and low CCR7. In contrast, mDC had high levels of transmigration to CCL19 (77% to 100ng/ml CCL19 vs 11% baseline), consistent with their high CCR7 and low CCR5 status. In preliminary experiments DC migration was also tested using twenty-four well transwell plates and modified Dunn chambers but these had poor intra-experimental reproducibility when using the low DC numbers required for proposed AqH-culture experiments, and so were discarded in favour of the 96 well transwell plates.

### 3.3.3 DC maturation under serum-free conditions alters cytokine profile

Under standard conditions, DC maturation is associated with alteration of the profile of cytokine production. To assess whether this change in cytokine profile occurred under serum-free conditions, I analysed DC culture supernatants by multiplex bead

Fig 3.7

immunoassay. Monocyte-derived dendritic cells which had differentiated under standard serum-supplemented conditions until day 6, were washed and then cultured for a further 48h with or without inflammatory cytokines (IL-1β, IL-6, TNFα, PGE2) under serum-free conditions (RPMI/1%ITS/1%NEAA/1%Na pyruvate). Supernatants were then harvested and cytokine levels for IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNFα, IFNα, IFNγ, GM-CSF, CCL2 (MCP-1), CCL3(MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL9 (MIG), CXCL10 (IP-10) and Eotaxin were measured by multiplex bead immunoassay.

DC maturation under serum-free conditions was associated with increased production of the Th1-inducing cytokine IL-12, the chemokine IL-8 and the inhibitory molecule soluble IL-2R (Figure 3.8). There were also trends towards increases in CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) but these did not reach conventional levels of statistical significance. The following were not elevated above baseline (limit of detection shown in parentheses): IL-4 (≤ 8pg/ml), IL-5 (≤ 0.5pg/ml), IL-7 (≤ 5pg/ml), IL-13 (≤ 1pg/ml), IL-15 (≤ 1pg/ml), IL-17 (≤ 15pg/ml), IFNα (≤ 10pg/ml), IFNγ (≤ 2.5pg/ml), CXCL9 (≤ 60pg/ml), CXCL10 (≤ 30pg/ml), Eotaxin (≤ 1pg/ml); since IL-1β, IL-6 and TNFα were added to the mDC cultures these were excluded from the analysis.

Fig 3.8

### 3.3.4 DC maturation under serum-free conditions increases DC capacity to induce T cell proliferation

The function of DC matured under serum-free conditions was then assayed in terms of their capacity to induce proliferation of CFSE labelled naïve CD45RA+CD4+ T cells using an allogeneic proliferation assay. The naive CD4+ compartment was of particular interest as representing the situation at the lymph node where it is proposed that a peripheral tissue-educated DC will present antigen to a naive T cell. CFSE labelled naïve CD45RA+CD4+ T cells were cultured for 4 days with or without monocyte-derived dendritic cells (either iDC or inflammatory cytokine-stimulated mDC). Number of proliferated cells in the cultures were estimated from the number of total live cells with submaximal CFSE (ie gate R2) which represent divided cells (Figure 3.9). Absolute cell numbers were estimated by the simultaneous use of 'counting beads', a known number of which had been added to each sample. mDC were observed to induce greater proliferation of naïve CD4+ T cells than iDC ($p=0.001$), and both iDC and mDC induced greater proliferation than unstimulated T cell cultures ($p<0.05$ and $p < 0.001$ respectively).

We also wished to assess whether there were significant differences in how the naive and memory compartments responded to DC in my model. CFSE labelled naïve (CD45RA+) or memory (CD45RO+) CD4+ T cells were cultured for 4 days with or without monocyte-derived dendritic cells as described previously. Memory CD4+ T cells showed a similar pattern of responses to naive CD4+ T cells, with mDC inducing higher levels of proliferation than iDC, and both inducing higher proliferation than T cells alone (mDC:T > iDC:T > T cells alone; Figure 3.10). There

Fig 3.9

Fig 3.10

was however a difference in the magnitude of the response to mDC with mDC-induced proliferation of naive CD4+ T cells being significantly higher than for memory CD4+ T cells ($p < 0.001$); absolute levels of proliferation for T cells cultured alone or with iDC were not significantly different between naive and memory compartments.

### 3.3.5 Cytokine production by DC-stimulated naive CD4+ T cells reflects their levels of proliferation

The interaction of the naïve CD4+ T cell with its activating dendritic cell is thought to be the primary factor governing T cell differentiation and eventual phenotype (Th1, Th2, Th17, etc). I therefore wished to investigate whether serum-free culture conditions resulted in DC which skewed T cell differentiation towards one particular phenotype (eg Th1, Th2 or Th17).

Naïve CD45RA+CD4+ T cells were cultured for 4 days with or without monocyte-derived dendritic cells (either iDC or inflammatory cytokine-stimulated mDC). Supernatants were then harvested and cytokine levels for IL-2, IL-5, IL-10, IL-12p40/p70, IL-13, IL-17, and IFNγ were measured by multiplex bead immunoassay (Figure 3.11). IL-2 levels were significantly higher in mDC stimulated cultures than iDC, associated with the increased levels of proliferation in these cultures. Indeed all cytokines considered showed a trend towards increasing levels in mDC rather than iDC stimulated cultures. Of interest the Th1-inducing cytokine IL-12 was elevated in mDC vs iDC: T cell co-cultures (mirroring the elevated IL-12 from mDC vs iDC cultures; Figure 3.8), but this did not translate into statistically significant higher

Fig 3.11

levels of IFNγ. Th2 cytokines IL-5 and IL-13 showed a non-significant trend to increase in the mDC vs iDC T cell cocultures; IL-17 levels were only just detectable for iDC:T cell co-cultures (16pg/ml) and remained low.

### 3.3.6 Elevated FoxP3 expression in naive CD4+ T cells is sustained at higher levels in iDC vs mDC: T cell co-cultures

We next wished to determine whether the state of DC maturation would differentially affect T cell expression of FoxP3, reflecting both the transient elevation seen with activation and the sustained FoxP3 elevation of regulatory T cells (reviewed Ziegler (Ziegler, 2006)). CFSE labelled naïve CD4+ T cells were cultured with iDC or mDC in an allogeneic proliferation assay as described previously. At various timepoints (d1, d2, d4, d8), the T cells were harvested and stained for intracellular FoxP3 (Figure 3.12). In the presence of DC (but not T cells alone) a $FoxP3^{hi}$ population of undivided cells could be identified from day 1. This population increased both in number and in FoxP3 expression from days 2 to 4 before subsiding either partially (for iDC) or completely (for mDC) to baseline (ie to the same levels as for T cells alone) at day 8. Divided cells were initially high for FoxP3 (day4) with FoxP3 levels dropping by day8 to medium (iDC) or low (mDC) levels. Further investigation confirmed these differences between iDC and mDC-stimulated cultures at day 8 (Figure 3.13) with significantly higher MFI in both divided (R11) and undivided (R10 + R12) iDC-stimulated CD4+ T cells.

Fig 3.12

Fig 3.13

## 3.4 The effect of inflammatory cytokines on circulating myeloid dendritic cells *in vitro*

Having used monocyte-derived DC to establish the *in vitro* model of dendritic cell maturation described above, I wished to establish whether myeloid DC would respond to inflammatory cytokines under similar serum-free conditions. I therefore investigated both the phenotype and function of myeloid DC in my *in vitro* model of dendritic cell maturation.

### 3.4.1 Myeloid DC upregulate MHC and costimulatory molecules in response to inflammatory cytokines

Myeloid DC were bead-purified from the blood of healthy donors using a two-stage magnetic separation involving (1) the removal of BDCA-1+ B-cells by an anti-CD19 depletion step and (2) the positive selection of BDCA-1+ myeloid DC via BDCA-1 biotin followed by anti-biotin microbeads. Myeloid DC were then cultured for 48h in serum-free conditions with or without the addition of inflammatory cytokines (IL-1β, IL-6, TNFα, $PGE_2$). The cells were then harvested and the expression of a range of key surface molecules was analysed by flow cytometry (Figure 3.14).

The addition of inflammatory cytokines to myeloid DC culture led to changes similar to those seen with monocyte-derived DC, with increased expression of MHC, the costimulatory molecules CD80 and CD86, and the marker of maturation CD83.

Fig 3.14

### 3.4.2 Inflammatory cytokines increase myeloid DC capacity to induce proliferation of CD4+ T cells

Myeloid DC were cultured as before in the presence or absence of the inflammatory cytokine cocktail (IL-1β, IL-6, TNFα, $PGE_2$), and tested for their capacity to induce proliferation of CFSE labelled naïve CD4+ T cells using an allogeneic proliferation assay. Monocyte-derived DC were included in this experiment by way of comparison.

Number of proliferated cells in the myeloid DC:T co-cultures were compared to T cells alone (Figure 3.15), and to monocyte-derived DC:T cell cocultures. For both monocyte-derived and myeloid DC, exposure to inflammatory cytokines (ie mDC) increased their capacity to induce proliferation of naïve CD4+ T cells vs unstimulated (ie iDC). There were no significant differences in myeloid or monocyte-derived DC capacity to induce T cell proliferation (iDC or mDC).

Fig 3.15

## 3.5 Discussion

The aim of the series of experiments described in this chapter was to establish an *in vitro* model of dendritic cell differentiation, maturation and function which could be used to investigate the effects of the ocular microenvironment, specifically human AqH and its components, on dendritic cells.

The importance of establishing a reliable *in vitro* model of DC function in the eye is underlined by the limitations of our current understanding of the nature and function of antigen presenting cells within the eye. MHC class II+ cells with dendritic cell or macrophage markers have been identified in the mouse, rat and human eye, but the extent and nature of their role in antigen presentation is unclear. It has been shown that MHC class II+ cells from the anterior segment of the rat eye can function effectively as antigen presenting cells (Steptoe et al., 1995), however neither dendritic cells nor macrophages have been observed to leave the eye, leading to the suggestion that antigen escapes in soluble form(Camelo et al., 2006; Dullforce et al., 2004). Indeed these APC could not be induced to migrate even after stimulation with LPS or anti-CD40 (Dullforce et al., 2004). Additionally the iris dendritic cells that have been identified express low CD11c, CD80 and CD86 (Steptoe et al., 1995) suggesting that they are relatively immature. We, like others, have hypothesized that it is the presence of immunosuppressive molecules in the anterior segment such as TGFβ and αMSH that inhibit maturation and at least partially repress migration of DC.

It is not possible to do functional studies on these ocular APC themselves due to the issues of isolating sufficient numbers in humans. In non-inflamed conditions they will

be resident in tissues bathed in aqueous humour rather than circulating in the aqueous humour, requiring biopsy of ocular tissue; such biopsies are scarce, would yield very low numbers of DC, and the tissue would usually be pathological. In uveitis (see chapter 5) I propose that the cellular infiltrate into the anterior chamber may well contain DC or at least DC-like APC, but not in sufficient numbers to perform any functional experiments. Even relatively large samples of AqH (150ul) taken from patients with severe uveitis may yield only 10 000 cells in total, the majority of which may be lymphocytes.

In my *in vitro* model of dendritic cell differentiation, maturation and antigen presentation, I am attempting to mimic the conditions that may lead to the generation of a functional ocular APC. In this model, peripheral blood monocytes are induced to differentiate to iDC in serum, recapitulating the differentiation of DC from monocytes in the peripheral blood thought to occur *in vivo*. Then, as would be anticipated to occur in the anterior segment, immature DC in this *in vitro* model are exposed to serum-free conditions where they are bathed in AqH, either non-inflammatory AqH (see chapter 4) or uveitis AqH (see chapter 5). In this environment they may also be subjected to inflammatory stimuli (in my model a cocktail of maturation cytokines IL1β, IL6, TNFα, and $PGE_2$). When modelling functional antigen presentation I co-culture with either allogeneic naive CD4+ T cells (to try to reflect the situation at the draining lymph node early on in inflammation) or allogeneic memory CD4+ T cells (to mimic re-stimulation).

It should be recognised that alternative models are possible and, whilst not the subject of this thesis, may well be worthy of further investigation. For example, one could test

the effect of AqH on monocytes in order to model the potential differentiation of monocytes to DC within the eye. Similarly it could be argued that to model restimulation of T cells by DC within the eye one should conduct the DC: T cell co-cultures (not just the DC cultures) within AqH; this would not, however, distinguish between the effects of AqH on the DC and on the T cell.

As a result of the series of experiments described in this chapter, I have adopted the monocyte-derived dendritic cell as the model dendritic cell for the *in vitro* studies on AqH. As outlined earlier these are widely used for modelling dendritic cell function, since monocytes are available in reasonable numbers (around $1x10^5$ /ml peripheral blood), and generate pure cultures of homogeneous DC of known history. In all the experiments described in this thesis I have used bead purification by positive selection (CD14+ microbead kit from Miltenyi Biotec) which in my hands gives CD14+monocyte purities of >99%. Alternative methods of monocyte isolation include isolation by adherence, differential centrifugation, Flow Assisted Cytometric Sorting (FACS) or alternative magnetic bead methods (either positive or negative selection). In my preliminary experiments I tested the adherence technique of isolation but found that the culture purities (70-90% CD14+) were not sufficiently high and gave lower yields, as has been observed by a number of other investigators (Thurner et al., 1999; Pickl et al., 1996). Differential centrifugation alone (Royer et al., 2006; Lehner et al., 2005) is also no longer commonly used due to low purities of cultures obtained (70-90%).

In contrast FACS techniques permit extremely pure cultures, but there is concern that the shear stresses on the cells and prolonged exposure to FACS buffer during sorting

adversely affect the health and viability of the cultures. Conversely negative selection bead-based techniques provide healthy cultures of untouched cells but at a cost of lower purity than positive selection techniques, whether these be bead-based or FACS. Isolation of monocytes by positive selection on CD14+ microbeads has thus become standard practice, whilst recognising that there is a theoretical possibility that ligating CD14 may modify function or induce partial activation. It should be noted also that only CD14+ monocytes will be selected by these positive selection techniques (ie excluding the CD14loCD16+ subset), although this is likely to promote the homogeneity of the final cultures.

In order to differentiate dendritic cells from these multipotent CD14+ monoctyes I cultured them in IL-4 and GM-CSF as first described by Sallusto and colleagues in 1994, and which has since become standard practice (Sallusto and Lanzavecchia, 1994; Jeras et al., 2005b). Most DC differentiation, like these early protocols, is carried out in FCS supplemented media, which, as described here, was found to give higher yields than other sera or serum-free alternatives (Figure 3.1). In addition to affecting the yield, I found, like others that the use of serum-free media often leads to more adherent and heterogeneous populations, with striking variations in the level of expression of a number of key molecules such as CD86 (Figure 3.1)(Scandella et al., 2002; Bender et al., 1996; Thurner et al., 1999).

We also observed that both CD80 and CD86 were affected by whether HS or FCS was used as the serum supplement. Thus HS supplementation was associated with high CD86 and low CD80 whereas FCS was associated with low CD86 and high CD80. A number of other investigators have reported similar findings. Pietschmann

and colleagues found that monocyte-derived DC differentiating in 1% human plasma had lower levels of CD80 and higher levels of CD83 and CD86 than FCS supplemented cultures(Pietschmann et al., 2000). Similarly Lehner and colleagues reported low CD80 in HS supplemented media(Lehner et al., 2005) and both Scandella(Scandella et al., 2002) and Royer (Royer et al., 2006) reported low CD86 expression in FCS-differentiated DC, although in both cases the comparison was to serum free AIM-V ((Lehner et al., 2005), (Scandella et al., 2002), (Royer et al., 2006)) or X-VIVO ((Royer et al., 2006)) media. Another interesting difference between FCS-grown DC and some other media is the level of CD1a expression observed. Whereas monocyte-derived DC are often described as being $CD1a^{hi}$ this is almost exclusively found with DC generated in FCS or some serum-free media(Sallusto and Lanzavecchia, 1994; Lehner et al., 2005). In contrast I, like others, found that DC grown under HS-or HP-supplemented conditions are $CD1a^{lo}$ (Lehner et al., 2005) (Pietschmann et al., 2000; Duperrier et al., 2000).

It is yet not known which molecules are of primary importance in the development of the CD80/CD86 profile of *in vitro* generated DC. Although the differences in the effects of HS, FCS and other media on these molecules have been noted by a number of investigators it does not appear to have been given serious study. My experiments showed that the key factors responsible are not inactivated by heat (1 hour at 56˚C in a water bath), and that there appears to be both (1) one or more factors in FCS and HS which promote CD80 and CD86 respectively, and (2) one or more inhibitory factors which reciprocally inhibit CD86 and CD80 expression (Figure 3.2,3). Further study of these phenomena though interesting was not a priority for this project, and thus these experiments were not carried further.

One of my fundamental concerns when selecting a technique for generating DC was whether there would be sufficient yields for the complex AqH-based experiments planned. Under standard conditions (ie with 10% human serum, IL-4, GM-CSF in T25 flasks) the (mean ± SD) yield was 23(±15) % with significant donor-to-donor variability. These compare reasonably with other investigators (Bender et al., 1996), who, as here, report higher yield with (1) the use of bead purification vs adherence technique and (2) the use of FCS rather than other media. Despite the potential advantages of a higher yield with FCS, HS supplementation was chosen in this thesis as being likely to better reflect the *in vivo* environment that a differentiating dendritic cell is exposed to.

An alternative method of generating *in vitro* dendritic cells is the use of human CD34+ cells, initially derived from cord blood, differentiated in the presence of GM-CSF and TNFα (Caux et al., 1992). The main disadvantage of deriving DC from CD34+ cells is that only 0.1% PBMC are CD34+. A similarly interesting but impractical technique for generating DC *in vitro* is the placement of PBMC on an unstimulated HUVEC monolayer, which results in selective transmigration of CD14+ cells within 1 hour (90% purity) and then reverse transmigration of some of these cells which have differentiated into a DC phenotype over the next 2 days(Randolph et al., 1998).

More recently it has become possible to isolate myeloid dendritic cells (MyDC) directly from peripheral blood mononuclear cells by bead-purification or by FACS. Since even the predominant BDCA-1+ population of myeloid DC are only present at

around 0.6% of PBMC, yield is around ten-fold lower than achieved with differentiation from monocytes ($3x10^5$ vs $2x10^6$ per 100ml blood); additionally the purity is significantly lower (75-85% vs 99% for monocyte-derived DC cultures). Despite these disadvantages, myeloid DC are a true *ex vivo* DC and therefore are an important comparator when considering whether any effects observed in monocyte-derived DC *in vitro* are likely to be translatable to the *in vivo* situation (Piccioli et al., 2007).

*In vivo* DC maturation occurs in the presence of an inflammatory stimulus such as local infection. The signal to the DC will therefore usually comprise pro-inflammatory cytokines with or without TLR stimulation. Similarly *in vitro* DC maturation is usually induced by recombinant human cytokines (eg TNFα, IL1β, IL6, $PGE_2$) and/or TLR ligands (eg LPS), although direct CD40 stimulation with CD40L may also be used (Sallusto and Lanzavecchia, 1994). Given that most uveitis is not infectious I elected to use a pure cytokine driven pro-inflammatory stimulus, however it should be recognised that some 'non-infective' uveitis is associated with recent systemic gram negative bacterial infection (specifically Yersinia and Salmonella)(Saari and Kauranen, 1980; Saari et al., 1980b; Saari et al., 1980a). Indeed it has been hypothesised that the precipitating event in acute anterior uveitis may be an inappropriate TLR-4 response either by LPS or by endogenous TLR4-ligands (Chang et al., 2004). Previously I have also used ligands for TLR1-9 as the maturation stimulus but have found that they were all less effective at inducing maturation than a standard cytokine cocktail of IL1β, IL6, TNFα and $PGE_2$; the additional effect of LPS combined with this cytokine cocktail was also relatively minor. Moreover LPS, which

was the most effective of the TLR ligands, showed considerable variation in effect depending on the monocyte donor.

Our standard cocktail of cytokines to induce maturation of DC (IL1β, IL6, TNFα and $PGE_2$) is in widespread usage(Jeras et al., 2005b), although alternative regimens include TNFα alone (Sallusto and Lanzavecchia, 1994), TNFα with IL-1β and IL-6, and TNFα with IL-1β and IFNγ(Jeras et al., 2005b). The predominant reason for including $PGE_2$ is that it significantly upregulates CCR7 (Scandella et al., 2002) important for its *in vivo* function of homing to lymph nodes;). Although some investigators have found that $PGE_2$ treated DC skew T cells to a Th2 phenotype (Jonuleit et al., 1997; Kalinski et al., 1998), I did not find the inclusion of $PGE_2$ to significantly bias to Th1 or Th2 subtypes. Conversely DC maturation by TNFα, IL-1β and IFNγ results in DC which are strongly polarising towards a Th1 phenotype(Mailliard et al., 2003; Mailliard et al., 2004).

A major uncertainty at the outset of this project was whether the serum-free conditions required by my model would prevent the normal DC maturation seen under standard conditions. Although slightly more heterogeneous than cultures grown under serum-supplemented conditions, DC matured in this serum-free model showed a normal mature phenotype in terms of surface molecules, cytokine behaviour, chemotactic behaviour, and induction of T cell responses. Typical surface molecule changes included upregulation of class II MHC, CD40, the costimulatory molecules CD80, CD86 and the accessory costimulatory molecule CD275 (ICOSL) consistent with published work(Jeras et al., 2005b; Scandella et al., 2002; Greenwald et al., 2005). I also found strong evidence of functional maturation in terms of upregulation

of the chemokine receptor CCR7and downregulation of CCR5, with accompanying chemotaxis to CCL19 and loss of sensitivity to CCL5 (RANTES) (Scandella et al., 2002)(Figure 3.9).

Maturation also induced a change in the cytokines and chemokines that were produced by the DC. mDC cultures produced higher levels than iDC of IL-12, soluble IL-2 receptor and the chemokine, IL-8. The high levels of IL-12 are interesting as this would usually promote T cell differentiation towards a Th1response, and thus contrasts with Kalinski and others' suggestion that $PGE_2$ treatment of DC may be Th2 polarising(Kalinski et al., 1998) It should be noted that despite the high levels of IL-12 from these DC there was no clear bias to Th1 phenotype from the corresponding T cell cultures, with significant levels of both Th1 and Th2 cytokines being produced. Soluble IL-2 receptor is the extracellular domain of the IL-2Rα shed from IL-2R expressing activated cells including lymphoctyes, macrophages and dendritic cells(Obara et al., 1992). It has been shown to be elevated in a number of inflammatory diseases such as ankylosing spondylitis (Wendling et al., 1991) and malignancy; it was not however shown to be altered in the serum of patients with idiopathic uveitis (Calder et al., 1999). It is not clear whether it has a biological function. The chemokine IL-8 is the ligand for CXCR1 and 2, and is chemotactic for neutrophils, basophils and T cells. Elevation of IL-8 production by mature DC is significant in attracting T cells in order to present antigen to them (Taub et al., 1996; Taub and Oppenheim, 1994).

The fundamental characteristic of dendritic cell maturation is their ability to present antigen and induce proliferation of naive T cells. As expected the mDC generated by

the inflammatory cytokine cocktail showed increased capacity to induce proliferation both of naïve and memory CD4+ T cells in an allogeneic proliferation assay. Interestingly although mDC always induced significantly more proliferation than iDC, the absolute levels of proliferation varied considerably between donors (Figure 3.9C). This is likely to reflect variations in allogenicity between donor pairs. An alternative method of measuring DC induction of T cell responses would have been to measure recall responses to tetanus toxoid pulsed DC (Duperrier et al., 2000; Pietschmann et al., 2000), however this would only be suitable for testing memory rather than naive CD4+ T cell responses. The use of CFSE as opposed to 3H-thymidine incorporation (Duperrier et al., 2000; Pietschmann et al., 2000) avoids the hazards of radioactivity, and enabled us to look at number of divisions and differences in variables such as side scatter or FoxP3 levels between generations of dividing cells.

It has been suggested that immature dendritic cells may deviate CD4+ T cells towards a regulatory rather than effector phenotype(Reis e Sousa, 2006; Lutz and Schuler, 2002). Although a number of extracellular markers have been identified, the most consistent marker of regulatory CD4+ T cells is the transcription factor FoxP3. Even the use of FoxP3 to define a Treg is problematic in a proliferating population, as activation *per se* also leads to an increase in FoxP3 levels. My studies of combined FoxP3/CFSE labelling showed that there are differences in the expression of FoxP3 expression by naïve CD4+ T cells in iDC vs mDC: T cell co-cultures, with elevated expression of FoxP3 being maintained out to day 8 for iDC co-cultures, but returning to near baseline for mDC co-cultures; this is the case for both divided and undivided cells. Whilst it is interesting to speculate that this may in part reflect iDC induction of a regulatory T cell phenotype it is likely that most, if not all, of the FoxP3 elevation

reflects the activation state of the T cells. Further elucidation would require purification of these FoxP3$^{hi}$ T cells using surrogate markers such as CD25hiCD127lo (since FoxP3 staining is intracellular and therefore not possible on live cells) followed by a functional test of their ability to suppress a normal T cell response (eg allogeneic, recall to tetanus toxoid or by direct stimulation with antiCD3/CD28) .

Comparison of *in vitro* generated monocyte-derived DC with isolated circulating myeloid dendritic cells revealed striking parallels in their responses under serum-free conditions to inflammatory stimuli, and in their ability to induce T cell responses. Both monocyte-derived DC and myeloid DC showed a broadly similar response to maturation cytokines, with increase in MHC and the costimulatory molecules CD80 and CD86 (Figure 3.14), and decrease in CCR5(data not shown). Interestingly Piccioli reported that BDCA-1+ myeloid DC were relatively unresponsive to TNFα alone(Piccioli et al., 2007), so it is likely that myeloid DC, like monocyte-derived DC, require all or most of the cytokines (IL1β, IL6, TNFα) and $PGE_2$. Additionally in an allogeneic proliferation assay, monocyte-derived and myeloid DC from the same donor generated similar levels of proliferation of naive CD4+ T cells. Whilst recognising that the myeloid DC studied here are themselves *ex vivo*, such parallel behaviour is supportive of the usefulness of monocyte-derived DC in modelling the function of myeloid DC *in vivo*. The ability of both DC populations to survive, mature and function normally in my serum-free *in vitro* model also suggests that these conditions would be suitable for studying the effects of AqH as I seek to understand the behaviour of dendritic cells in the ocular microenvironment.

# 4 DENDRITIC CELL FUNCTION IN THE OCULAR MICROENVIRONMENT: THE SUPPRESSIVE ROLE OF NON-INFLAMMATORY AQUEOUS HUMOUR

## 4.1 Introduction

In the ocular microenvironment, DC, macrophages or any other potential antigen presenting cells, are exposed to a number of molecules which have been reported to have immunomodulatory effects. *In vivo* and *in vitro* animal studies have suggested that TGFβ2 (Takeuchi et al., 1997; Takeuchi et al., 1998) and αMSH (Taylor et al., 1992; Taylor et al., 2000) are the dominant immunosuppressive components of AqH, with well-documented inhibitory effects on macrophage inflammatory activity and on the generation of Th1 responses. Since almost all functional studies have used either rabbit AqH (Shen et al., 2007; Taylor et al., 1992; Taylor et al., 1997; Taylor and Yee, 2003; Taylor et al., 1994b; Cousins et al., 1991a; Nishida and Taylor, 1999; Kaiser et al., 1989) or murine AqH(Kaiser et al., 1989; Granstein et al., 1990), it has not been clear to what extent these molecules are significant mechanisms of APC regulation in man, nor whether other mechanisms such as endogenous glucocorticoids (notably cortisol) (Knisely et al., 1994; Rauz et al., 2003) might contribute. In addition studies of AqH components on APC function have tended to focus on the macrophage (Takeuchi et al., 1998; Takeuchi et al., 1998) rather than the DC, even though the DC is the most potent APC of peripheral tissues.

Our central hypothesis was that the normal resting ocular microenvironment maintains a relatively immature DC phenotype. We propose that this contributes to immune privilege by reducing their capacity to induce T cell proliferation, and by

changing the nature of the T cells produced. We show here that, as seen in animal models (Shen et al., 2007), human AqH inhibits DC-induction of naïve T cell responses associated with reduced expression of MHC and costimulatory molecules on the DC. We observe that the principal inhibitors of DC function in human AqH are cortisol and TGFβ2, with cortisol having the dominant effect.

## 4.2 Investigation of the effects of human non-inflammatory AqH on monocyte-derived dendritic cell function

Having established an *in vitro* serum-free model within which to test the effects of the ocular microenvironment, we conducted a series of experiments to investigate whether non-inflammatory human AqH modulated human dendritic cell (DC) function under either resting conditions or in response to a strong cytokine-driven inflammatory stimulus previously shown to induce full DC maturation (see Chapter 3).

### 4.2.1 Non-inflammatory AqH reduces expression of MHC and costimulatory molecules on immature dendritic cells and prevents normal dendritic cell maturation

We wished to determine whether DC surface expression of MHC and key costimulatory molecules was affected by the presence of AqH under resting conditions or when exposed to a potent cocktail of inflammatory cytokines. Monocyte-derived DC were cultured under standard conditions (RPMI/10%HS) as described previously. They were then washed and placed in serum-free culture for 48h in the presence or absence of 50% pooled human AqH, with or without a cocktail of inflammatory cytokines (IL-1β, IL-6, TNFα, $PGE_2$). At the end of this period they were harvested, labelled and analysed by flow cytometry (Figure 4.1).

Under resting conditions the presence of AqH was associated with significant reduction in DC expression of class II MHC and of the costimulatory molecule CD86.

Fig 4.1

In the presence of inflammatory cytokines normal maturation was prevented, with downregulation of MHC (class I and class II) and CD86, and failure to upregulate CD80 and CD83.

### 4.2.2 Non-inflammatory AqH alters chemokine production by dendritic cells under resting conditions and in response to inflammatory cytokines

Having identified changes in key surface molecules of DC, we wished to determine whether AqH modulated DC function, notably in terms of cytokine production, migration or induction of T cell responses. Monocyte-derived DC generated under standard conditions were placed in serum-free culture for 48h in the presence or absence of 50% pooled human AqH, with or without a cocktail of inflammatory cytokines (IL-1β, IL-6, TNFα, $PGE_2$) as described previously. Supernatants were then harvested and cytokine levels for IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNFα, IFNα, IFNγ, GM-CSF, CCL2 (MCP-1), CCL3(MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL9 (MIG), CXCL10 (IP-10) and Eotaxin were measured by multiplex bead immunoassay (Figure 4.2).

Under resting conditions the presence of AqH was associated with significant reduction in the levels of the chemokines, CCL3 (MIP-1α) and CCL4 (MIP-1β). In response to an inflammatory stimulus (ie to generate mDC), production of IL-12 and the chemokine IL-8 increased (as noted previously; Figure 3.8). Addition of non-inflammatory AqH to these mDC cultures did not significantly affect levels of IL-12 or IL-8 but did lead to significant reduction in the chemokines CCL4 (MIP-1β) and CCL5 (RANTES) (Figure 4.2). Analytes not shown were either not significantly

Fig 4.2

affected by the addition of non-inflammatory AqH (IL-1β, IL-1RA, IL-2, IL-2R, IL-8, IL-10, IL-12, CCL-2, CXCL9, CXCL10, Eotaxin) or were at or below the limits of detection IL-4, IL-5, IL-7, IL-13, IL-15, IL-17, IFNα, IFNγ, CXCL9, CXCL10, Eotaxin (see section 3.3.3). Since IL-1β, IL-6 and TNFα were added to the mDC cultures these were excluded from the analysis.

### 4.2.3 Non-inflammatory AqH does not alter chemokine receptor expression and chemotactic function in response to inflammatory cytokines

I wished to determine whether the presence of non-inflammatory AqH affected chemokine receptor expression and chemotactic function, both under resting conditions and in the presence of a cytokine-driven maturation stimulus, such as would normally cause a downregulation of CCR5 and upregulation of CCR7 (see chapter 3).

Monocyte-derived DC generated under standard conditions were placed in serum-free culture for 48h in the presence or absence of 50% pooled human AqH, with or without a cocktail of inflammatory cytokines (IL-1β, IL-6, TNFα, $PGE_2$) as described previously. Cells were harvested and either stained for chemokine receptor expression and analysed by flow cytometry or entered into a transmigration assay as below. Ninety-six well plates with transwell inserts were used to assess DC migration to a range of concentrations of either CCL5 (RANTES; ligand for CCR5 expressed on iDC) or CCL19 (ligand for CCR7 expressed on mDC). They were then harvested and placed in fresh serum-free medium in the top chambers of the 96-well insert, with serum-free medium or 1 -100ng/ml chemokine placed in the bottom chamber. After

three hours of culture both chambers were harvested and cell number was counted by flow cytometry (Figure 4.3).

Non-inflammatory AqH did not affect baseline levels of CCR5 and CCR7 and did not prevent the normal upregulation of CCR7 and downregulation of CCR5 observed with DC maturation. The upregulated CCR7 was shown to be equally functional both for DC matured under normal conditions, and for DC matured in the presence of non-inflammatory AqH.

### 4.2.4 Non-inflammatory AqH inhibits the capacity of dendritic cells to induce proliferation of allogeneic naive CD4+ T cells

To investigate whether non-inflammatory AqH alters DC capacity to induce T cell responses, AqH-treated DC were cultured in an allogeneic proliferation assay. Monocyte-derived DC were cultured in serum-free medium for 48h in the presence or absence of 50% pooled human AqH, with or without a cocktail of inflammatory cytokines (IL-1β, IL-6, TNFα, $PGE_2$) as described previously. These DC were then washed and placed in fresh culture medium with CFSE labelled naïve CD45RA+CD4+ T cells for 4 days. As previously, the number of proliferated cells in the cultures was calculated from the number of live cells with submaximal CFSE (ie gate R2) counted on flow cytometry, with counting beads enabling absolute cell numbers to be calculated (Figure 4.4).

AqH-treatment significantly inhibited DC capacity to induce proliferation of naive CD4+ T cells, regardless of whether these DC were cultured under resting (iDC) or

Fig 4.3

Fig 4.4

inflammatory conditions (mDC). As previously noted, mDC were observed to induce greater proliferation of naïve CD4+ T cells than iDC ($p<0.001$), and both iDC and mDC induced greater proliferation than unstimulated T cell cultures (both $p < 0.001$). Proliferation of CD4+ naive T cells was found to correlate with CD86 expression of DC (Figure 4.5; $r = 0.86$; $p = 0.006$)), although there was also a weaker correlation with HLA-DR levels.

### 4.2.5 Cytokine production by naive CD4+ T cells stimulated by AqH-treated DC reflects their reduced levels of proliferation

Having noted that AqH treatment of DC reduced the magnitude of the T cell response induced, I wished to determine whether the differentiation of T cells was also affected, with phenotype being skewed for example to Th1, Th2, Th17 or Treg. Naïve CD45RA+CD4+ T cells were cultured for 4 days with or without monocyte-derived DC (either iDC or inflammatory cytokine-stimulated mDC). Supernatants were then harvested and cytokine levels for IL-2, IL-5, IL-10, IL-12p40/p70, IL-13, IL-17, and IFNγ were measured by multiplex bead immunoassay (Figure 4.6A).

In general, cytokine concentrations reflected the levels of proliferation in each culture with higher concentrations in T cell co-cultures with mDC rather than iDC. AqH-treated mDC induced significantly lower levels of IL-2 and IL-13 in DC: T cell cocultures than untreated mDC. Concentrations of IL-2, IL-13, IFNγ and TNFα all correlated with levels of proliferation (Figure 4.6B). IL-17 was near baseline from all cultures and showed no significant effect of AqH treatment.

Fig 4.5

Fig 4.6

## 4.3 Investigation of the effects of human non-inflammatory AqH on myeloid dendritic cell function

Having established that non-inflammatory AqH significantly altered function and inhibited normal maturation in monocyte-derived DC, I wished to identify whether AqH induced similar effects on myeloid DC purified directly from peripheral blood.

Myeloid DC were bead-purified from the blood of healthy donors using a two-stage magnetic separation as described previously. Myeloid DC were then cultured for 48h in serum-free conditions with or without the addition of inflammatory cytokines (IL-1β, IL-6, TNFα, $PGE_2$). The cells were then harvested and the expression of a range of key surface molecules was analysed by flow cytometry (Figure 4.7A,B).

The addition of inflammatory cytokines to myeloid DC culture led to dramatic elevation in CD86 expression which was largely prevented by the presence of AqH (Figure 4.7). A similar but less dramatic effect was seen with HLA-DR (2004 $\pm$ 58) for iDC, 1968 $\pm$ 0) for AqH-treated iDC, 2628 $\pm$ 0) for mDC and 1865 $\pm$ 48) for AqH-treated mDC (mean $\pm$ SD)).

Myeloid DC were then cultured as before in the presence or absence of 50% pooled human AqH, with or without the addition of the inflammatory cytokine cocktail (IL-1β, IL-6, TNFα, $PGE_2$), and tested for their capacity to induce proliferation of CFSE labelled naïve CD4+ T cells using an allogeneic proliferation assay.

Fig 4.7

As had been observed for monocyte-derived DC, treatment with AqH significantly inhibited myeloid mDC ability to induce T cell proliferation. A mild non-significant inhibitory effect was also seen for myeloid iDC (Figure 4.7 C).

## 4.4 Investigation of the mechanism by which human aqueous humour inhibits dendritic cell function

Having demonstrated that whole human AqH could significantly alter the behaviour of human dendritic cells, I wished to determine which components of human AqH were chiefly responsible for this effect. Possible candidates suggested by animal studies included TGFβ, αMSH and VIP, however our own work amongst others also suggested that the endogenous glucocorticoid cortisol might be present at sufficient levels to play an inhibitory role (Knisely et al., 1994; Rauz et al., 2003).

### 4.4.1 Cortisol downregulates CD86 expression by dendritic cells and contributes to CD86 downregulation by aqueous humour

Cortisol has previously been measured by radioimmunoassay to be present at levels of between 5-50 $x10^{-9}$M in non-inflammatory AqH (Knisely et al., 1994; Rauz et al., 2003). In order to determine these levels in our own cohort of patients, an ultrasensitive ELISA was used to measure cortisol levels in a series of 17 non-inflammatory AqH samples (non-pooled).

Human AqH was diluted 25-fold prior to testing in a cortisol acetylcholinesterase competitive EIA assay (Cayman) as per the manufacturer's instructions. As the

ELISA is a competitive assay, absorbance values are proportional to concentration of competitive bound cortisol tracer (and inversely proportional to concentration of cortisol). Standard curves were plotted using a 4-parameter logistic equation fitted to the logarithmic transformation of the standard concentrations vs the percentage cortisol bound. The concentration of cortisol in AqH was found to range from 755 to 6402pg/ml with a median (IQR) of 2820 (1650-4297) pg/ml.

In order to determine whether cortisol would affect human dendritic cell function, monocyte-derived DC were cultured in the presence of $10^{-9}$ to $10^{-6}$M cortisol with or without the glucocorticoid receptor antagonist, RU486, with or without the addition of the inflammatory cytokine cocktail (IL-1β, IL-6, TNFα, $PGE_2$). CD86 expression, which I had previously been determined to be a sensitive marker of overall DC function, was measured by flow cytometry. Percentage inhibition of CD86 expression was used as the chief measure of the inhibitory effect of AqH or its constituents. In a second set of experiments monocyte-derived DC were cultured in the presence of medium, 50% AqH or $10^{-7}$M cortisol, with or without RU486 and with or without the addition of the inflammatory cytokine cocktail.

Cortisol treatment of DC caused dose-dependent inhibition of CD86 expression (Figure 4.8A). This was present under both resting conditions (iDC) and in the presence of inflammatory cytokines (mDC), although mDC were ten-fold less sensitive to the effects of cortisol than iDC. The addition of RU486 blocked the effect of cortisol for cortisol concentrations up to $10^{-7}$M for iDC and up to $10^{-6}$M for mDC.

Fig 4.8

AqH-treatment of DC showed, as previously, significant inhibition of CD86 expression for iDC which was partially reversed by glucocorticoid blockade with RU486 (Figure 4.8B). As observed with the addition of exogenous cortisol earlier, mDC were less sensitive to the effects of AqH, and, although there was a trend towards reversal with RU486, this was not statistically significant.

### 4.4.2 TGFβ2 but not αMSH or VIP downregulates CD86 expression by dendritic cells

TGFβ2 and the neuropeptides αMSH and VIP are known immunomodulatory compounds which are present at measurable levels in AqH (see Introduction). I therefore wished to establish whether these molecules were contributing to AqH inhibition of DC function.

Monocyte-derived DC were cultured in the presence or absence of 0.1 – 100ng/ml TGFβ2, αMSH or VIP with or without the addition of the inflammatory cytokine cocktail (IL-1β, IL-6, TNFα, $PGE_2$). CD86 expression was measured by flow cytometry. TGFβ2 treatment of DC caused dose-dependent inhibition of CD86 expression (Figure 4.9A). This was present under both resting conditions (iDC) and in the presence of inflammatory cytokines (mDC). iDC were significantly inhibited from 10ng/ml; mDC were only sensitive to the effects of TGFβ2 from 100ng/ml. αMSH and VIP caused no significant inhibition of CD86 expression in iDC or mDC even up to doses of 100ng/ml (Figure 4.9B). Similarly the alternative TGFβ isoforms TGFβ1 and TGFβ3 were also not found to have significant inhibitory effects on DC expression of CD86 or induction of T cell responses (data not shown).

Fig 4.9

### 4.4.3 Cortisol and TGFβ2 have additive downregulatory effects on CD86 expression by dendritic cells

Having established that cortisol and TGFβ2 can independently downregulate CD86 expression, I wished to establish whether these molecules demonstrated synergistic (or at least additive) effects when present together, as would occur in AqH. Monocyte-derived DC were cultured in the presence of $10^{-9}$ to $10^{-6}$M cortisol in combination with 0, 1 or 10ng/ml TGFβ2. The addition of 1ng/ml TGFβ2 caused significant additional inhibition at cortisol doses of $10^{-7}$ and $10^{-6}$M (Figure 4.10A); the same additive effect was also observed at the higher TGFβ2 dose of 10ng/ml (data not shown).

In view of the additive effect described above, we hypothesised that the residual inhibitory effect of AqH not accounted for by cortisol alone (Figure 4.8) might be due to the additive or even synergistic effects of TGFβ2. To establish that I could effectively inhibit TGFβ function, I tested the Activin Receptor-Like Kinase (ALK) inhibitor SB431542 on TGFβ alone (data not shown) and then in combination with RU486 to block a combination of cortisol and TGFβ2 (Figure 4.10B).

Fig 4.10

### 4.4.4 Cortisol and TGFβ2 jointly contribute to AqH-induced downregulation of CD86 on dendritic cells and reduction in their capacity to induce T cell proliferation

In order to test my hypothesis that the inhibitory effect of AqH on DC was due to cortisol and TGFβ2 in combination, I studied the effects of blocking both these molecules in whole non-inflammatory AqH.

Monocyte-derived DC were cultured in the presence or absence of 50% AqH with or without the addition of the inflammatory cytokine cocktail (IL-1β, IL-6, TNFα, $PGE_2$). Reversal of any AqH effect was tested with combined glucocorticoid and TGFβ blockade (RU486 and SB431542). The combination of cortisol (10-7M) and TGFβ2 (10ng/ml) was used as a positive control. CD86 expression was measured by flow cytometry, and capacity of DC to induce T cell proliferation was tested in an allogeneic proliferation assay.

Combined glucocorticoid and TGFβ blockade with RU486 and SB431542 caused significant reversal of AqH-induced CD86 inhibition (Figure 4.11A). As noted earlier when studying the effect of cortisol alone this effect was more dramatic for iDC than mDC, with mDC being relatively less sensitive to the effects of both the exogenous molecules and AqH itself. CD86 was shown earlier to correlate with capacity of DC to induce T cell proliferation. In addition to their reversal of the AqH effects on CD86 expression, combined glucocorticoid and TGFβ blockade led to a significant recovery of DC function as demonstrated by their improved induction of T cell proliferation (Figure 4.11B).

Fig 4.11

## 4.5 Discussion

The aim of the series of experiments described in this chapter was to use my *in vitro* model of DC differentiation, maturation and function (described in chapter 3) to investigate for the first time the effects of AqH on DC in an entirely human system. I have shown that the presence of non-inflammatory AqH induces significant suppression of human DC function, with reduction in MHC and costimulatory molecule expression, cytokine production, chemotaxis and induction of T cell responses. It is of note that AqH inhibition of DC function occurred even in the presence of a potent cocktail of inflammatory cytokines that would normally drive DC to a highly immunogenic mature DC phenotype(Jonuleit et al., 1997). Thus would suggest that in the human eye, DC are constitutively kept in a suppressed state and, even in the presence of a strong inflammatory stimulus, full maturation is inhibited.

Although evidence for the anterior chamber of the eye being an immunologically privileged niche dates back to the observations of van Dooremaal and Medawar ((Medawar, 1948); reviewed Niederkorn (Niederkorn, 2006)), direct evidence of the modulatory properties of AqH on the cells of the immune system stems from the work of Kaiser and colleagues in which they described the inhibitory effects of rodent AqH on lymphocyte proliferative responses *in vitro*. Specifically they found that murine and lapine AqH inhibits T cell proliferation to antigens, and lymphocyte responses to either polyclonal mitogens or growth factors. They found that AqH-induced immunosuppression was not achieved through direct cytotoxicity, and that it was distinct from the effects of murine serum (Kaiser et al., 1989). Shortly afterwards Cousins and colleagues identified TGFβ2 in lapine and human AqH, and

demonstrated that the inhibitory effects of rabbit AqH were at least partially reversed by neutralising TGFβ2 (Cousins et al., 1991a).

Indirect evidence of an immunomodulatory role of AqH on ocular antigen presenting cells, was provided by experiments which sought to define the mechanisms underlying ACAID. Streilein and colleagues showed in mouse, that iris and ciliary body contained bone-marrow derived cells which were mostly F4/80+ (found on mature murine macrophages and macrophage-like cells), and demonstrated that iris/ciliary body preparation (intact tissue or single cell suspensions) were not allostimulatory but in fact inhibited a Mixed Lymphocyte Reaction (Williamson et al., 1989). Subsequently the same group showed that whilst the induction of ACAID was dependent on iris/ciliary body F4/80+ cells, injection of non-ocular F4/80+ cells into the anterior chamber and subsequent pulsing with antigen also resulted in the antigen-specific suppression of DTH reactions characteristic of ACAID (Wilbanks et al., 1991). Critically they then showed that similar ACAID-inducing characteristics could be generated in non-ocular macrophages by exposure to either aqueous humour or to culture supernatants from iris and ciliary body cells, and that this also was TGFβ-dependent (Wilbanks and Streilein, 1992).

The identification of TGFβ2 as *the* molecule of ocular immune privilege in mouse led many in the field to abandon the study of whole AqH in favour of the use of TGFβ and in due course the various neuropeptides identified in AqH. Whilst this is perhaps not surprising given the challenges of working with whole AqH, it has led to a disparity between the extensive literature on the identification and subsequent characterisation of various components of AqH, and a rather sparse body of work on

the effects of the complex biological fluid itself. As noted earlier, the series of experiments described in this chapter attempt to investigate the wide-ranging effects of whole human AqH, with particular regard to the role of the dendritic cell and its implications for the influence of the ocular microenvironment on antigen presentation.

For the first time in an entirely human system I have shown that the presence of non-inflammatory AqH maintains an immature DC phenotype, associated with reduction in MHC and costimulatory molecule expression and significant suppression of normal human DC function in terms of cytokine production, chemotaxis and induction of T cell responses. Previous investigations of the effects of whole AqH on antigen presenting cells include: the induction of the ability to induce ACAID (mouse AqH on non-ocular macrophages cells (Wilbanks and Streilein, 1992), suppression of nitric oxide generation by macrophages(Taylor et al., 1998), suppression of HLADR, CD80 and CD86 and inhibition of a Mixed Lymphocyte Reaction (Mouse BM-derived DC with rabbit AqH)(Shen et al., 2007). In addition there are a number of studies which did not directly look at the effects of AqH on APC (as they are sometimes reported to have done (Stein-Streilein and Watte, 2007)), but which consider the effects of AqH or iris/ciliary body supernatants on T cell and APC together. Cousins showed that IL-2 secretion of the T cell hybridoma (DG11) in response to an I-$A^d$-expressing B cell lymphoma (A20) was inhibited by up to 20% in the presence of rabbit AqH(Cousins et al., 1991a). Similarly Streilein found that iris-ciliary body supernatants inhibited both the same DG11/A20 model used by Cousins, and a murine mixed lymphocyte reaction (C3H responder spleen cells with irradiated BALB/c stimulator spleen cells)(Streilein and Bradley, 1991).

In animal studies the constitutive AqH components implicated in immunosuppression are TGFβ2, and αMSH and to a lesser extent VIP, CGRP, somatostatin and cortisol. In AqH the concentration of TGFβ2 is 1-10ng/ml with >90% being in latent form, bound to the latency associated peptide (LAP)(Taylor, 2007). In the mouse, TGFβ2 treatment of APC inhibits their production of IL12 (ie deviates them away from a TH1 response) and reduces CD40 activation accessory signals(Takeuchi et al., 1997; Takeuchi et al., 1998). Additionally TGFβ2 prevents macrophage pro-inflammatory functions such as secretion of inflammatory cytokines or generation of reactive oxygen species. Interestingly Tsukahara observed that TGFβ treatment of peritoneal exudate cells with 5ng/ml of porcine TGFβ2 did not alter expression of CD80 or CD86, but that blockade of these two molecules prevented TGFβ2-treated PEC from inducing ACAID. TGFβ2 treatment was also noted to divert the PEC to produce IL4 and IL10 but not IFNγ (Tsukahara et al., 2005). Recently however Shen *et al* have demonstrated that murine bone-marrow derived DC are maintained in an immature $MHC^{lo}$ $CD80/86^{lo}$ state by the addition of rabbit aqueous humour, and that this effect can be recapitulated by recombinant TGFβ2 or blocked by AqH pre-treatment with anti-TGFβ2(Shen et al., 2007). In my studies I found TGFβ2 to have a weak inhibitory effect on DC maturation at levels of $\geq$10ng/ml for iDC but only at the supraphysiological dose of 100ng/ml for mDC, suggesting that under conditions of intraocular inflammation TGFβ2 would no longer be a significant regulator of DC function. I did not find the alternative isoforms TGFβ1 and TGFβ3 to have any effect on DC expression of CD86 or induction of T cell proliferation. Although neither of these isoforms are present at measurable levels in AqH under resting conditions, they are potentially relevant to the immunity of the ocular microenvironment as mRNA of all three isoforms have been identified in the eye (Pasquale et al., 1993) and

interestingly whole AqH induces TGFβ1 production by T cells under serum-free conditions.

It is interesting that in my study αMSH did not significantly affect human DC maturation. This finding is supported by the recent study by Shen(Shen et al., 2007) which found that despite their well-documented inhibitory effects on murine macrophage function (Taylor, 2005; Taylor et al., 1998) αMSH and CGRP were not significantly inhibitory for murine DC. The finding that VIP similarly had no effect at physiological levels is perhaps to be expected since its main immunomodulatory effects are thought to be directly against T cells(Taylor et al., 1994b).

In contrast, the endogenous glucocorticoid cortisol (hydrocortisone) did have a significant suppressive effect on DC maturation, inhibiting both CD86 expression and induction of naive CD4+ T cell proliferation. Cortisol has been observed at biologically significant levels in human and rodent AqH. Levels in human AqH as measured by radioimmunoassay ranging from 1.7 to 18.0 ng/ml equating to 4.8 to 49.7nmol/l (Rauz et al., 2003) (Weinstein et al., 1991; Rozsival et al., 1981; Knisely et al., 1994) . Interestingly the binding protein Cortisol Binding Globulin (CBG) was not present at detectable levels (by radioimmunoassay) (Knisely et al., 1994). Thus it would appear that the vast majority of cortisol in AqH exists in free form, in contrast to the situation in serum where 80-90% is bound to CBG, 4-10% is bound to albumin and the remaining 6-10% is free cortisol.

The potential immunoregulatory function of cortisol in AqH has received little attention previously, and there are no previous studies on its potential role on APC

function in the eye. Knisely noted that cortisol at the levels present in AqH could inhibit PHA/IL-1 stimulated thymocyte proliferation, and interestingly that there was an additive effect with physiological levels of TGFβ(Knisely et al., 1994). *In vitro* studies of human DC have shown that cortisol and other glucocorticoids may interfere with DC differentiation and maturation. The addition of dexamethasone during DC differentiation from monocytes reduces Class II MHC, CD80/86 and CD83 expression with consequent reduction in T cell proliferation in a mixed lymphocyte reaction (Piemonti et al., 1999). Freeman observed that the addition of $10^{-8}$M cortisol during DC differentiation from monocytes inhibited DC upregulation of CD83, IL-12 production and allostimulatory activity in response to a maturation stimulus (TNFα) (Freeman et al., 2005).

The consequences of glucocorticoid treatment on DC post-differentiation is more complex. Vieira reported that when monocyte-derived DC were cultured in FCS-supplemented media and stimulated with LPS, treatment with cortisol or clobetasol-17-propionate inhibited production of IL-12 but did not affect expression of HLA-DR or CD80 or their allostimulatory activity (Vieira et al., 1998). In contrast when monocyte-derived DC were stimulated with IL-1β and TNFα (similar to my own model of DC maturation), the presence of cortisol or clobetasol-17-propionate did inhibit upregulation of CD80, CD83 and CD86, with consequent reduced ability to simulate naive CD4+ T cells (De Jong et al., 1999). Similarly addition of hydrocortisone or the synthetic glucocorticoid dexamethasone to monocyte-derived DC activated by nickel sulphate hexahydrate, prevented normal upregulation of CD86, and reduced IL-12 and TNFα production (Toebak et al., 2008). These studies are supportive of my observations that cortisol at the levels present in AqH can inhibit

DC maturation, and that blockade of endogenous glucocorticoids within the AqH leads to a significant (albeit partial) reduction in the inhibitory effect of AqH.

It should be noted that there is also evidence that AqH has a number of immunosuppressive properties directly on T cells (ie not via its effects on APC). These effects include inhibition of Th1 function (Taylor et al., 1992; Cousins et al., 1991b), induction of T reg function (Taylor et al., 1997) and induction of apoptosis. However in this study I wished to specifically investigate the effects of AqH on the DC, and so carefully removed AqH from the DC cultures before commencing DC: T cell coculture.

Although distinct, the role of AqH within the eye shares some striking similarities to the role of the CSF in the brain. In a parallel series of experiments to their induction of ACAID with AqH-treated APC, Wilbanks and colleagues showed that CSF treatment of PEC also induced ACAID (Wilbanks and Streilein, 1992). DC, both myeloid and plasmacytoid, were shown to be present in the CSF of patients with both inflammatory and non-inflammatory disease (Pashenkov et al., 2001). The same group then studied the effects of inflammatory and non-inflammatory CSF on monocyte-derived DC, and observed that, in contrast to my observations with AqH, non-inflammatory CSF did not cause significant downregulation of HLA-DR, CD80 or CD86 and did not inhibit DC induction of T cell proliferation in an allogeneic proliferation assay (Pashenkov et al., 2002). Whilst this may be a real difference between CSF and AqH, it may also reflect the disparity between ‘normal’ fluids from completely healthy volunteers for which sampling usually cannot be justified and the ‘non-inflammatory’ fluids which are used as a surrogate for normal. In my

experiments the 'non-inflammatory' group have no ocular or significant systemic disease other than mild to moderate lens opacity, whilst in Pashenkov's CSF series, the non-inflammatory group are patients with wide-ranging active 'Non-inflammatory neurological diseases' such as ischaemic stroke, depression and pseudotumor cerebri. Given that the range of effect of CSF for the non-inflammatory group was so wide (ie from >30% inhibition to >30% increase of an allogeneic proliferation assay (Pashenkov et al., 2002)) it would be interesting to know the effect of non-inflammatory CSF on DC in the context of a more homogeneous 'normal' population.

Our central hypothesis is that the normal resting ocular microenvironment maintains a relatively immature DC phenotype. I propose that this contributes to immune privilege by reducing their capacity to induce an adaptive immune response. The experiments described in this chapter provide evidence that human AqH inhibits DC-induction of naïve T cell responses associated with reduced expression of MHC and costimulatory molecules on the DC, and that in human AqH the key immunoregulators are cortisol and TGFβ2, with cortisol having the dominant effect.

# 5 DENDRITIC CELL FUNCTION IN UVEITIS: SUSTAINED SUPPRESSION OF DENDRITIC CELL FUNCTION DESPITE IFNγ MEDIATED UPREGULATION OF MHC

## 5.1 Introduction

One of the fundamental paradoxes of ocular immunology is the occurrence of potentially devastating intraocular inflammation (uveitis) in an immune privileged site. There has been considerable debate as to whether uveitis constitutes a failure of immune privilege, or whether immune privilege persists but is simply overwhelmed ((Ohta et al., 2000b; Ohta et al., 2000a; Mo et al., 2003; Mo and Streilein, 2005; Mo and Streilein, 2001).

In the body of work described in the previous chapter I presented evidence supporting my hypothesis that under normal resting conditions the ocular microenvironment maintains DC in a relatively immature state, reducing their capacity to induce an adaptive immune response. In contrast I proposed that during uveitis alterations in the ocular microenvironment result in uveitic AqH becoming stimulatory to DC function, resulting in a loss or at least a diminution of immune privilege.

Although APC populations resembling macrophages and dendritic cells have been identified in tissues of the anterior segment in rodents and human cadaveric studies, they have not previously been isolated from humans *ex vivo*. We hypothesised that whilst usually resident in the tissue, dendritic cells might be found in the anterior chamber during severe intraocular inflammation. We therefore conducted a series of

experiments first to test the feasibility of identifying myeloid dendritic cells in the AqH, then to characterise them and finally to test the extent to which their phenotype could be recapitulated by exposure of monocyte-derived DC to uveitis AqH *in vitro*. In light of the debate on the persistence or otherwise of immune privilege during uveitis, we then sought to establish whether the effect of uveitis AqH on DC *in vitro* was primarily regulatory (as observed for non-inflammatory AqH; see chapter 4) or stimulatory (as per our hypothesis). Previous analysis of the cytokine profile of uveitis AqH has identified increased levels of a number of pro-inflammatory cytokines (such as IL-6 and IFNγ (Curnow et al., 2005; Ooi et al., 2006)). This, coupled with a fall in TGFβ levels (Curnow et al., 2005), pointed to the likelihood that uveitic AqH would be stimulatory, however there remained the possibility that AqH might continue be immunosuppressive due to the persistence of molecules present during resting conditions (eg cortisol) or due to the influx or production of other molecules in response to the inflammation.

In summary, the aims of the series of experiments described in this chapter was (1) to identify and characterise myeloid dendritic cells in human AqH and (2) to investigate whether dendritic cell regulation by the ocular microenvironment persists during human uveitis. We show here that myeloid DC can be successfully isolated from the uveitic anterior chamber. In addition we refute our hypothesis, finding that, during uveitis, AqH continues to be predominantly immunosuppressive as evidenced by the phenotype of myeloid DC isolated directly from the uveitic eye. We have shown *in vitro* that this phenotype is a result of the effects of the AqH itself and that the consequences of exposure to AqH, even during uveitis, is a functionally immature population of DC within the eye.

## 5.2 The identification of myeloid dendritic cells in human AqH during uveitis with a distinct MHChi CD86lo phenotype

Matched peripheral blood and AqH were taken from patients presenting with uveitis. Myeloid DC and CD14+ monocyte/macrophages were identified by flow cytometry of both the PBMC fraction and the AqH. Myeloid DC were defined according to Forward/Side scatter profile and characteristic expression of BDCA-1(CD1c)+CD19-CD56-CD14-/low (Figure 5.1). Monocyte and macrophage populations were also identified by high expression of CD14 (Figure 5.1). Nineteen patients were recruited, sixteen of which had acute anterior uveitis and three of which had panuveitis (Table 5.1).

Myeloid DC could be identified in the AqH of patients with uveitis representing 0.6 $\pm$ 0.7% (mean $\pm$ SD) of total AqH cells. This compares with the peripheral blood where 0.3 $\pm$ 0.6% (mean $\pm$ SD) of the PBMC fraction were myeloid DC. CD14+ cells (monocytes/ macrophages) were more numerous than myeloid DC, being present at a 3.3 $\pm$ 3.9% (mean $\pm$ SD) of AqH cells and at 7.9 $\pm$ 5.5% (mean $\pm$ SD) of the PBMC fraction. The relative cellular profile for anterior and panuveitis are compared in tables 5.2 (for AqH) and 5.3 (for peripheral blood).

Fig 5.1

| | **Anterior Uveitis** | **Panuveitis** |
|---|---|---|
| **Number of samples** | 16 | 3 |
| **Aetiology** | 14 Idiopathic<br>1 Ankylosing spondylitis<br>1 Ulcerative colitis | 2 Fuchs Heterochromic Uveitis<br>1 Vogt-Koyanagi-Harada syndrome |
| **Activity (AC cells)** (Mean ± SD) | 2.4 ± 0.2 | 1.0 ± 0.6 |
| **Unilateral vs bilateral disease** | 15 Unilateral<br>1 Bilateral | 2 Unilateral<br>1 Bilateral |
| **First episode vs recurrent disease** | 5 First episodes<br>11 Recurrences | 3 Recurrences |
| **Duration of current episode (days) ***** (Mean ± SD) | 3.9 ± 0.6 | 17.5 ± 3.5 |
| **Treatment** | 1 Topical glucocorticoid<br>15 Untreated | 1Systemic glucocorticoid<br>1 Topical glucocorticoid<br>1 Untreated |

**Table 5.1 Clinical profile of Anterior and Panuveitis samples analysed by flow cytometry for the presence of myeloid dendritic cells.**
Anterior chamber cellular activity was graded as per Standardization of Uveitis Nomenclature 2005 classification criteria(Jabs et al., 2005). Unpaired t-test for comparison of anterior and panuveitis for activity and duration (normality of distribution tested by Kolmogorov-Smirnov test); *** $p < 0.001$; all else NS.

| | Anterior Uveitis | Panuveitis |
|---|---|---|
| **Total cell count** (Mean ± SD) | 3870 ± 2645 | 6185 ± 5666 |
| **Neutrophils (%)** (Mean ± SD) | 60.6 ± 19.8 | 29.5 ± 5.9 |
| **Lymphoctyes (%) **** (Mean ± SD) | 35.1 ± 18.8 | 68.7 ± 5.7 |
| **Monocyte/macrophages (%)** (Mean ± SD) | 3.7 ± 4.2 | 1.0 ± 0.5 |
| **Myeloid DC (%)** (Mean ± SD) | 0.6 ± 0.7 | 0.8 ± 0.8 |

**Table 5.2 Cellular profile of Anterior and Panuveitis AqH samples analysed by flow cytometry for the presence of myeloid dendritic cells**
Monocyte/macrophages defined by CD14+; myeloid DC defined as BDCA-1+CD14-/lowCD19-CD56-; lymphocytes and neutrophils defined on the basis of forward/side scatter and being negative for the above markers.
Unpaired t-test for comparison of anterior and panuveitis aqueous humour samples for cellular composition (normality of distribution tested by Kolmogorov-Smirnov test); ** $p < 0.01$; all else NS.

| | Anterior Uveitis | Panuveitis |
|---|---|---|
| **Monocyte/macrophages (%)** (Mean ± SD) | 8.6 ± 5.7 | 4.3 ± 2.4 |
| **Myeloid DC (%)** (Mean ± SD) | 0.37 ± 0.69 | 0.14 ± 0.06 |

**Table 5.3 Cellular profile of Anterior and Panuveitis matched blood samples analysed by flow cytometry for the presence of myeloid dendritic cells**
Monocyte/macrophages defined by CD14+; myeloid DC defined as BDCA-1+CD14-/lowCD19-CD56-.
Unpaired t-test for comparison of anterior and panuveitis blood samples for cellular composition (normality of distribution tested by Kolmogorov-Smirnov test); all NS.

For characterisation with regard to expression of key surface molecules, the homogeneous group of untreated idiopathic acute anterior uveitis were considered. Myeloid DC in the AqH expressed significantly higher levels of Class I and Class II MHC, but lower levels of the costimulatory molecule CD86 compared to the matched peripheral blood samples (Figure 5.2); they also showed significant elevation in their expression of CCR5 but no difference in levels of CD11c (data not shown). CD14+ monocyte/macrophages showed similar elevations in MHC (class I and class II) and CCR5 but unlike myeloid DC did not show a significant difference in CD86 expression (Figure 5.3).

## 5.3 Investigation of the role of the uveitic microenvironment on dendritic cell phenotype *in vitro*

Having identified that dendritic cells in the uveitic anterior chamber appear to have a distinct phenotype, we wished to identify whether this was due to differential recruitment of MHChiCD86lo expressing DC or a consequence of the ocular microenvironment on normal DC. To investigate the role of the ocular environment we first tested the effects of uveitis AqH on the phenotype of monocyte-derived DC *in vitro*, and then sought to identify the molecules present in uveitis AqH that were responsible for these changes.

### 5.3.1 Uveitis AqH can recapitulate an MHCIhi CD86lo phenotype in monocyte-derived DC *in vitro*

Monocyte-derived DC were cultured in serum-free medium for 48h in the presence or absence of either 50% uveitic AqH or non-inflammatory AqH. Uveitis AqH was

Fig 5.2

Fig 5.3

taken from patients with active uveitis and ≥ 1+ anterior chamber cells. Non-inflammatory AqH was taken from patients attending for routine cataract surgery as described previously. At the end of 48h the cells were harvested, labelled and analysed by flow cytometry.

Treatment of monocyte-derived DC with uveitis AqH supernatant resulted in significant upregulation in class I MHC and downregulation of class II MHC (specifically HLA-DR) and the costimulatory molecule CD86 (Figure 5.4). Upregulation of class I MHC was greatest when treated with uveitic AqH from patients with severe anterior uveitis (3/4+ anterior chamber cells), whereas class II MHC and CD86 levels were not affected by severity. CCR7 upregulation did not occur (Figure 5.5) and CCR5, rather than being downregulated as observed during normal DC maturation (Figure 3.6B) was further upregulated (Figure 5.5).

### 5.3.2 Uveitis AqH-induced upregulation of MHCI on DC is IFNγ dependent

In order to identify the mechanism by which uveitis AqH induced upregulation of class I MHC, we investigated the role of a number of molecules which had previously been noted to be elevated in active uveitis, notably IFNγ and IL-6 ((Curnow et al., 2005; Ooi et al., 2006)); additionally IFNγ is known to be capable of MHC upregulation (Steimle et al., 1994). We therefore tested the effects both of the recombinant molecule and the consequence of blocking its effects in AqH. Monocyte-derived DC were cultured in serum-free medium for 48h in the presence or absence of (1) 0.1 -100 ng/ml IFNγ with or without an IFNγ blocking antibody or (2) 50% uveitic

Fig 5.4

Fig 5.5

AqH vs non-inflammatory AqH with or without an IFNγ blocking antibody. At the end of 48h the cells were harvested, labelled and analysed by flow cytometry.

Recombinant IFNγ caused a dose-dependent increase in MHC (class I and II) and CD86 (Figure 5.6). There was differential sensitivity of molecular expression to IFNγ with upregulation of class I MHC occurring from concentrations of IFNγ of 0.1ng/ml, HLA-DR from 1ng/ml and CD86 upregulation only being present at the higher concentrations of 10 and 100ng/ml. AqH-induction of class I MHC was shown to be IFNγ dependent with significant reversal with the addition of an IFNγ blocking antibody. As previously observed AqH-treatment downregulated HLA-DR but interestingly HLA-DR expression was further reduced in the presence of IFNγ blockade suggesting that there is an IFNγ-dependent drive to upregulate HLA-DR (as seen with class I MHC) but that this is insufficient to overcome the inhibitory effects of other components of AqH. CD86 levels which were reduced in the presence of AqH (as previously noted) were unaffected by IFNγ blockade.

In a similar set of experiments investigating the possible contribution of IL-6, the effects of uveitis AqH on DC phenotype were (1) not recapitulated by the addition of recombinant IL-6 and were not reversed (or augmented) by IL-6 blockade: MFI for CD86 was 26.1 (15.5-40.3) with uveitis AqH alone (median (IQR) vs 28.3 (20.1-45.8) with matched uveitis AqH and IL-6 blockade (median (IQR); $p = 0.56$; Wilcoxon matched pairs analysis).

Fig 5.6

### 5.3.3 Cortisol levels are elevated in uveitis AqH, and increase with anterior chamber activity

Despite the pro-inflammatory IFNγ-dependent upregulation of class I MHC, the predominant effect of uveitis AqH appeared to be inhibitory, with downregulation of class II MHC and CD86, as previously noted for non-inflammatory AqH. In order to establish the mechanism by which this was achieved, we first considered the role of the molecules which we had already demonstrated to be important in the immunosuppressive function of non-inflammatory AqH, namely cortisol and TGFβ2. To investigate the role of cortisol we first established by ELISA whether it was present at significant levels during uveitis, before proceeding to attempt to block its function in uveitis AqH. Our group had already demonstrated that TGFβ levels in AqH fall markedly during uveitis from a median of 353 (range <40 to 497) to a median of 86 (<40 to 667) pg/ml (Curnow et al., 2005). Since this is significantly below the level at which we have found TGFβ to inhibit DC function (Chapter 4), TGFβ was not considered further in this context.

The presence of cortisol in human AqH during uveitis had not previously been determined. We therefore used an ultrasensitive ELISA to measure cortisol levels in a series of 13 untreated uveitic AqH samples, as described previously. As the ELISA is a competitive assay, absorbance values are proportional to concentration of competitive bound cortisol tracer (and inversely proportional to concentration of cortisol). The standard curve (Figure 5.7A) was plotted using a 4-parameter logistic equation fitted to the logarithmic transformation of the standard concentrations vs the percentage cortisol bound. The concentration of cortisol in uveitic AqH was found to

range from 1884 to 25536 pg/ml, with a median (IQR) of 5468 (3905 – 12300) pg/ml. This compares with a median (IQR) of 2820 (1650-4297) pg/ml for non-inflammatory AqH (Figure 5.7B). Cortisol level in AqH increased with severity of uveitis as measured by anterior chamber activity (Figure 5.7C; linear trend test $p < 0.01$).

### 5.3.4 In uveitis, AqH-induced inhibition of CD86 loses glucocorticoid-dependency which is restored in the presence of therapeutic glucocorticoids

Having established (1) that cortisol levels were elevated during active uveitis (Figure 5.7), (2) that addition of exogenous cortisol at these levels significantly inhibited DC expression of CD86 and induction of T cell responses (Chapter 4) and (3) that blockade of the glucocorticoid receptor with RU486 reversed the inhibitory properties of non-inflammatory AqH (Chapter 4), we hypothesised that the persistence of immunosuppression by AqH during uveitis was also likely to be due to endogenous cortisol, supplemented by exogenous (ie therapeutic) glucocorticoids in the case of 'treated' samples. We therefore tested the inhibitory effects of uveitis AqH in the presence or absence of glucocorticoid blockade, recognising that for treated uveitis AqH samples glucocorticoid reversal would be blocking the combined effect of endogenous cortisol and exogenous glucocorticoids (such as dexamethasone). Monocyte-derived DC were cultured in the presence or absence of non-inflammatory or uveitis (untreated or treated) AqH with or without the glucocorticoid receptor antagonist, RU486; $10^{-7}$M cortisol was included as a positive control. After 48h, surface expression of HLA-A,B,C, HLA-DR and CD86 was determined by flow cytometry (Figure 5.8).

Fig 5.7

Fig 5.8

As noted previously for non-inflammatory AqH, both untreated and treated uveitis AqH caused significant reduction in CD86 expression. Glucocorticoid blockade with RU486 caused significant reversal of inhibition for treated uveitis AqH (as also seen for non-inflammatory AqH), but did not cause significant reversal of the inhibitory effect of untreated uveitis AqH. Class I and class II MHC were not affected by the addition of RU486.

## 5.4 Investigation of the role of the uveitic microenvironment on dendritic cell function

Having identified that the inflamed ocular microenvironment induced both a 'stimulatory' upregulation of MHC (class I and II *in vivo*; class I only *in vitro*) and an 'inhibitory' downregulation of CD86, we wished to investigate the functional consequences of this altered phenotype, in particular the possibility of selective inhibition of CD4+ vs CD8+ T cell responses, and the consequences of treatment on DC function.

### 5.4.1 Uveitis AqH inhibits DC capacity to induce proliferation of CD4+ and CD8+ T cells

Monocyte-derived DC were cultured in serum-free medium for 48h in the presence or absence of 50% uveitic AqH vs non-inflammatory AqH. These DC were then washed and placed in fresh culture medium with CFSE labelled naïve CD45RA+CD4+ T cells, or memory CD45RO+CD4+ T cells or CD8+ T cells for 4 days. As previously, the number of proliferated cells in the cultures was calculated from the number of live

cells with submaximal CFSE (ie gate R2) counted on flow cytometry, with counting beads enabling absolute cell numbers to be estimated (Figure 4.3).

Uveitis AqH-treatment significantly inhibited DC capacity to induce T cell proliferation, whether naive or memory CD4+ or CD8+ (Figure 5.9). Inhibition with uveitis AqH was of a similar magnitude to that seen with non-inflammatory AqH. As noted previously for non-inflammatory AqH (Figure 4.5), induction of proliferation was found to correlate with CD86 expression of DC (Figure 5.10; $r = 0.67$; $p = 0.006$).

### 5.4.2 *In vivo* glucocorticoid treatment does not affect the capacity of uveitic AqH to inhibit DC induction of T cell proliferation, but is associated with lower IL-10 levels in DC: T cell supernatants

Having noted that uveitis AqH treatment caused similar inhibition of DC function to non-inflammatory AqH, we wished to observe whether the inhibitory effects of uveitis AqH were affected by the presence of glucocorticoid treatment. In addition we wished to determine whether the differentiation of T cells was also affected, with phenotype being skewed for example to Th1, Th2, Th17 or Treg.
Naïve CD45RA+CD4+ T cells were cultured for 4 days with or without monocyte-derived dendritic cells in the presence or absence of non-inflammatory AqH, untreated uveitis AqH or treated uveitis AqH (ie from patients who had already commenced topical glucocorticoid treatment at the time of sampling). Supernatants were then harvested and cytokine levels for IL-2, IL-5, IL-10, IL-12p40/p70, IL-13, IL-17, IFNγ and TNFα were measured by multiplex bead immunoassay (Figure 5.11).

Fig 5.9

Fig 5.10

In general, cytokine concentrations reflected the levels of proliferation in each culture. Uveitis AqH treatment of DC resulted in lower levels of proliferation regardless of whether the AqH was a 'treated' or 'untreated' sample, with no significant difference between them (Figure 5.11A). Similarly exposure of DC to uveitis AqH, whether untreated or treated, resulted in lower levels of IL-2, IL-10, IL-13, IFNγ and TNFα (figure 5.11B); correlation with proliferation was high for all these cytokines (Figure 5.11C). IL-17 was near baseline from all cultures and showed no significant effect of AqH treatment.

### 5.4.3 In uveitis, AqH inhibition of DC function loses glucocorticoid-dependency which is restored in the presence of therapeutic glucocorticoid

Having established that (1) during uveitis downregulation of CD86 by AqH was no longer glucocorticoid-dependent, but that (2) this was restored in treated uveitis AqH, we wished to establish whether this was reflected in the glucocorticoid dependency (or otherwise) of the AqH inhibition of DC induction of T cell responses. We therefore tested the inhibitory effects of uveitis AqH in the presence or absence of glucocorticoid blockade on DC induction of proliferation and T cell cytokine production; again it was noted that for treated uveitis AqH samples, the glucocorticoid receptor antagonist RU486 would be blocking the combined effect of endogenous cortisol and exogenous glucocorticoids (such as dexamethasone).

Monocyte-derived DC were cultured in the presence or absence of non-inflammatory or uveitis (untreated or treated) AqH with or without the glucocorticoid receptor

Fig 5.11

antagonist, RU486; 10-7M cortisol was included as a positive control. After 48h, DC were then washed and placed in fresh culture medium with CFSE labelled naïve CD45RA+CD4+ T cells for 4 days. The level of proliferation was estimated from the number of live cells with submaximal CFSE counted on flow cytometry (Figure 5.12A).

As noted previously for non-inflammatory AqH, both untreated and treated uveitis AqH caused significant impairment in DC capacity to induce T cell proliferation. Glucocorticoid blockade with RU486 caused significant reversal of inhibition for treated uveitis AqH (as also seen for non-inflammatory AqH), but did not cause significant reversal of the inhibitory effect of untreated uveitis AqH. Although it was not possible to directly measure the levels of therapeutic glucocorticoids in these AqH samples, it was noted that RU486 reversal of their inhibitory effect was not related to the intensity of treatment which they had received prior to sampling (5.12B).

Fig 5.12

## 5.5 Discussion

The aim of the series of experiments described in this chapter was first to identify and characterise myeloid DC from the human anterior chamber, and second to investigate whether, with regard to the dendritic cell, immune privilege persists during human uveitis. We have shown that myeloid DC can be identified in human AqH during active uveitis, that they are MHChiCD86lo and that when this phenotype is recapitulated *in vitro* by the exposure of monocyte-derived DC to uveitis AqH supernatant, the function of these DC is generally inhibited despite the opposing effects of inflammatory molecules such as IFNγ. Furthermore the regulatory effects of uveitis AqH prior to treatment appear to be distinct from the cortisol and TGFβ2-dependent mechanism we demonstrated for non-inflammatory AqH (chapter 4).

The identification and characterisation of a defined BDCA-1+ myeloid DC population in human AqH is important in the context of the evolving debate over the identity and function of the ocular APC populations. As reviewed earlier (see Introduction) an ocular DC-like population was identified in human cadaveric studies, both by Flugel who described a population of CD45+ HLA-DR+ dendritiform cells(Flugel et al., 1992) and by Chang who noted that these dendritiform cells were HLADR+ TLR4+ CD68- (ie typical of DC rather than macrophages) but which also expressed CD14 (suggestive of immature DC or macrophages)(Chang et al., 2004; Chang et al., 2004). Detailed studies in rodents have identified separate populations with both dendritic cell and macrophage-like characteristics (reviewed in Introduction). For example in the mouse the important F4/80 population responsible for ACAID can be divided into a class II MHC (Ia+) subset (typical of DC) and class II MHC (Ia-) subset (typical of

macrophage). In our flow cytometric analysis of whole uveitic AqH we have similarly been able to identify both a CD14+ monocyte/macrophage population and a separate CD14-/low BDCA-1+ myeloid DC population. We found that in the uveitic anterior chamber, DC were less abundant than CD14+ monocyte/macrophages; similarly in mouse the macrophage population outnumbered the DC population(McMenamin et al., 1994). Functionally, however, the role of these DC should not be under-estimated as they are far more potent than macrophages in their antigen presentation capacity, are unique in their ability to induce naive T cell responses and can fulfil regulatory as well as stimulatory roles.

One of the most important findings of this entire thesis is that the regulatory properties of AqH observed under resting conditions persist during uveitis. Although in the context of our model we have only considered the effects of uveitis AqH on the afferent arm of the immune system (specifically DC function), this refutation of our hypothesis is very interesting in the context of the wider debate over whether immune privilege is maintained or lost during uveitis. We, and others, have analysed the cytokines present in human AqH during uveitis, finding increases in IFNγ and IL-6 and a fall inTGFβ2 suggestive of an anterior chamber switch from a regulatory to an inflammatory microenvironment (Curnow et al., 2005; Ooi et al., 2006). Rodent models of uveitis reflect a similar change in profile, and provide some additional information as to how these levels change over time during an episode of uveitis. For example in the murine model of experimental autoimmune uveitis, Ohta found that at onset of disease (day 11 after inoculation of IRBP) there is an increase of AqH IL-6, IL-1β and IL-2; that at the peak of disease (day 17) there is a sharp increase in IFNγ and TNFα, a fall in IL-6 and IL-1and maintenance of IL-2; and that at disease

resolution (day 28) most cytokine levels had returned to baseline, although IL-2 and TNFα were still found at low levels(Ohta et al., 2000a).

A number of groups have investigated the functional consequences of these changes in the ocular microenvironment in rodents, with different conclusions. Most early investigators argued that immune privilege was lost during ocular inflammation (at least in the early phase), and that this was primarily due to a rise in IL-6. In the experimental autoimmune uveitis (EAU) model outlined above, Ohta observed that the ability of the ocular microenvironment to suppress anti-CD3 driven T cell proliferation *in vitro* was lost at the onset of disease (d11; associated with blood ocular barrier breakdown and elevated AqH IL-6), and that recovery of this suppressive function occurred at the peak of disease (d17; associated with a fall in IL-6 and elevated TGFβ2 (locally produced) and TGFβ-1 (from blood/infiltrating leucocytes)) (Ohta et al., 2000a). Neutralisation of IL-6 was associated with maintenance of normal AqH suppression at day 11, whereas neutralisation of TGFβ2 blocked the recovery of this suppressive function(Ohta et al., 2000a). Subsequently using the alternative model of endotoxin induced uveitis(EIU) the same group found that 6 to 24 hours after systemic LPS injection into the footpads of C3H/HeN mice, AqH lost its capacity to suppress *in vitro* T-cell activation (again associated with an increase in AqH IL-6), and that this could be recovered by neutralization of IL-6(Ohta et al., 2000b). Finally they noted that the induction of antigen-specific anterior chamber–associated immune deviation (ACAID) in normal mice was prevented by the addition of IL-6 into the anterior chamber (Ohta et al., 2000b).

This model of a loss of immune privilege at the onset of uveitis, and its restoration coincident with the onset of recovery seemed reasonably robust, supported as it was by convincing evidence in two different animal models. More recently however two alternative animal models of uveitis have demonstrated that severe intraocular inflammation is compatible with maintenance of immune privilege, a finding which we have now demonstrated for the first time in human. In the first of these models, a variant of the Endotoxin Induced Uveitis model in which LPS is injected, not systemically, but into the vitreous cavity of BALB/c mice, Mo and colleagues found that even at the peak of intraocular inflammation (9h; associated with high levels of IL-6) these eyes would permit the proliferation of allogeneic tumour cells and support ACAID *in vivo*, and their AqH would still strongly inhibit T cell activation *in vitro* (Mo and Streilein, 2001). In a second model of Mycobacterium tuberculosis adjuvant-induced uveitis (MTU), intravitreal injection of Mycobacteria tuberculosis adjuvant into the eyes of BALB/c mice resulted in an intense anterior uveitis which again supported the growth of allogeneic tumour cells, and their AqH again inhibited T cell activation *in vitro*. Interestingly, however, the early phase of disease was associated with a temporary failure in the ability of the eye to induce ACAID(Mo and Streilein, 2005).

Our finding that uveitis AqH continues to be inhibitory to DC function is an important addition to the predominantly murine literature regarding the retention or otherwise of immune privilege during uveitis. The four animal models described above (see also Table 1.9) have important immunological differences to each other, reflected in their different profiles of disease severity, time-course and eventual outcome .Whilst none are a perfect model for spontaneous disease in humans, between them they reflect

something of the puzzling spectrum of human disease. Mo *et al* argue that maintenance of immune privilege is normal during inflammation, but that (1) a strong Th1dominated inflammation can lead to the retention of a DTH response (ie loss of ACAID) associated with elevated IL-12 levels during EAU and the early phase of MTU; and that (2) whilst IL-6 can abrogate TGF-β driven immune privilege in systemically immunized models which are characterised by lower grade ocular inflammation (ie EAU and systemic EIU), the more extensive blood ocular barrier breakdown seen with intraocular immunization (ie local EIU and MTU models) results in an influx of plasma proteins which neutralize the effects of IL-6 ((Mo and Streilein, 2007)). It is interesting to note that whilst in humans we have observed elevated IL-6 levels in uveitis AqH to be functional in terms of preventing T cell apoptosis (via a sIL-6R trans-signalling pathway(Curnow et al., 2004)), we have recently shown that IL-6 is auto-regulated by an influx of sgp130, a natural inhibitor of IL-6 trans-signalling(Simon et al., 2008). We hypothesise that differences in sgp130associated with variable blood-ocular barrier breakdown may account for the varying responses to IL-6 seen in the different animal models of uveitis.

With regard to DC function, we had demonstrated in preliminary experiments that IL-6 alone did not significantly affect DC function . When used in combination with IL-1β, TNFα and $PGE_2$ it caused characteristic changes of maturation such as elevation of MHC and costimulatory molecules and allostimulatory function, but that these changes could be blocked by the addition of normal AqH (chapter 3); similarly blockade of IL-6 in uveitis AqH did not significantly affect DC phenotype. In contrast IFNγ was associated with the limited pro-inflammatory activity of uveitis AqH. We have previously shown IFNγ levels to increase in uveitis and to correlate with disease

activity(Curnow et al., 2005). Here we have demonstrated that recombinant IFNγ upregulates class I and class II MHC, and that IFNγ-blockade reduces the uveitis AqH-driven upregulation of class I MHC and leads to further downregulation of class II MHC.

Our observation of the persistence of the regulatory properties of human AqH during uveitis is also intriguing since it appears to be operating by an alternative mechanism to the dominant cortisol/TGFβ2 pathway of non-inflammatory AqH. Again this is controversial in the animal literature. It would appear that in mouse the predominant regulatory molecules are neuropeptides such as αMSH and VIP, with TGFβ playing a relatively minor role due to most of it being in the latent state. In the EAU and systemic EIU models, levels of active TGFβ1 and TGFβ2 increase and it appears that TGFβ quickly becomes the dominant mechanism of immune privilege (hence regulation can be reversed by the addition of IL-6)(Ohta et al., 2000b; Ohta et al., 2000a).

In contrast, whilst the local EIU and MTU models do not discount a role for TGFβ, the lack of IL-6 effect suggests that other factors must be involved. In parallel to the animal studies we found that the dominant regulatory mechanism we had identified in non-inflammatory human AqH (predominantly cortisol with TGFβ2 contributing) was not operational, or at least not dominant, in uveitis AqH, despite the presence of increasing levels of cortisol in these samples; in humans TGFβ levels have been shown to fall precipitately in uveitis and hence was discounted as a significant contributor in these experiments. We have been unable to determine the novel factor(s) that appear in uveitic AqH and contribute to the maintenance of regulation. It

is likely that the potential regulatory role of cortisol is maintained (or even enhanced given its increasing levels), but that the presence of novel alternative regulatory molecules provide for redundancy in the system, resulting in AqH inhibition of DC function no longer being reversible by glucocorticoid blockade alone. This seems plausible given that we have shown up to ten-fold increases in the level of cortisol during uveitis, and have shown *in vitro* that at these higher concentrations cortisol can inhibit DC function even under inflammatory conditions. An alternative explanation of the loss of cortisol-dependency is that rather than simply being redundant, the cortisol is no longer effective, either due to elevated cortisol binding globulin or due to inactivation of cortisol to cortisone by 11β-HSD2 dehydrogenase activity. This explanation seems less likely given that we have measured the levels of free cortisol (rather than total) and have shown that cortisol at these doses is effective. Furthermore with relation to 11β-HSD expression, monocyte-derived dendritic cells and ocular tissue predominantly express the oxo-reductase enzyme 11β-HSD1 (which activates cortisone to cortisol) rather than the 11β-HSD2 isoform (Freeman et al., 2005; Rauz et al., 2001).

The origin and identity of these novel regulatory molecules is uncertain. Possibilities include locally produced molecules such as regulatory cytokines (eg TGFβ, IL-10) or mediators of resolution (resolvins and other lipid-derived small molecules such as lipoxin A4), or serum-borne molecules (such as sgp130 or 1α,25-dihydroxyvitamin D (Simon et al., 2008; Hewison et al., 2003)). As alluded to earlier we have already established that in humans neither TGFβ nor IL-10 are elevated in most uveitis; in fact TGFβ levels fall, and IL-10 is maintained at non-inflammatory levels in all forms of uveitis other than Herpes-viral (Curnow et al., 2005). Evidence that the key

molecule(s) may be present in the serum come from animal and our own studies which have shown that whilst low concentrations of uveitic serum was stimulatory, higher concentrations of serum (uveitic or normal) was significantly immunosuppressive. Whilst we hypothesise that in the animal models (and in human T cell survival) sgp130 regulation of IL-6 trans-signalling may be a key factor, the lack of IL-6 effect in our experiments argues against any significant role of AqH sgp130 in terms of human DC function. The recently described 'resolvins' and related lipid-derived molecules are of interest. Although the effects on DC are as yet poorly characterised, Lipoxin A4 has been shown to inhibit dendritic cell function in mouse by inhibiting IL-12 production, downregulating CCR5 and inhibit migration, although they do not comment on surface molecule expression of MHC and CD86(Aliberti et al., 2002).

Our observation that treatment of uveitis is associated with restoration of a glucocorticoid-dependent mechanism of regulation is fascinating and has clear parallels with the recovery of immune privilege in the animal models. Whilst in our experiments it was not possible to differentiate between the effects of exogenous and endogenous topical steroids, it is interesting that the degree of inhibitory effect and subsequent reversal by RU486 was independent of the intensity of glucocorticoid therapy suggesting that this is at least partly due to recovery of the normal cortisol mechanism of regulation. In any event, the return to glucocorticoid dependency implies that the unknown mechanism of immunoregulation operational in uveitis AqH prior to treatment, is no longer a significant mechanism once treatment is underway. This may reflect restoration of the blood-ocular barrier (untreated and treated groups were matched for cellular activity but not for flare) which would result in falling

levels of any serum-borne mediator of regulation, or may be a direct effect of the treatment (eg glucocorticoid therapy has been associated with a reduction in resolvins). We wish to pursue this work by analysing a number of recently described bioactive molecules such as resolvins in non-inflammatory and uveitis AqH. Additionally by performing time-course studies of the change in cytokine levels (and other modulators of inflammation) during an episode of uveitis and correlating this with the effects of AqH on DC and related cell types, we hope to refine our understanding of the role of DC in the onset, progression, resolution and recurrence of human uveitis.

# 6 FINAL DISCUSSION

## 6.1 Introduction

During the course of this thesis I have shown that myeloid DC can be isolated from the anterior chamber of the human eye during uveitis, that human AqH regulates DC function and that even in the presence of the pro-inflammatory milieu of uveitis this regulatory function predominates. I have also provided evidence that in the normal human eye this suppressive function is cortisol and TGFβ2 dependent, but that during uveitis novel regulatory mechanisms are introduced, with apparent redundancy of the cortisol/TGFβ system.

In the previous chapters I have set these results in the context of two of the great debates in ocular immunology: first, the identity of the ocular APC and the location and nature of its interaction with the target T cell; second, the maintenance (or otherwise) of immune privilege during intraocular inflammation. In this final discussion I will consider the broader implications of these findings, first with regard to the concept of immune privilege and second with regard to the aetiology, pathogenesis and treatment of ocular inflammatory disease.

## 6.2 Immune privilege: an extreme of normal regulation

Like many homeostatic processes, the immune system is tightly regulated to operate within the narrow compass of what is both effective and safe. An inadequate immune response leaves the organism vulnerable to surrounding pathogens. A misdirected or

excessive response may itself lead to damage to the organism, either due to specific anti-self responses or due to collateral damage to healthy tissue as the pathogen is cleared. For the immune response to be effective, all parts of the organism must be accessible to it. For it to be appropriate, each tissue must have regulatory mechanisms (whether specific or generic) to control the magnitude and direction of that response.

The concept of immune privilege as originally described by Medawar was as an exception to the normal immune response (Medawar, 1948). 'Normal' immunity could be demonstrated by the usual rejection of foreign grafts except when placed in a few 'immune privileged' sites, such as the eye. Since then, however, the number of sites described as being immune privileged has grown. Thus whilst only a limited number of tissues exhibit prolonged tolerance of foreign grafts (as per the original definition), relative degrees of immune privilege are present in most tissues in order (at least in teleological terms) to 'tailor' the immune system to the requirements of that site. In this discussion I argue that the literature points to regulation of the immune system as a normal property of all tissues, varying according to the needs of that site. In this context 'immune privilege' *per se* simply represents the extreme of a continuum of normal regulation exhibited by all tissues. In line with this model, I propose that the mechanisms of DC regulation that I have been investigating in the AqH may be more generally applicable and are likely to occur in other sites outside of the eye.

Although ocular immune privilege is remarkable as a phenomenon, there is nothing unique in its component parts. Careful consideration of other tissues demonstrates that every mechanism of immune regulation found in the eye may be observed to some

extent in other sites. Some, such as complement regulatory proteins to prevent inappropriate cascade initiation, are almost ubiquitous. Similarly Fas:Fas ligand interactions to induce leukocyte apoptosis, though originally described in the eye, has since been recognised in the testis, brain, lung, kidney, liver, pancreas, heart, skin, thyroid and blood vessels (reviewed (Green and Ferguson, 2001)).

Interestingly the regulatory mechanisms surrounding antigen presentation and the dendritic cell may be more limited in their distribution. These mechanisms include: (1) limited drainage of antigen or APC due to either absent or inefficient lymphatic drainage (eye, brain(Yamada et al., 1991), hamster cheek pouch(Barker and Billingham, 1971) or due to DC 'entrapment' (as seen in the feto-placental unit (Collins et al., 2009)); (2) immune deviation responses such as ACAID (seen in the eye) and similar phenomena (seen in the brain and possibly by the feto-placental unit); and (3) central to this thesis, the presence of immunosuppressive molecules such as (a) TGFβ (present in the eye, brain, feto-placental unit, tumours, sites of inflammation), (b) the neuropeptides αMSH, VIP, CGRP and somatostatin (present in the eye, brain, feto-placental unit) and (c) steroid hormones such as cortisol (also eye, brain (Popp et al., 2009), feto-placental unit (Nolten and Rueckert, 1981)) or progesterone (feto-placental unit).

Our finding that human DC may be regulated jointly by cortisol and TGFβ2 (chapter 4) and that regulation continues even in the presence of inflammation, is thus relevant both to the specific situation in the eye, but also more generally to other sites where these molecules are present. Whilst the parallels to the CNS and the feto-placental unit are most evident, it is possible that cortisol and TGFβ2 contribute to DC

regulation in the many other tissues where these molecules may occur constitutively or be found under inflammatory conditions.

## 6.3 Aetiology and pathogenesis of uveitis: common pathways from varied insults

The extent to which autoimmunity (ie a self-directed immune response) is an inevitable consequence of a highly sensitive amplificatory immune system or whether dysregulation only occurs in the context of an external stimulus is still a matter of debate(Cooke, 2003; Kivity et al., 2009). In reviewing the aetiology and pathogenesis of uveitis we will first consider the extent to which uveitis (or some forms of uveitis) may be entirely due to the 'spontaneous' recognition of unique uveitogenic antigens within the eye, before turning to the putative role of infectious agents (both extra-ocular and intra-ocular) in triggering ocular autoimmunity.

Since the DC is regarded as the key player in the induction of an antigen-specific adaptive immune response, it is strongly implicated in the development of the autoreactive T cells which are thought to mediate much 'idiopathic' uveitis. These models of the initiation of uveitis are therefore considered within our evolving understanding of DC immunology, and in the light of my findings regarding modulation of DC function within the ocular microenvironment. Previous discussion of the ocular microenvironment (chapters 4 and 5) arising from my findings has been primarily limited to the anterior segment of the eye, in recognition that all the experimental work described within this thesis is based on AqH, both its contents (cellular and molecular) and immunodulatory effects. In this final discussion, I wish

to reflect more broadly on the probable role of DC within the aetiology and pathogenesis of uveitis, a process which will highlight both the similarities and some of the differences between types of uveitis found in both animal models and human disease.

### 6.3.1 Model 1: 'Spontaneous' ocular autoimmunity

A number of different ocular antigens have been proposed to be uveitogenic. In animal models, immunization with retinal-S antigen and interphotoreceptor retinoid binding protein (IRBP), among others, have been shown to induce uveitis. It is interesting that in most uveitis models, and many other models of autoimmunity, disease only develops when immunisation occurs with an appropriate adjuvant. Such models include Experimental Autoimmune Anterior Uveitis ( EAAU; antigen is a fragment of type I collagen) which primarily causes an anterior uveitis and Experimental Autoimmune Uveitis (EAU; antigen is retinal S-Ag or IRBP) which primarily generates a panuveitis ((Bora et al., 2004; Roberge et al., 1993; Lipham et al., 1990) and recently reviewed (Kerr et al., 2008)). Extrapolating to the human eye would suggest that most 'spontaneous' human uveitis does in fact require an additional infective (or at least inflammatory) stimulus in order for uveitis to develop. Given that ocular antigen is, to at least some extent, sequestered (resulting in immunological ignorance) this is perhaps to be expected. In passing it should be noted that the model Endotoxin Induced Uveitis (EIU) is sometimes described as being 'adjuvant-free' depending as it does on systemic LPS administration to induce a hyperacute neutrophilic ocular infiltrate (Rosenbaum et al., 1980). Since this model does not induce an antigen-specific adaptive immune response it cannot be regarded

as a helpful model of autoimmune uveitis, although it is of interest in providing a mechanism by which extraocular exposure to Gram negative bacteria (and their cell wall component, LPS) can selectively induce intraocular inflammation (considered later).

One naturally occurring animal model of uveitis, recurrent equine uveitis, has been proposed as a model of spontaneous human disease (Forrester, 2007; Deeg et al., 2002). It should be noted however that this form of uveitis appears to be associated with environmental exposure, particularly to Leptospira sp.(Faber et al., 2000).

A number of models of 'spontaneous uveitis' have now been developed. Some of these models involve compromising thymic tolerance such as by the reconstitution of nude mice with rat embryonic thymus(Ichikawa et al., 1991)or by deletions of the autoimmune regulator (AIRE) gene (Anderson et al., 2002). Other proposed models involve the insertion into the mouse genome of MHC susceptibility genes such as HLA-A29 (associated with bird-shot retinochoroidopathy in humans) or HLA-B27 (associated with acute anterior uveitis). Interestingly whilst HLA-A29 does lead to a spontaneous retinochoroidopathy resembling human disease in up to 80% of transgenic mice(Szpak et al., 2001), HLA-B27 transgenic mice do not spontaneously develop uveitis (whereas they do develop colitis and spondylarthropathy as discussed below).

An alternative strategy is to generate mice which are transgenic for a foreign antigen expressed under ocular tissue promoters (such as the rhodopsin promoter) and then 1) inoculate with that antigen, 2) adoptively transfer with T cells specific to the foreign

antigen or 3) cross with mice transgenic for the TCR to the foreign antigen. Gregerson and colleagues observed the effect of differing levels of expression of E. Coli β-galactosidase in the retina by expressing it under different promoters. They observed that mice were refractory to the induction of uveitis by inoculation or adoptive transfer except when β-galactosidase was expressed at very high levels (β-gal-hi-arrestin) and immunization was with a strong adjuvant (CFA and Pertussis) (Gregerson and Dou, 2002; Gregerson, 2002; Gregerson et al., 1999); this underlined the role of antigen sequestration in the tolerance of ocular antigens.

Conversely the role of central tolerance was demonstrated in two studies of 'spontaneous' uveitis using double transgenic mice. Ham *et al* used a double transgenic model (HEL expressed under the rhodopsin promoter (rho-HEL) with anti-HEL TCR) to demonstrate that there was central deletion of anti-HEL T cells. Interestingly since deletion was partial, a degree of 'spontaneous' uveitis was seen in the double transgenic model which was comparable to that seen with adoptive transfer of anti-HEL T cells into a single transgenic (rho-HEL); HEL immunisation with CFA into the single transgenic (rho-HEL) mice was not successful in inducing uveitis (Ham et al., 2004). Similarly Zhang *et al* observed central deletion of anti-HEL T cells in a double transgenic model (membrane bound and soluble forms of HEL expressed under the α-crystallin promoter with anti-HEL TCR). Again uveitis still occurred due to escape of anti-HEL T cells (Zhang et al., 2003).

While these models of spontaneous uveitis are ingenious, they serve rather to underline the strength of the immunoregulatory mechanisms which maintain ocular tolerance. Inducing uveitis in the absence of an infective or inflammatory trigger (as

postulated by this model) is difficult. This is entirely consistent with my finding that the ocular microenvironment strongly suppresses DC function in human. This strong inhibition of DC by the ocular microenvironment is also supported by data from animal models, notably Shen's observations that rabbit AqH inhibits mouse DC maturation and function (Shen et al., 2007). Spontaneous uveitis due to failure of these mechanisms is possible, but is likely to be a rare event *in vivo*. In summary, my data and evidence from the wider literature suggests that it is very unlikely that this model of 'spontaneous' ocular autoimmunity is a frequent cause of non-infective uveitis.

### 6.3.2 Model 2: Extraocular infection triggering ocular autoimmunity

Although external aetiological agents have been proposed for many autoimmune diseases, there are relatively few for which there is robust evidence of cause and effect. One of the best-described examples of an infection-triggered autoimmune disease is reactive arthritis in which there is strong evidence for the role of Gram negative bacteria. In outlining the possible role of extraocular infection in triggering ocular autoimmunity, I will first outline the evidence for the role of Gram negative bacteria in the development of ReA before considering the evidence for a similar mechanism in uveitis.

#### 6.3.2.1 Reactive arthritis as a model for infection-triggered autoimmunity

Reactive arthritis (also known as Reiter's syndrome) comprises a spectrum of clinical features including arthritis, urethritis and conjunctivitis which follows an infectious episode, either a genitourinary infection (commonly Chlamydia trachomatis) or gastro-intestinal (Salmonella sp., Shigella, Campylobacter jejuni and Yersinia enterocolitica). In most cases clinical features start 2-4 weeks post-infection, and resolve by 6-12 months post-infection, but around 15% may relapse and develop a progressive course. The presence of HLA-B27 is a risk factor for ReA, and is particularly common amongst those developing a progressive course or exhibiting extra-articular manifiestions (Brewerton et al., 1973b; Rihl et al., 2006a).

In addition to clinical evidence associating prior Gram negative infection with the subsequent disease, there is reasonable immunological evidence to suggest cause and effect. Chlamydia, Yersinia and Salmonella antigens and Chlamydia bacterial RNA (but not cultivatable organisms) have all been identified from synovial tissue or fluid in ReA, sometimes years after the initial infection(Rihl et al., 2006b; Gerard et al., 2000). The role of HLA-B27 in promoting susceptibility is not clear. It is possible that when HLA-B27 processes peptides from Gram negative bacteria, it results in an MHC-peptide complex which resembles self, and can thus via ' molecular mimicry' induce an anti-self response. Interestingly HLA-B27 restricted T cell responses are observed both to Chlamdyia and Klebsiella antigens and to human aggrecan (a proteoglycan found in cartilage). An alternative explanation is that the B27 molecule itself is immunogenic. This might be through misfolding (it is noted to have a relatively slow folding rate) and accumulation in the endoplasmic reticulum(Turner et al., 2005); or through the formation of immunogenic homodimers (Allen et al., 1999); or through molecular mimicry (ie resemblance between HLA-B27 and Gram negative

bacteria antigens (Chang et al., 2006; Chang et al., 2005; Chang et al., 2004). Other susceptibility genes identified include functional polymorphisms of IL-10 and TNFα.

#### 6.3.2.2 Uveitis and the role of extraocular infection

In most uveitis, the evidence for an external trigger leading to a dysregulated immune response is less clear. For acute anterior uveitis, like ReA, there is a strong association with HLA-B27 (50% of those presenting with AAU are HLA-B27+)(Chang et al., 2005). Furthermore AAU has been reported to develop after both Yersinia and Salmonella infection, and specific cellular and humoral immune responses to a number of Gram negative bacteria have been identified in AAU patients (reviewed Wakefield (Wakefield et al., 1991)). It has been suggested, particularly in light of the organisms implicated, that chronic mucosal inflammation may be an important factor in disease development(Chang et al., 2005). Interestingly two-thirds of those with AAU have been shown to have chronic asymptomatic bowel inflammation on ileocolonoscopy(Banares et al., 1995). This finding parallels the observation that whilst HLA-B27 transgenic mice develop spondylarthropathy and colitis in the presence of commensal gut bacteria, they fail to do so in pathogen free conditions (Rath et al., 1996).

The idea that much uveitis is an autoimmune response to an ocular antigen is an attractive hypothesis. In the context of AAU it is generally suggested that, as proposed for ReA, an ocular-antigen specific immune response arises through molecular mimicry to a pathogenic antigen (presented by DC or other professional APC). Autoreactivity may be present from the outset, or occur via epitope/antigen spreading in which priming to self-antigens released during tissue damage may occur;

this arises most commonly in the context of persistent immune response to the original antigen such as during chronic infection. It should be noted that, unlike the situation in ReA, conclusive identification of intact antigen from Gram negative bacteria has not been identified in the target tissue (ie the eye) itself, nor is there clear evidence for DC traffic from the eye (Dullforce et al., 2004; Camelo et al., 2003; Camelo et al., 2006; Camelo et al., 2004a); indeed my own data suggests that the required CCR7 upregulation/CCR5 downregulation is not induced by uveitis AqH. This provides further support to the concept that the encounter of APC and antigen may have occurred at an extra-ocular site of infection, such as the gastrointestinal or genitourinary tract.

### 6.3.3 Model 3: Intraocular infection triggering ocular autoimmunity

Infective uveitis due to intraocular invasion by pathogen may be caused by a wide range of organisms including viruses (eg Herpes simplex family), bacteria (eg Mycobacteria tuberculosis, Treponema pallidum), protozoa (eg Toxoplasma gondii), nematodes (eg Toxocara canis, Onchocerca volvulus) and fungi (eg Candida albicans, Histoplasma capsulatum). It is increasingly evident that much of the pathology of these diseases is mediated less by the organism itself and more by the immune response to chronic infection or possibly persistent antigen. It is interesting to speculate that some of the idiopathic uveitis syndromes such as Fuchs' heterochromic uveitis (FHU) and Posner-Schlossman syndrome (PSS) may represent a persistent immune response to a latent infection or to persistent antigen from a cleared infection. Each of these syndromes share clinical features with recognised presentations of viral anterior uveitis. In the past chronic infection with Herpes simplex and more recently

Rubella have been postulated for FHU (Bonfioli et al., 2005); trabeculitis due to Herpes simplex family members are still the leading candidates for PSS (Chee and Jap, 2008).

An extension of this hypothesis is that the intraocular infection leads to the generation of autoreactive to T cells to an ocular antigen (by molecular mimicry, epitope spreading and/or bystander activation) so inducing a purely autoimmune uveitis which would continue after clearing of the pathogen. Although speculative, such a mechanism could account for the discordance between the reported falling incidence of FHU in line with rubella vaccination and the lack of clear evidence of persistent rubella pathogen (or even antigen) in ocular tissues/fluid (Birnbaum et al., 2007). Similarly in the context of posterior or panuveitis, it has been proposed that sarcoidosis (and its related uveitis) reflects a persistent immune response to chronic atypical mycobacteria infection; however it is also possible that such an infection generates a purely autoimmune syndrome which persists even after successful clearing of the mycobacteria.

My data does not support the hypothesis that ocular DC, whether under resting or inflammatory conditions, migrate to draining lymph node tissue to induce a naive T cell response. As noted earlier my own data suggests that uveitis does not induce the normal CCR7 upregulation/CCR5 downregulation required for migration to the draining lymph nodes (indeed CCR5 was upregulated). Additionally ocular DC expression of the costimulatory molecule CD86 was reduced, and there was concomitant diminution of T cell responses (naive and memory; CD4+ and CD8+), suggesting that if any DC had trafficked to the draining lymphoid tissue (or if DC

encounter memory T cells in the ocular tissue itself) that this is unlikely to result in a strong antigen-specific effector immune response.

### 6.3.4 T cell trafficking to the eye

Although the mechanisms are very ill-defined, it seems increasingly likely that the initiating events in most cases of uveitis involve the extra-ocular/systemic priming of autoreactive T cells as a by-product of a normal response to pathogen(Forrester, 2007). Indeed the capacity of a systemic injection of retinal antigen in conjunction with adjuvant to induce uveitis (as in EAU), is a clear demonstration that these mechanisms can operate. The next unresolved question is: regardless of where T cells are primed, how do they encounter antigen in the eye?

#### 6.3.4.1 Extraocular priming and site specific homing

In the context of the model of ocular autoimmunity being triggered by extra-ocular mucosal inflammation (discussed earlier), it would follow that priming of naive antigen-specific T cells would occur in draining secondary lymphoid tissue. Thus in the presence of gastrointestinal inflammation, this would constitute Peyers patches and the mesenteric lymph nodes. The majority of T cells primed in this lymphoid tissue would home back to the gut due to expression of CCR9 and α4β7-integrin (responding to gut-specific CCL25 and mucosal vascular addressin cell-adhesion molecule (MADCAM-1) respectively), but such site-specific recirculation is not complete, allowing for the possibility that gut-DC primed T cells may circulate (Mora

et al., 2003)through other tissues such as the eye (reviewed Adams (Adams and Eksteen, 2006)). Mora *et al* found that the ratio of gut-DC primed T cells to peripheral lymph node-DC primed T cells was 17 times higher in the small intestine than the blood, but they also showed that these gut-DC primed T cells circulated to a range of other tissues including liver, lung and peripheral lymph nodes. Furthermore there is evidence that site-specific homing can be reprogrammed by subsequent encounters with DC from other tissues as has been demonstrated between skin and gut-DC primed T cells (Dudda et al., 2005). Whilst unique homing signals have not been identified for ocular tissues, this does at least provide a mechanism by which gut or skin-DC primed T cells may be diverted to other peripheral tissues including the eye.

#### 6.3.4.2 Hypothesis 1. Routine T cell trafficking through the non-inflamed eye leads to encounter of ocular antigen-specific T cell with cognate Ag/resident APC

It is not clear to what extent there is any circulation of T cells through the eye under non-inflamed conditions, although during active uveitis we have found the anterior chamber infiltrate to have a high proportion of CD4+T cells which are almost exclusively the CD45RO+ memory (rather than CD45RA+ naive) population. Studies of non-inflamed CSF have however demonstrated a significant number of memory CD4+ and CD8+ T cells circulating through the CSF which appear to provide a degree of 'immune surveillance' to the adjacent CNS tissues (Hickey, 1999). Although this runs somewhat counter to its status as an immunologically-ignored antigen-sequestered organ, it seems likely that the eye may experience a similar degree of routine lymphocyte trafficking. Such trafficking would then permit the encounter of recirculating memory CD4+ or CD8+ T cell with APC in the eye

allowing reactivation of the antigen-specific T cell by either resident dendritic cell or macrophage, activation of macrophages by Th1 effector cells or direct cytotoxic function of effector CD8+ T cells. Interestingly Prendergast *et al* suggest that activated T cells are not restricted by the blood-ocular barrier. They observed that activated T cells, whether antigen specific (a retinal S-Ag specific T cell line) or non-specific (concanavalin-A stimulated T cells), accessed the retina within 24h and that this preceded any visible signs of inflammation (Prendergast et al., 1998).

An alternative explanation for this model comes from the findings of Xu *et al* who showed that adoptive transfer of non-Ag-specific activated T cells (concanavalin-A stimulated) into the normal mouse can themselves cause a transient local breakdown in the blood ocular barrier with passage of activated T cells across the endothelium. This mechanism, which is dependent on ICAM-1 upregulation on the endothelium and its interaction with LFA-1 on the T cell (Xu et al., 2003), provides an explanation by which activated autoreactive T cells may encounter cognate antigen within the eye in the absence of pre-existing ocular inflammation.

A central feature of this hypothesis is it places the interaction of effector T cell and its cognate Ag-bearing APC (or Ag-bearing parenchymal cell) within the eye *before* there has been any local inflammation or breakdown in the blood-ocular barrier. In the context of this model, my findings that DC function is generally inhibited in the presence of non-inflammatory AqH (see chapter 4), would suggest that most encounters with circulating ocular-antigen specific T cells will not result in an effective (auto)immune response. Indeed, as discussed previously, the encounter may result in T cell anergy or even regulatory function, although I have not been able to

investigate this conclusively within this thesis. In the light of my findings, it seems unlikely that this non-specific routine circulation of T cells through the eye under resting conditions is the dominant mechanism by which uveitis-associated ocular-Ag specific T cell recruitment to the eye occurs.

#### 6.3.4.3 Hypothesis 2. Non-specific systemic inflammation leads to generalised endothelial activation and blood-ocular-barrier breakdown enabling encounter of ocular antigen-specific T cell with cognate Ag/resident APC

The blood-ocular barrier and limited lymphatic drainage reduces leukocyte trafficking to and from the eye, resulting in a degree of immunological ignorance. It is therefore argued that any primed ocular antigen-specific T cells may simply never encounter antigen that is sequestered exclusively in the eye. A systemic inflammatory response, such as occurs during a generalized viral infection (eg influenza), leads to systemic release of IL-1β, IL-6 and TNFα. Release of these cytokines results in generalised activation of endothelial barriers, and may also affect the blood-ocular barrier. This in turn would lead to recruitment of memory T cells to the site of inflammation; it has also been suggested that under extreme inflammation naive T cells may also circulate through.

Support for this hypothesis comes from the data of Xu *et al* who, in contrast to the conclusions of Prendergast *et al* (discussed earlier), suggest that activated T cells cannot routinely cross the uninflamed blood-ocular barrier. Using a scanning laser ophthalmoscope to track labelled leucocytes, they observed that under normal circumstances the high shear stress of the retinal vessels and lack of appropriate

adhesion molecules, precluded lymphocyte rolling, sticking and egress. When EAU was induced, they observed that early on in the disease (d9) reduction in shear stress, and upregulation of ICAM-1, P- and E-selectin on the endothelium permitted IRBP Ag-primed cells to leave the vessels and infiltrate the retina. Interestingly at the peak of disease (d16), naive cells also were able to infiltrate the retina(Xu et al., 2004). The numbers of these naive cells identified in retinal tissue were significantly fewer than the Ag-primed cells at all time points which may, in addition to the observed reduction in recruitment, reflect differential retention (or selective apoptosis).

One molecule which may be released systemically and which has been shown to cause blood-ocular barrier breakdown is LPS. In EIU, injection of LPS alone (either systemic or intravitreal) leads to an anterior uveitis characterized by severe blood-ocular barrier breakdown and neutrophilic infiltrate. The selective sensitivity of iris and ciliary body to LPS is interesting, and is possibly mediated via TLR-4 which has been shown to be expressed on APC in these tissues(Chang et al., 2004). The sensitivity of the blood-ocular barrier to LPS is important both in terms of the possible link between extraocular mucosal infection and anterior uveitis and more generally in demonstrating how a systemic stimulus can cause selective blood-ocular barrier breakdown.

A central feature of this hypothesis is that the effector T cell does not enter the eye until inflammation (albeit generalised) is already present. The encounter of autoreactive T cell with cognate antigen as presented by antigen presenting cells (or on parenchymal cells) would thus occur in the context of inflammatory cytokines such as IL-1, IL-6 and TNFα. Given that AqH continues to inhibit DC function even in the

presence of inflammation, whether actual uveitis (chapter 5) or exogenous cytokines (chapter 4), it is likely that antigen presentation within the eye (at least by DC) does not result in a strong effector response, either for naive or for memory T cells.

## 6.4 The natural history of uveitis and the role of treatment

One of the unresolved issues of this thesis (and of our understanding of human uveitis in general) concerns how the immune response evolves during the clinical course of uveitis. To further characterise the uveitic immune response would require longitudinal AqH samples from a cohort of patients. Ideally one would take samples at onset and regularly throughout any acute episode (for first episodes and recurrences) or through any exacerbation of chronic disease, as is possible in animal models. Although such regular exposure to an invasive technique would not be justified in humans, ethical approval has been granted to take up to three serial AqH samples in appropriately consented patients. Although serial sampling was not carried out as part of this series of experiments, such longitudinal information regarding the ocular infiltrate and microenvironment could be extremely valuable. Specifically it should provide further insight into: the impact of treatment (note the advantage that one is comparing within the same patient cf between patients in this thesis); signals of resolution vs persistence; and the immunological difference between a first episode and recurrence (and possibly what determines recurrences).

In the context of this thesis it is interesting to speculate as to whether a rapidly resolving uveitis represents non-specific breakdown in the blood-ocular barrier (such as associated with LPS) but without DC priming of ocular antigen specific T cells,

whereas persistent (and possibly recurrent) disease represents a true autoimmune phenomenon mediated by DC (either ocular or extraocular). One way in which this might be tested would be to investigate the Ag-specificity of the T cells present in the AqH during episodes of uveitis, and look for restriction to ocular antigens occurring over time during a single recurrence and from first episode to subsequent recurrences.

Patients frequently inform ophthalmologists that they have had previous episodes of uveitis that resolved spontaneously. It would be interesting to know how spontaneous resolution differs from 'treatment-assisted' resolution, but since one could not with-hold treatment from any patient with active uveitis it is difficult to see how this could be tested. It is possible however that progress may be made by observing the differences in resolution between patients on different forms of therapy.

## 6.5 Treatments for uveitis: do ocular APC have a role?

Regulation of inflammation in the eye may potentially be achieved by the use of two different DC-based strategies: first, treatment directed at the ocular DC population; and second, treatment with *in vitro* generated 'tolerogenic' DC populations. We will consider each of these in turn.

The dominant finding of this thesis is that the ocular microenvironment is suppressive to normal DC function, and continues to be so even in the presence of uveitis. It should be noted, however, that despite this suppression, these DC are still capable of inducing some degree of T cell proliferation, with progeny expressing both Th1 and Th2 cytokines. It is therefore interesting to speculate that pharmacological ablation of

DC function or deviation towards a tolerogenic DC phenotype might lead to further reduction in the effector T cell response and/or an increase in the regulatory T cell response within the eye. This would translate into more rapid resolution of clinical inflammation, and, more speculatively, might also reduce disease recurrence. Such potential pharmacological agents include molecules to which the eye is already exposed such as glucocorticoids and TGFβ.

As discussed previously (Chapter 4) endogenous glucocorticoid (cortisol) and TGFβ2 are already present within the eye at physiologically significant levels. It is interesting that the first-line treatment of uveitis (therapeutic glucocorticoids such as dexamethasone) has, among its protean effects, a profound dose-dependent suppression of DC expression of CD86 and associated loss in induction of T cell responses (chapter 5). Additionally Woltman *et al* have shown that stimulation of naive or memory T cells by dexamethasone treated of human monocyte-derived DC results in T cells that are anergic and have mild regulatory properties (Woltman et al., 2000; Woltman et al., 2006) .

The suppressive role of TGFβ induced tolerogenic APC has been demonstrated in a number of murine models of inflammation, including the suppression of EAU by TGFβ2-treated IRBP-pulsed PEC, the delayed onset and reduction in severity of Experimental Autoimmune Encephalomyelitis (EAE) by TGFβ2-treated Myelin Basic Protein (MBP)-pulsed PEC, and the reduction in severity of a model of autoimmune pulmonary fibrosis induced by hapten 2,4,6-trinitrobenzene-sensitized T effector cells by the addition of TGFβ2-treated hapten-pulsed APC. As discussed previously (see chapter 4), TGFβ2 in normal AqH appears to have an inhibitory role on DC, although

it has been suggested that this effect is reduced by the high proportion of the molecule in latent form. It is interesting to speculate whether exogenous treatment with TGFβ2 or even *in vivo* activation of the molecule could further promote toloerogenicity in the ocular DC.

In a review written in 2001, de Smet noted that 'the functional characteristics of ocular DC have yet to be determined, particularly in the setting of acute inflammation' and that 'modulation of DC function ... may prove to be an effective therapeutic approach in years to come' (de Smet and Chan, 2001). Since then *in vitro* generated tolerogenic DC have been used to induce suppression in a wide variety of animal models and, to a much more limited extent, in human. Strategies include the use of immature DC, or DC treated with IL-10 (with or without TGFβ), dexamethasone, $1,25(OH)_2$ Vitamin D3, $PGE_2$, VIP, cyclosporin A, rapamycin and mycofenolate mofetil(Thomson and Robbins, 2008).

In mouse such tolerogenic DC have been successful in prolonging allograft survival, in protecting from disease in models of colitis, arthritis, asthma and endotoxaemia and in reducing DTH response. In human healthy volunteers, immature DC pulsed with influenza matrix protein and keyhole limpet haemocyanin led to selective inhibition of MP-specific CD8+ T cell function, and generation of IL-10 producing MP-specific cells (Gad et al., 2004).

Tolerogenic DC trials for human autoimmune disease are currently underway: these include one trial for diabetes mellitus using DC pulsed with antisense oligodeoxyribonucleotides to CD40, CD80 and CD86 and two trials for rheumatoid

arthritis using DC, one with DC pulsed with citrullinated peptides and one with non-pulsed DC treated with dexamethasone and vitamin D3 (reviewed (Thomson and Robbins, 2008)). Although there are many practical challenges to the use of tolerogenic DC in the clinic, progress may be rapid due to the extensive experience gained from vaccination protocols for immunogenic DC use in cancer (reviewed (Palucka et al., 2008)). Whilst it is unlikely that DC vaccination is ever first-line therapy for mild disease, it remains an exciting possibility that, in refractory uveitis, tolerogenic DC (perhaps generated by endogenous ocular molecules or their analogues) may finally enable targeted resolution of the adaptive immune system in the eye.

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# 8 APPENDIX

## 8.1 Patient Information Sheet

See pages I to V.

## 8.2 Patient Consent Form

See page VI.

## 8.3 Study letter to GP

See page VII.

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# Immune mechanisms in the ocular microenvironment

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

**PART 1.**

**What is the purpose of the study?**

We are studying how the fluid inside the front of the eye (known as aqueous humour) is involved in a range of diseases that can cause the eye to become inflamed. We wish to look at a number of cells and molecules in the aqueous humour of people who have eye inflammation and compare these with people who do not have eye inflammation. By studying these differences we hope it will give us new information about how the inflammation is caused. In the longer term this information may lead to the development of better treatments for these conditions.

**Why have I been chosen?**

You have been invited to participate in this study because you have a condition that has caused your eye to become inflamed.

**Do I have to take part?**

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

**What will happen to me if I take part?**

If you decide to take part, we will take a very small sample of the fluid (aqueous humour) from the front of your eye. This is done in the Out-Patient Clinic or Casualty Department. First we use a few anaesthetic drops to numb your eye and then some antiseptic drops to clean the eye. With you sitting on our slit-lamp microscope (this is the machine we always use to look at your eyes) we introduce a very fine needle through the outside edge of the window of the eye to take a tiny sample of fluid (about the size of a drop of water) from the front of the eye. As the procedure is

undertaken under local anaesthesia there should be no pain but there might be a sensation of a slight pulling feeling for a few seconds as the sample is taken.
We often perform this test in people who we think an infection has caused their eye inflammation. Only a doctor highly experienced in this technique and a member of Professor Murray's team will take the sample.

We also wish to take a blood sample (about six teaspoonfuls) so we can compare the cells and molecules in it with your aqueous humour sample.

Overall, it should take about 30 minutes to have the aqueous humour and blood samples taken.

In most people we will only take the aqueous humour and blood samples once. Some people can get more than one attack of eye inflammation and if it does comes back we may ask to take the samples again. We will not ask to take samples more than a total of three times.

**What do I have to do?**

During the procedure you should keep as still as possible and follow any instructions from the doctor, for example to look in a particular direction.

After the procedure we ask you to use some antibiotic drops for a few days. You will also need to use the treatment prescribed for your eye inflammation, and attend your follow-up clinic appointment as directed by the doctor who has seen you.
You do not have to make any extra visits because you have had a sample of aqueous humour taken from the eye.

**What is being tested?**

After the procedure we examine your fluid (aqueous humour) and blood sample in the laboratory to look at a number of cells and molecules that might be responsible for the eye becoming inflamed.

**What are the potential side effects of the procedure?**

Complications are unusual from taking aqueous humour samples. Theoretical risks include the fluid from the front of the eye leaking out of the wound, reduced eye pressure, infection getting into the eye, clouding of the focusing lens, and bleeding into the front of the eye. We have performed hundreds of these procedures and have never had any of these complications.

Complications from having a blood sample taken are rare, other than the brief discomfort of the needle. Some people may get some bruising of the skin around where the sample was taken.

**What are the other possible disadvantages and risks of taking part?**

Your appointment may take a few minutes longer than usual, but all other treatment and follow-up arrangements are unchanged.

**What are the possible benefits of taking part?**

We cannot promise that our research will help you directly but we hope that it will help us understand why some people get eye inflammation and that this may lead to improvements in treatment for these conditions.

**What happens when the research study stops?**

Your direct involvement in this study only lasts for the time taken to take the aqueous humour and blood samples. Sometimes the samples may be kept for several years before the research is completed. At this point the samples will be carefully disposed of.

**What if there is a problem?**

Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The details are included in Part 2.

If you have any complaints please contact the Chief Investigator, Prof Philip I. Murray on 0121 507 6851. If you wish to contact someone independent please contact the Patient Advice and Liaison Services on 0121 507 4396.

**Will my taking part in the study be kept confidential?**

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

**Contact Details:**

For further information about the study or should you have any concerns about your involvement please contact either:

| **Chief Investigator:** | **Principal Investigator:** |
|---|---|
| Prof Philip I. Murray<br>Academic Unit of Ophthalmology<br>Birmingham & Midland Eye Centre<br>Sandwell & West Birmingham NHS Trust<br>Dudley Rd<br>Birmingham<br>B18 7QU | Miss Saaeha Rauz<br>Academic Unit of Ophthalmology<br>Birmingham & Midland Eye Centre<br>Sandwell & West Birmingham NHS Trust<br>Dudley Rd<br>Birmingham<br>B18 7QU |
| Tel: 0121 507 6851 | Tel: 0121 507 6849 |
| Fax: 0121 507 6853 | Fax: 0121 507 6853 |
| Email: P.I.Murray@bham.ac.uk | Email: S.Rauz@bham.ac.uk |

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**This completes Part 1 of the Information Sheet.**

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

**Part 2**

**What will happen if I don't want to carry on with the study?**

You can withdraw from the study at any point, even after we have taken your samples. If you withdraw from the study, and you wish us to destroy your samples, we will do so but we would need to use the data collected up to your withdrawal.

**What if there is a problem?**

**Complaints**: If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (0121 507 6851). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

**Harm**: In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against Sandwell & West Birmingham Hospitals NHS Trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

**Will my taking part in this study be kept confidential?**

All information which is collected about you during the course of the research will be kept strictly confidential. This information will be gathered by one of the clinical members of staff either directly from you at the time you enrol in the study or from your clinical notes at a later date. This information is anonymised, and only clinical members of staff involved directly with this research will have access to any identifiable data. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it

Our procedures for handling, processing, storage and destruction of your data are compliant with the *Data Protection Act 1998*. You have the right to view the data we have on record about you and to correct any errors.

With your permission we would like to inform your GP that you have participated in this study. We will inform the GP that you have had a sample of aqueous humour and blood taken today but that this does not affect your treatment or follow-up arrangements.

**What will happen to any samples I give?**

The samples are stored in a secure environment on the Birmingham & Midland Eye Centre Site and are only removed to the Academic Unit of Ophthalmology Laboratory (University of Birmingham site) when they need to be analysed. Only members of Professor Murray's research team will have access to the samples. The samples will

eventually be destroyed in a safe manner following clinical waste protocol. All these conditions are compliant with the MRC guidance 'Human *Tissue and Biological Samples for Use in Research*' and the *Human Tissue Act 2004*

**Will any genetic tests be done?**

No

**What will happen to the results of the research study?**

It is intended that the results of the research will be presented at scientific meetings, and published in relevant clinical and academic journals. We also feed these results back to participants through patient support groups and information in clinic. You will not be identified in any report or publication.

**Who is organising and funding the research?**

The Academic Unit of Ophthalmology of the University of Birmingham is organising this study. Our funding is derived from several ocular charities including the *Birmingham Eye Foundation*. Your doctor will be not be paid for including you in this study, and you will not receive any payment for participating in the study.

**Who has reviewed the study?**

This study was given a favourable ethical opinion for conduct in the NHS by the Dudley Local Research Ethics Committee.

Patient Information Leaflet: Immune Mechanisms in the Ocular Microenvironment 31.08.06 version 1.1

**And finally …**

You will be given a copy of the information sheet and a signed consent form. Thank you for taking the time to read this sheet and considering involvement in this research study.

Sandwell and West Birmingham Hospitals NHS
NHS Trust

Birmingham and Midland Eye Centre
Dudley Road
Birmingham
B18 7QH
Tel: 0121 554 3801
www.cityhospital.org.uk

Centre Number: BMEC
Study Number: UKCRN 4654
Patient Identification Number for this trial:

**CONSENT FORM: Immune mechanisms in the ocular microenvironment**

Name of Chief Investigator: Professor Philip I. Murray
Name of Principal Investigator: Miss Saaeha Rauz

**Please initial box**

1. I confirm that I have read and understand the information sheet dated 31.08.06 (version 1.1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to my GP being informed of my participation in the study. ☐
5. I agree to take part in the above study. ☐

| Name of Patient | Date | Signature |
|---|---|---|
| Name of Person taking consent (if different from researcher) | Date | Signature |
| Researcher | Date | Signature |

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Sandwell and West Birmingham Hospitals NHS
NHS Trust

Birmingham and Midland Eye Centre
Dudley Road
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B18 7QH
Tel: 0121 554 3801
www.cityhospital.org.uk

Centre Number:BMEC
Study Number: UKCRN4654

**Title of Project: Immune mechanisms in the ocular microenvironment**

Name of Chief Investigator: Professor Philip I. Murray
Name of Principal Investigator: Miss Saaeha Rauz

**Dear Doctor,**

This is to inform you that your patient:

| Insert Addressograph here |
|---|

has today participated in our study on “Immune Mechanisms in the Ocular Microenvironment”. Their involvement included having samples of aqueous humour and peripheral blood taken. Neither of these procedures should affect their clinical care in any way. A separate letter will be sent to you regarding their clinical condition.

Yours faithfully,

Philip I. Murray PhD FRCP FRCS FRCOphth
Professor of Ophthalmology

# CHAPTER 3
# The development of an in-vitro model to study dendritic cell function in the ocular microenvironment

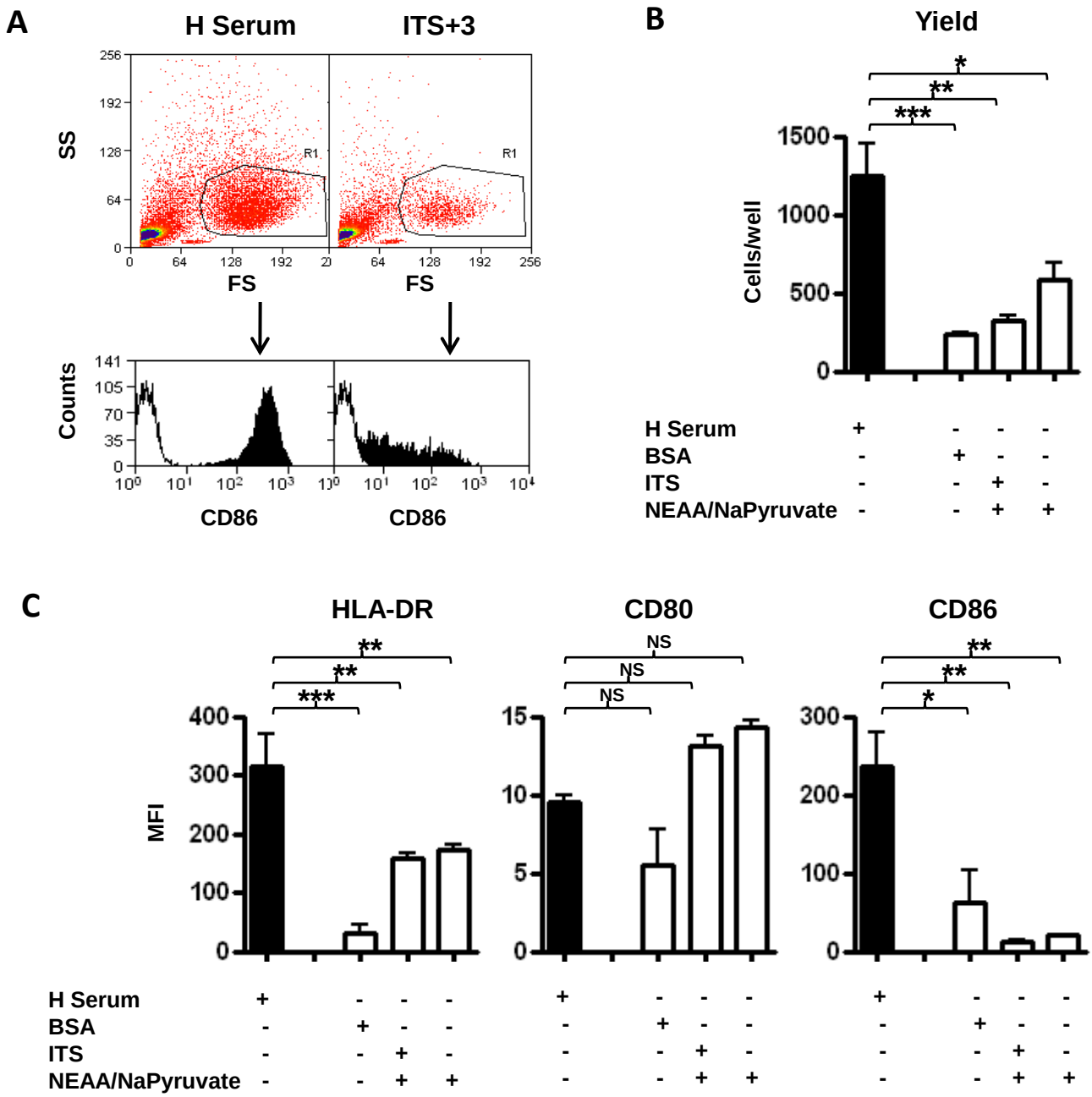



**Figure 3.1 Differentiation of dendritic cells from monocytes is inhibited in the absence of serum, with a lower yield and altered surface profile.**
Bead purified CD14+ monocytes were cultured for 6d in the presence of 10% human serum or a serum-free alternative (1% BSA, ITS+3 or ITS+3/Non-essential amino-acids/Sodium pyruvate). (A) Forward scatter vs Side scatter and CD86 expression for representative cultures in human serum vs serum-free (here ITS+3). (B) Yield and (C) MFI of HLA-DR, CD80 and CD86 for mean ± SD of triplicate cultures; representative of three separate experiments.
One-way ANOVA with Bonferroni post-hoc test for each column vs human serum
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant

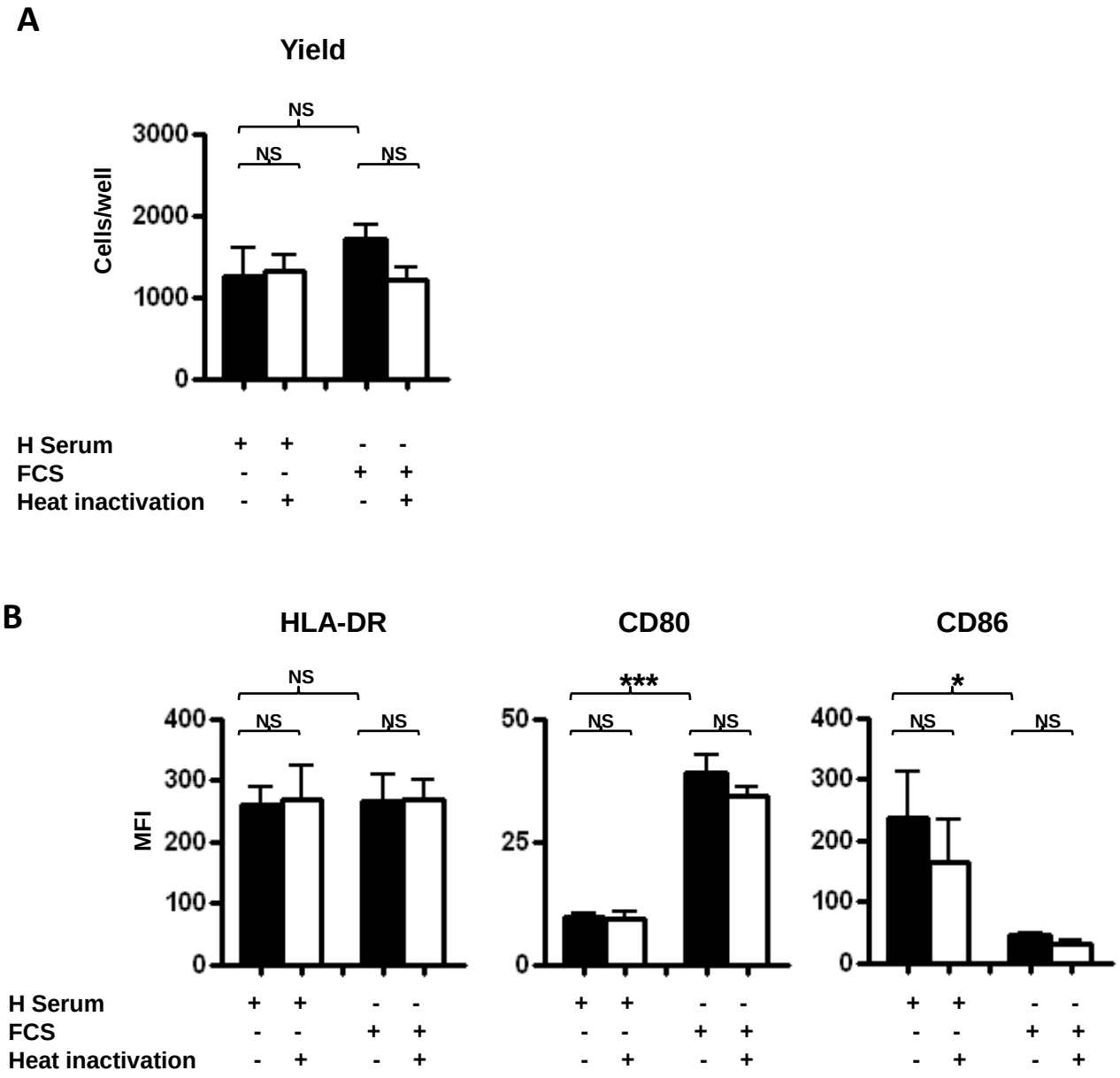



**Figure 3.2 Differentiation of dendritic cells from monocytes is affected by serum type, with reciprocal changes in CD80 and CD86.**
Bead purified CD14+ monocytes were cultured for 6d in the presence of 10% human serum or fetal calf serum (FCS), which was either standard or heat inactivated. (A) Yield and (B) MFI of HLA-DR, CD80 and CD86 for mean ± SD of triplicate cultures; representative of three separate experiments.
One-way ANOVA with Bonferroni post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

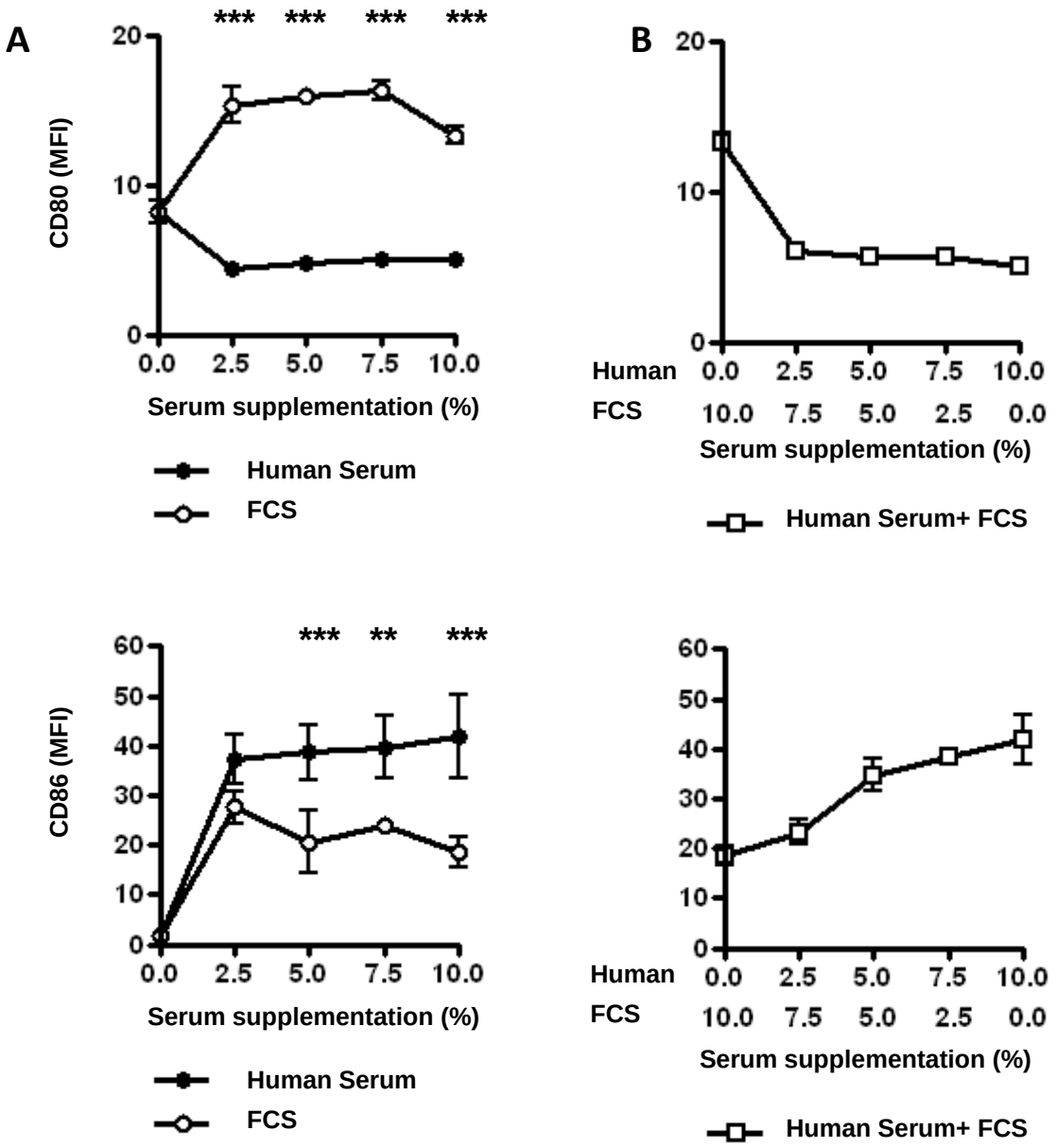



**Figure 3.3 Fetal calf serum (FCS) induction of CD80 is prevented by the presence of human serum (HS), whilst FCS inhibits normal CD86 upregulation by HS.**

Bead purified CD14+ monocytes were cultured for 6d in the presence of (A) 0-10% human serum or fetal calf serum or (B) both sera. MFI of CD80 and CD86 for mean ± SD of triplicate cultures; representative of two separate experiments. One-way ANOVA with Bonferroni post-hoc test for matched concentrations shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

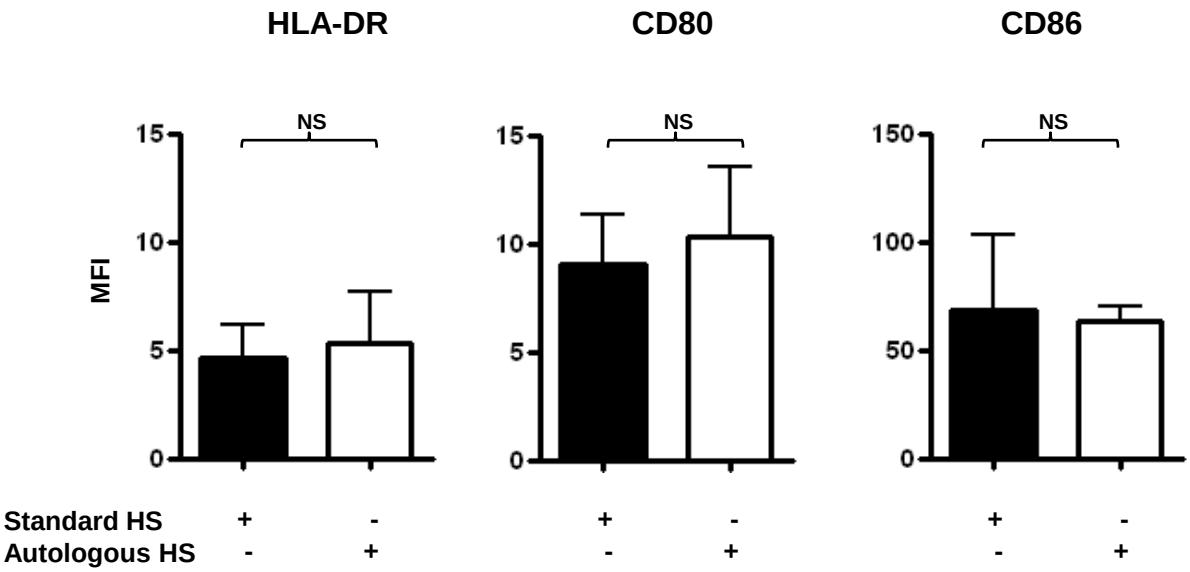



**Figure 3.4 Dendritic cell differentiation in human serum is not affected by whether the serum is autologous or standard pooled AB male serum.**
Bead purified CD14+ monocytes were cultured for 6d in the presence of 10% human serum (HS; standard pooled male AB+ vs autologous). MFI of HLA-DR, CD80 and CD86 for mean ±SD of triplicate cultures; representative of three experiments. Student's t-test (unpaired); NS = not significant.

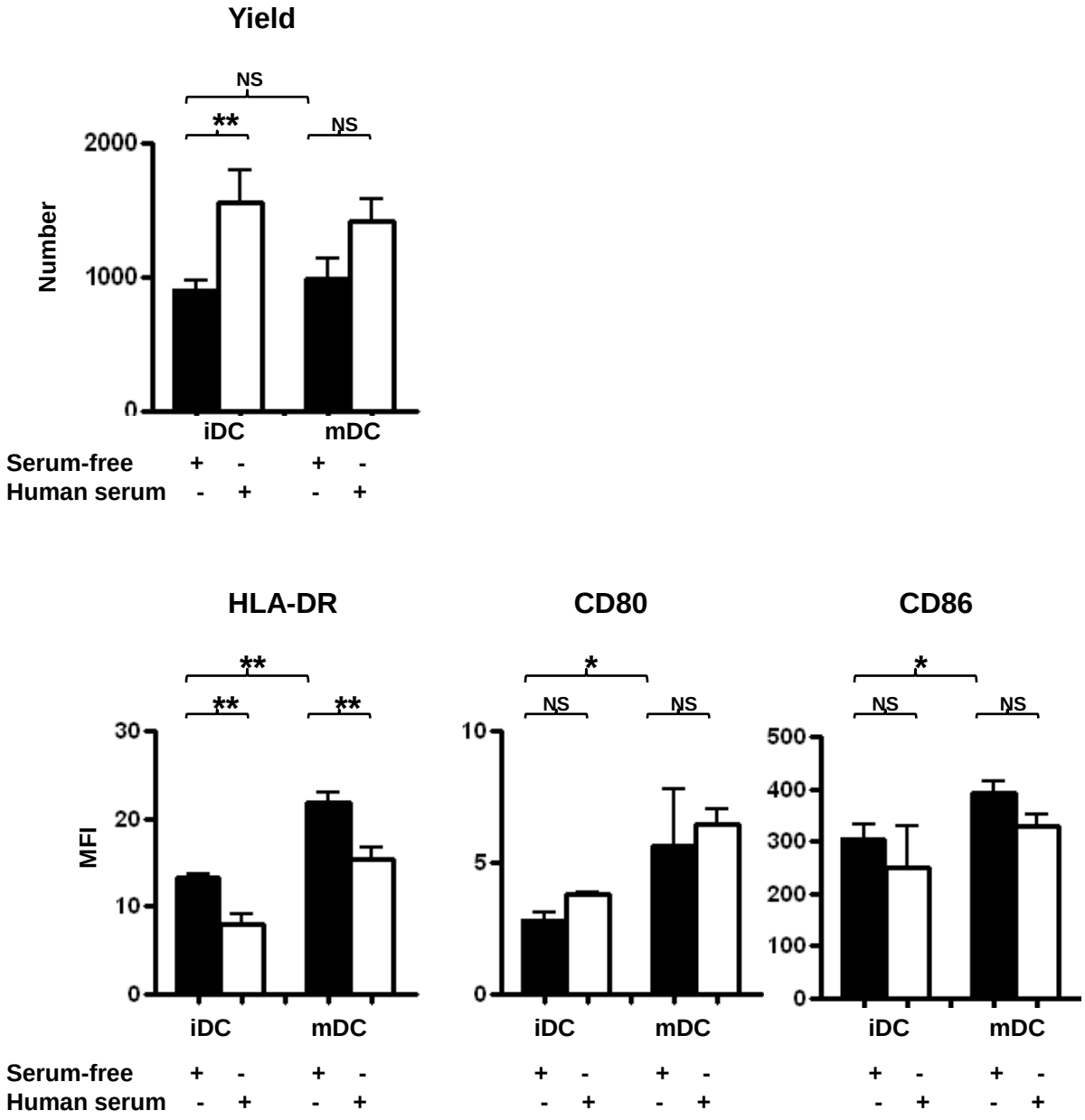



**Figure 3.5 Serum-free conditions are compatible with dendritic cell maturation in response to inflammatory cytokines but are associated with lower yields.**

Monocyte-derived dendritic cells were cultured for 48h with or without inflammatory cytokines (IL1β, IL6, TNFα, $PGE_2$ to generate mature mDC vs immature iDC) in the presence or absence of serum. Histograms for (A) yield (B) MHC and key costimulatory molecules for mean ± SD MFI of triplicate cultures; representative of three separate experiments.

One-way ANOVA with Bonferroni post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

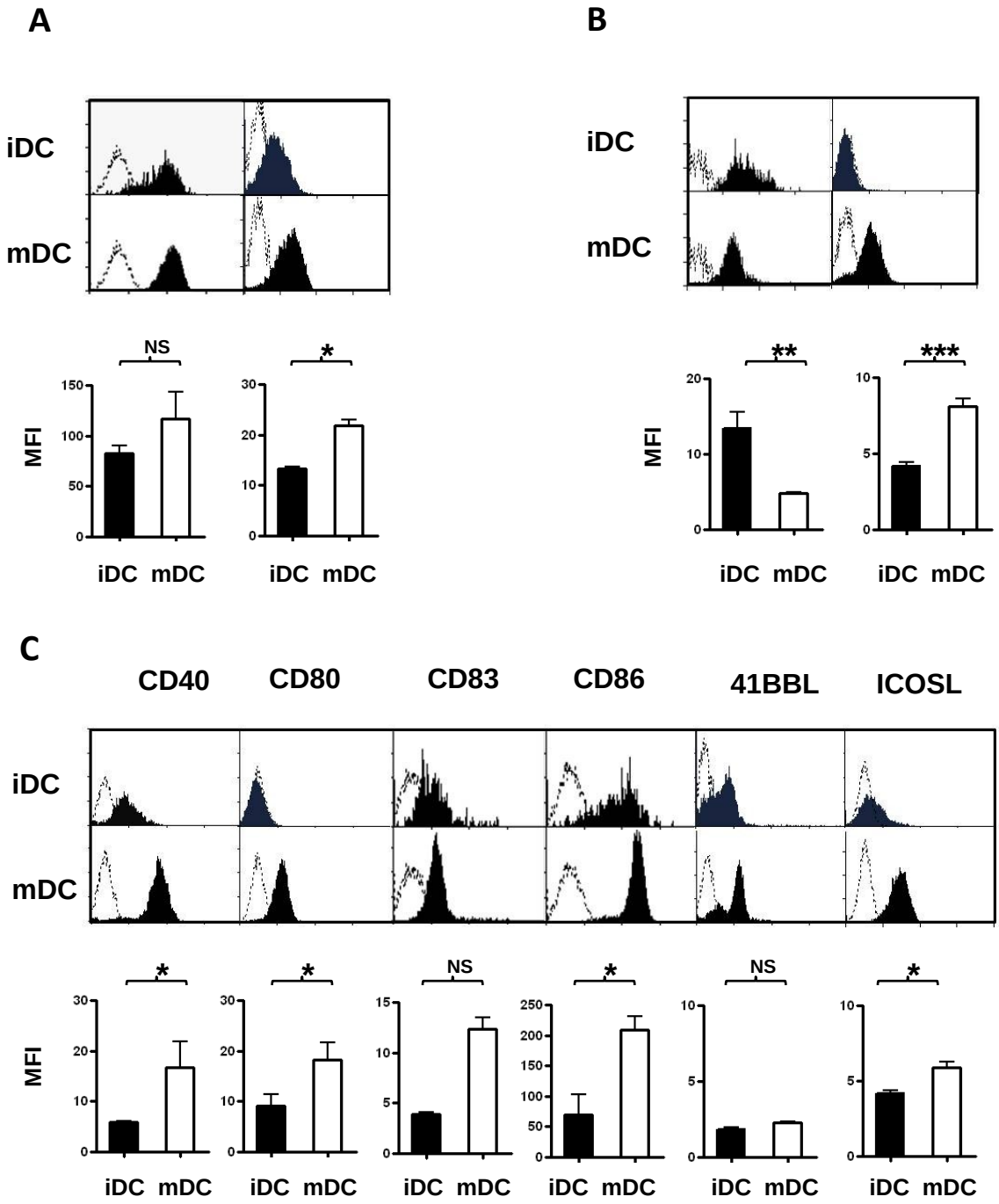
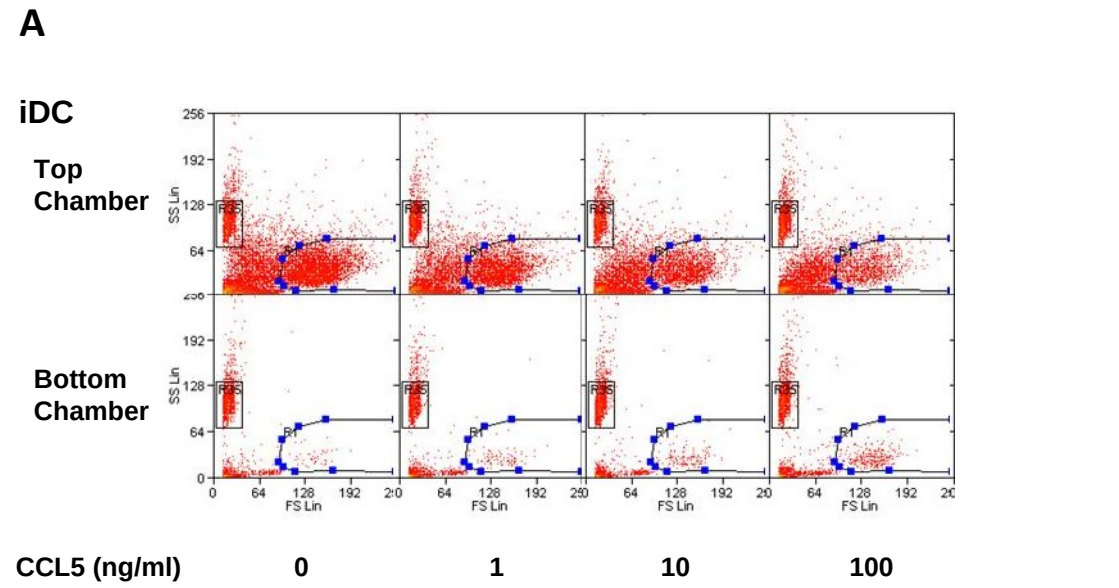



**Figure 3.6 Inflammatory cytokines under serum-free conditions induce changes in key surface molecules characteristic of dendritic cell maturation**
Monocyte-derived dendritic cells were cultured for 48h in the presence or absence of a cocktail of inflammatory cytokines (IL1β, IL6, TNFα, $PGE_2$). Flow cytometry plots and mean ±SD MFI of triplicate cultures for (A) MHC (B) selected chemokine receptors and (C) key costimulatory molecules; representative of three separate experiments.
Student's t-test (unpaired); * $p < 0.05$; NS = not significant.



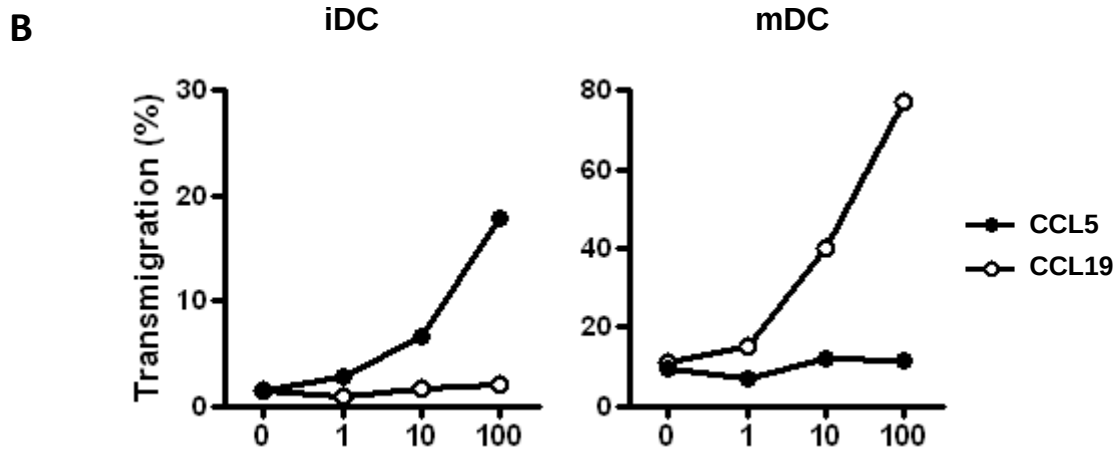




**Figure 3.7 Changes in chemokine receptors induced by inflammatory cytokines under serum-free conditions affect transmigration in response to CCL5 (RANTES) and CCL19.**

Monocyte-derived dendritic cells were cultured for 48h in the presence or absence of a cocktail of inflammatory cytokines (IL1β, IL6, TNFα, $PGE_2$). Migration to chemokine was assayed in a 96-well transwell plates with (A) flow cytometric counting of both chambers after 3h incubation to estimate (B) percentage transmigration. Representative of three separate experiments. Region R1 corresponds to live cells; R35 to counting beads.

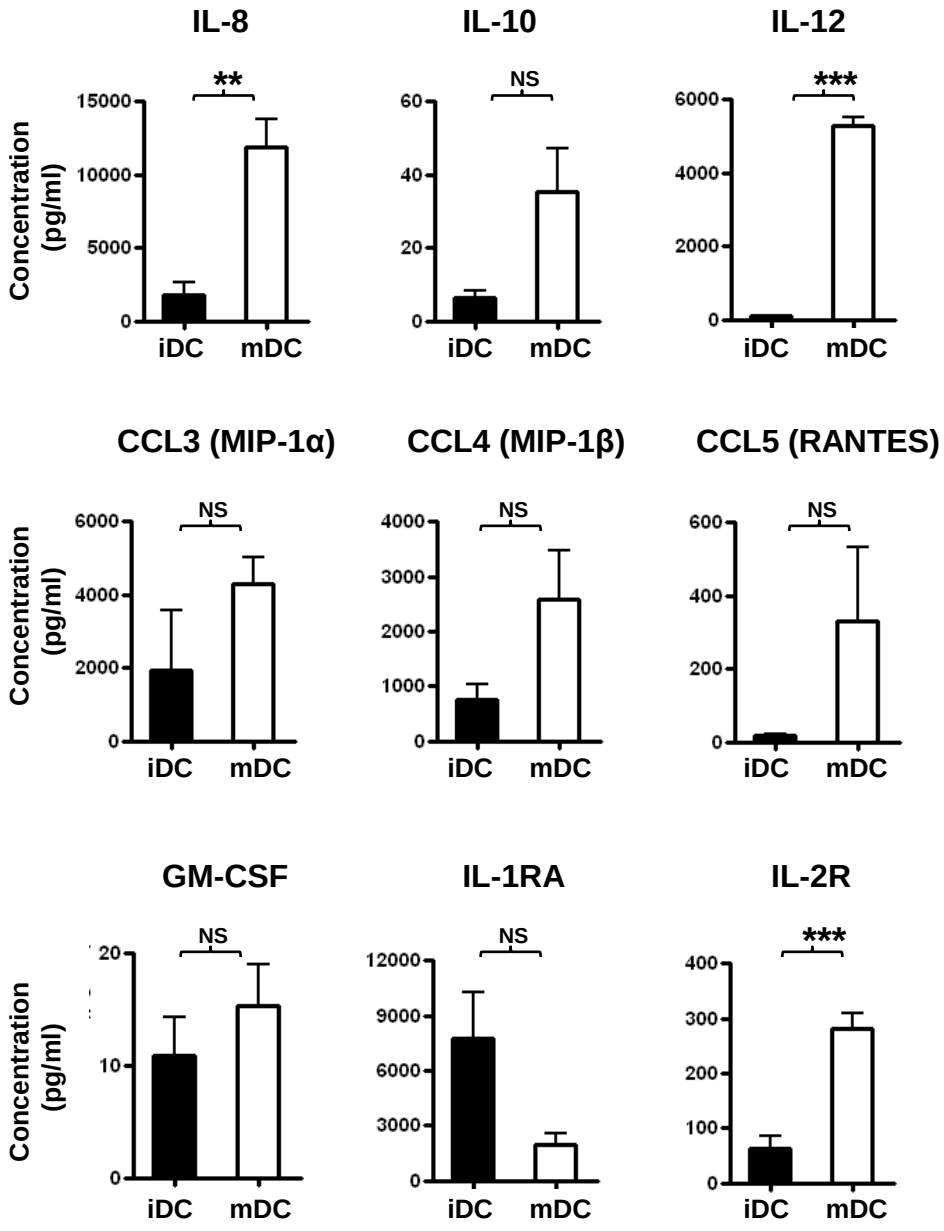



**Figure 3.8 Cytokine production induced by inflammatory cytokines under serum-free conditions is selectively upregulated**

Monocyte-derived dendritic cells were cultured for 48h in serum-free conditions with or without inflammatory cytokines (IL1β, IL6, TNFα, $PGE_2$). Culture supernatants were analysed by multiplex bead immunoassay. Mean ± SEM concentrations for triplicate cultures; representative of four separate experiments. Student's t-test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

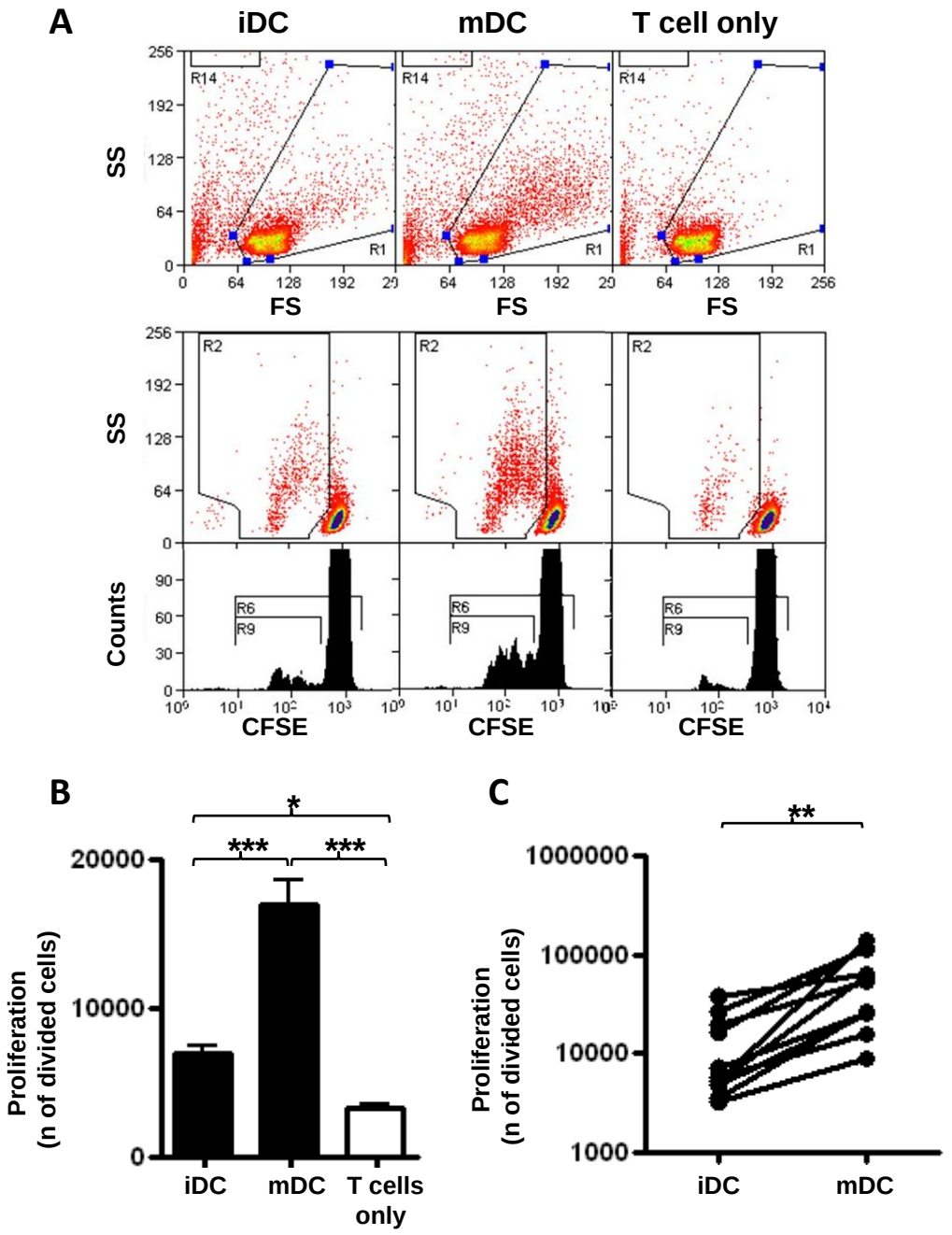



**Figure 3.9 Dendritic cell maturation under serum-free conditions is compatible with highly stimulatory DC in an allogeneic proliferation model**
CFSE labelled naive CD4+ T cells were cultured for 4 days in the presence or absence of monocyte-derived dendritic cells (either untreated (iDC) or pre-treated with inflammatory cytokines (mDC)). (A) Flow cytometric detection of CFSE levels in counted cells enables estimation of (B) number of divided cells with mean ± SD of triplicate cultures shown. (A) and (B) are representative of ten separate experiments as shown in (C).
One-way ANOVA with Bonferroni post-hoc test for selected columns (B) and Wilcoxon matched pairs test (C). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

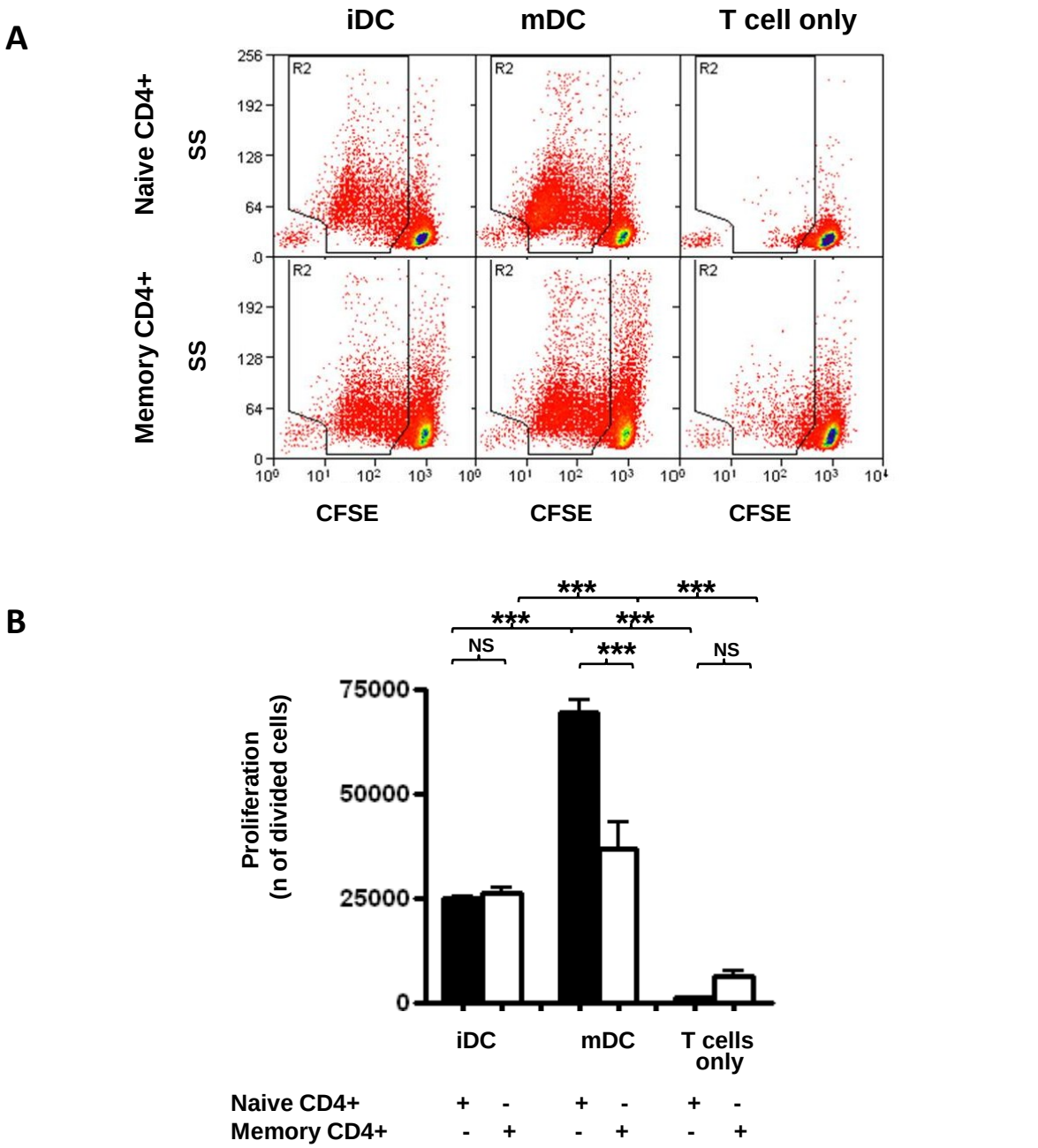



**Figure 3.10 Dendritic cells matured under serum-free conditions are highly stimulatory to both naive and memory CD4+ T cells in an allogeneic proliferation model**

CFSE labelled naive or memory CD4+ T cells were cultured for 4 days in the presence or absence of monocyte-derived dendritic cells (either untreated (iDC) or pre-treated with inflammatory cytokines (mDC)) (A) Flow cytometric detection of CFSE levels (B) number of divided cells with mean ± SD of triplicate cultures shown. (A) and (B) are representative of three separate experiments.
One-way ANOVA with Bonferroni post-hoc test for selected columns shown;
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

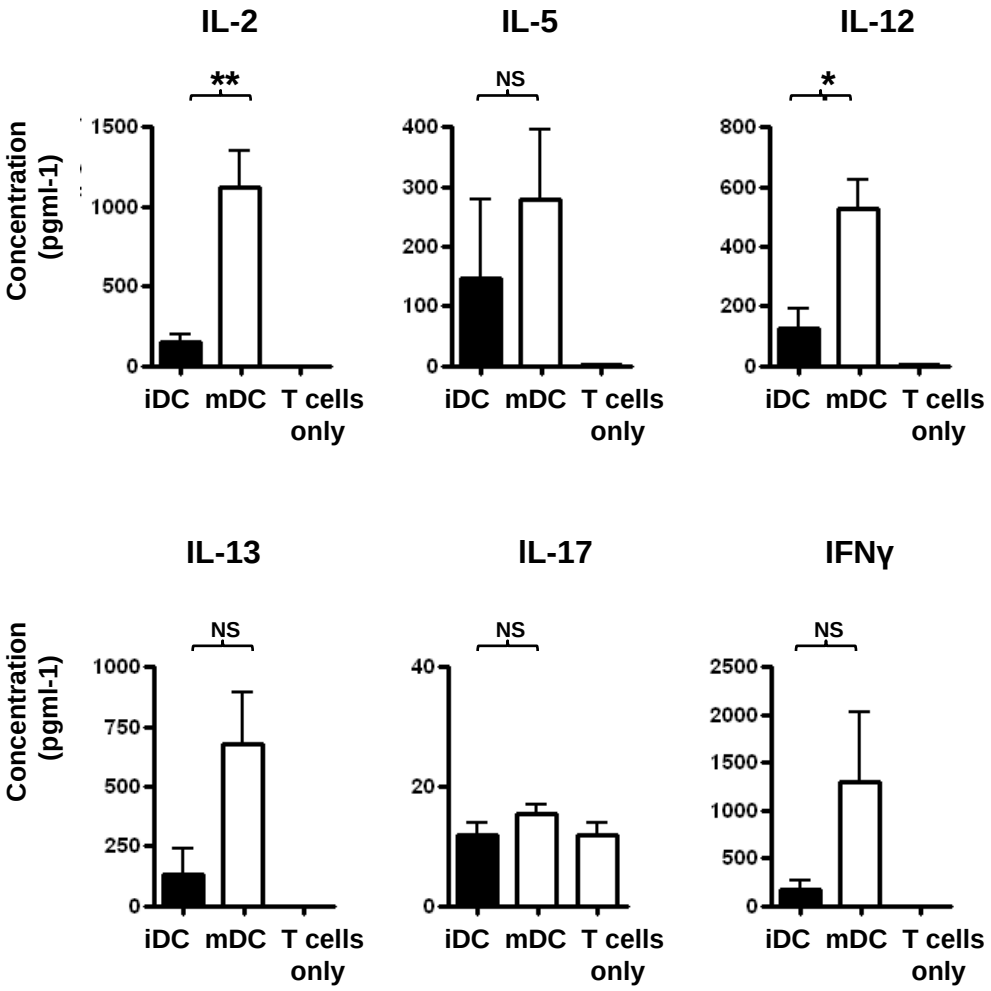



**Figure 3.11 Dendritic cells cultured in serum-free conditions do not selectively skew T cell differentiation as measured by T cell cytokine production**

CFSE labelled naive or memory CD4+ T cells were cultured for 4 days in the presence or absence of monocyte-derived dendritic cells (either untreated (iDC) or pre-treated with inflammatory cytokines (mDC)), and cytokine concentration in culture supernatants measured by multiplex bead immunoassay. Mean ± SEM concentrations for four separate experiments shown.

One-way ANOVA with Bonferroni post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

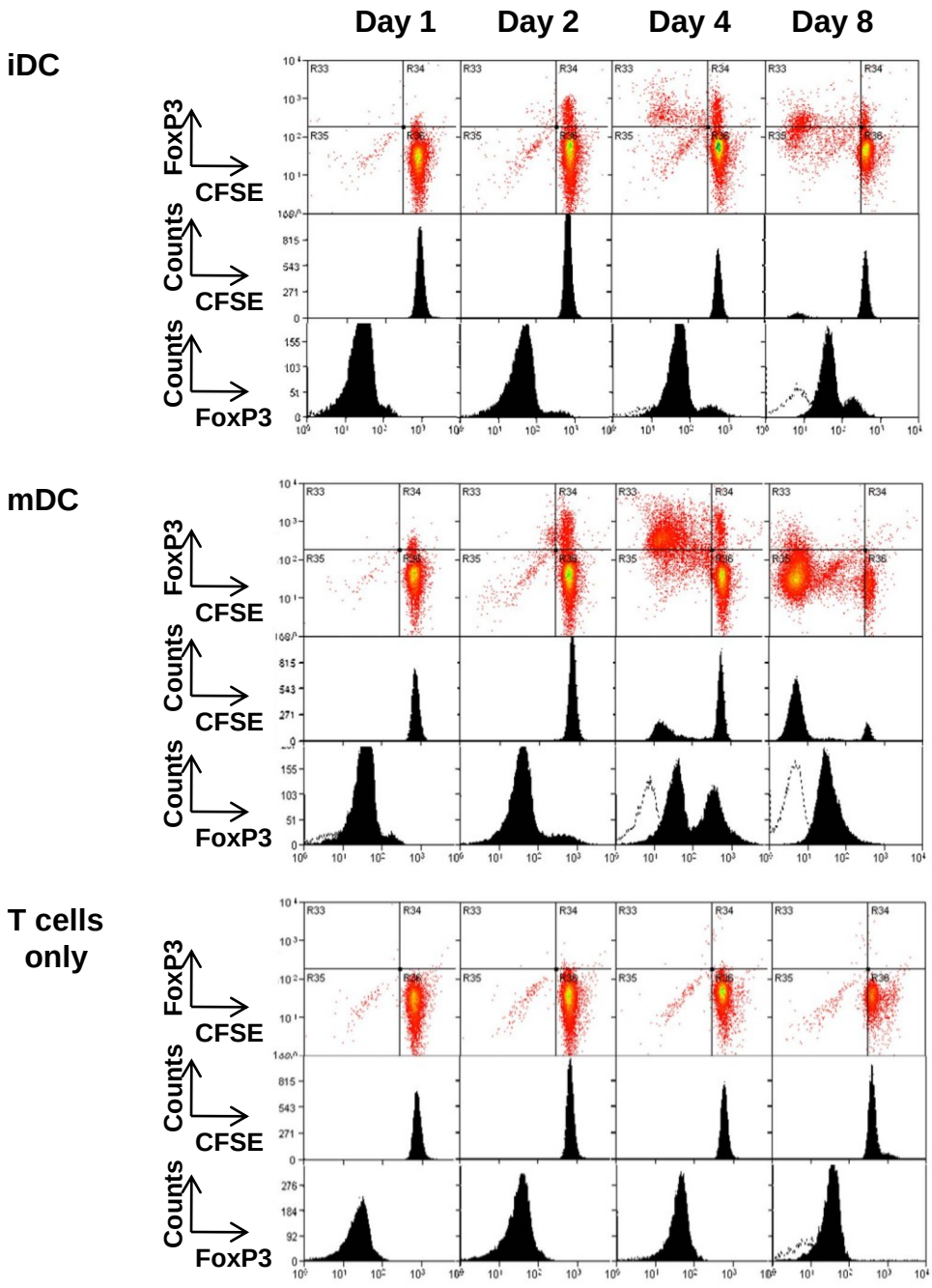



**Figure 3.12 Activation by dendritic cells induces FoxP3 elevation in naive CD4+ T cells which peaks at day 4, but is only present in a minority by day 8.** CFSE labelled naive CD4+ T cells were cultured for 4 days in the presence or absence of monocyte-derived dendritic cells (either untreated (iDC) or pre-treated with inflammatory cytokines (mDC)) and stained for intracellular FoxP3 expression.

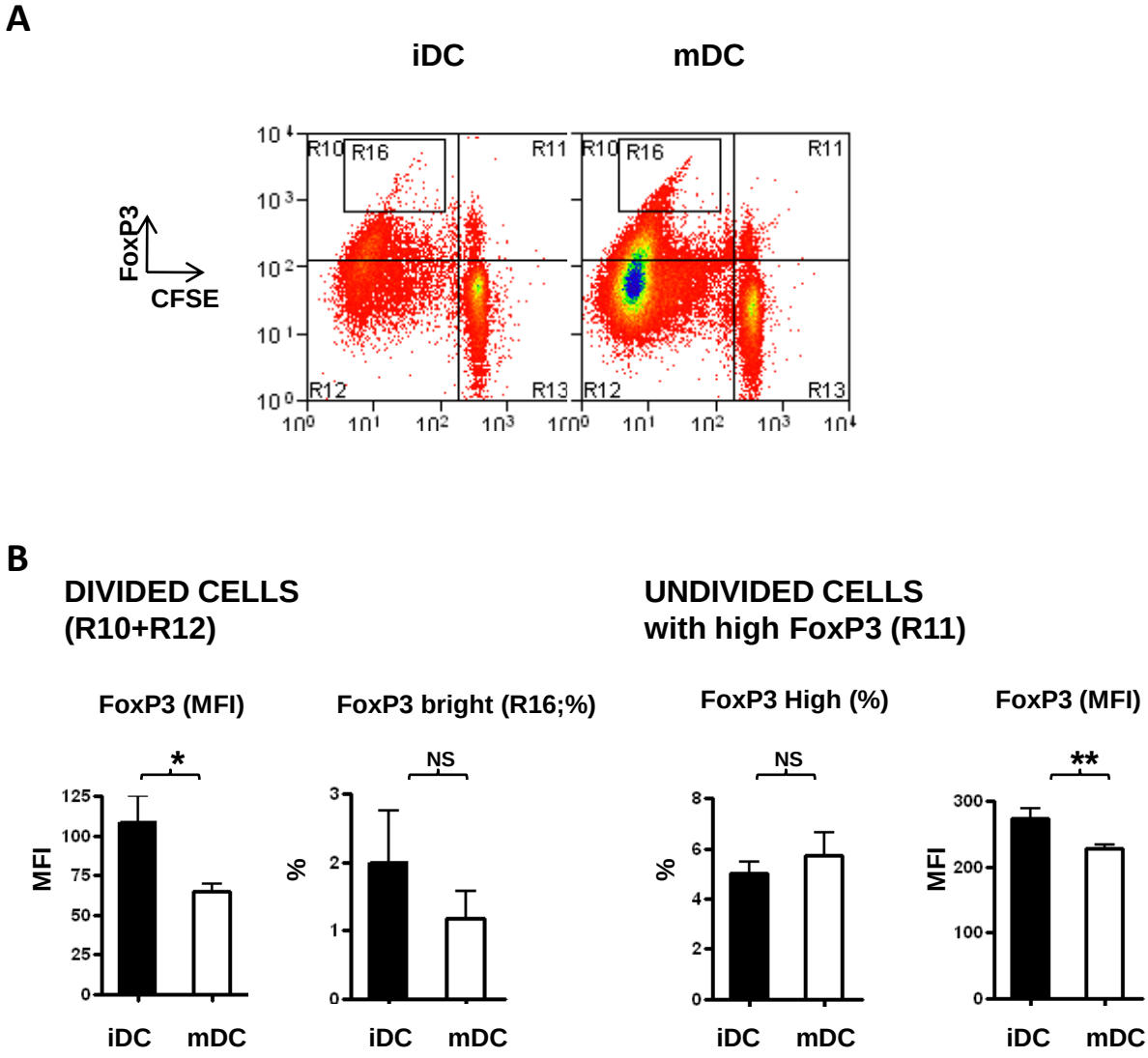



**Figure 3.13 FoxP3 expression in dendritic cell-activated naive CD4+ T cells is sustained at higher levels when stimulated by immature rather than mature dendritic cells**

CFSE labelled naive CD4+ T cells were cultured for 8 days in the presence or absence of monocyte-derived dendritic cells (either untreated (iDC) or pre-treated with inflammatory cytokines (mDC)) and stained for intracellular FoxP3 expression. (A) CFSE vs FoxP3 expression. R11 represents undivided cells with high FoxP3 expression; R10 and R12 represent divided cells; R16 represents FoxP3 bright divided cells. (B) MFI or percentage expression for mean ±SD triplicate cultures; representative of three separate experiments.
Student's t-test (unpaired); * $p < 0.05$; ** $p < 0.01$; NS = not significant.

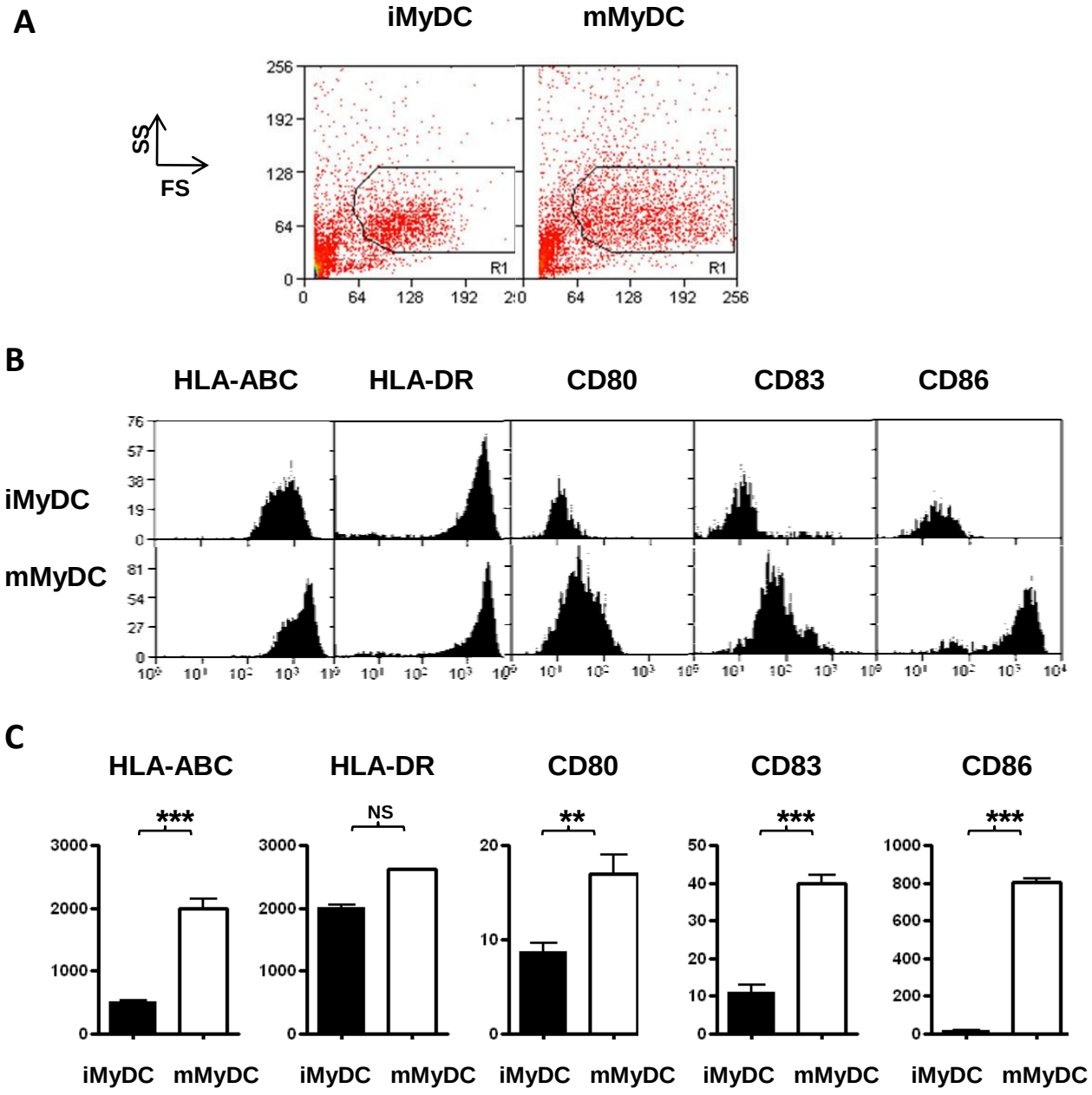



**Figure 3.14 Myeloid dendritic cells upregulate MHC and costimulatory molecules in serum-free conditions in response to an inflammatory stimulus**
Bead-purified myeloid dendritic cells (MyDC) were cultured for 48h in the presence or absence of a cocktail of inflammatory cytokines (IL1β, IL6, TNFα, $PGE_2$). (A) Forward scatter vs side scatter, (B) surface expression (C) mean ±SD MFI for MHC and other key surface molecules for triplicate cultures; representative of three separate experiments.
One-way ANOVA with Bonferroni post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

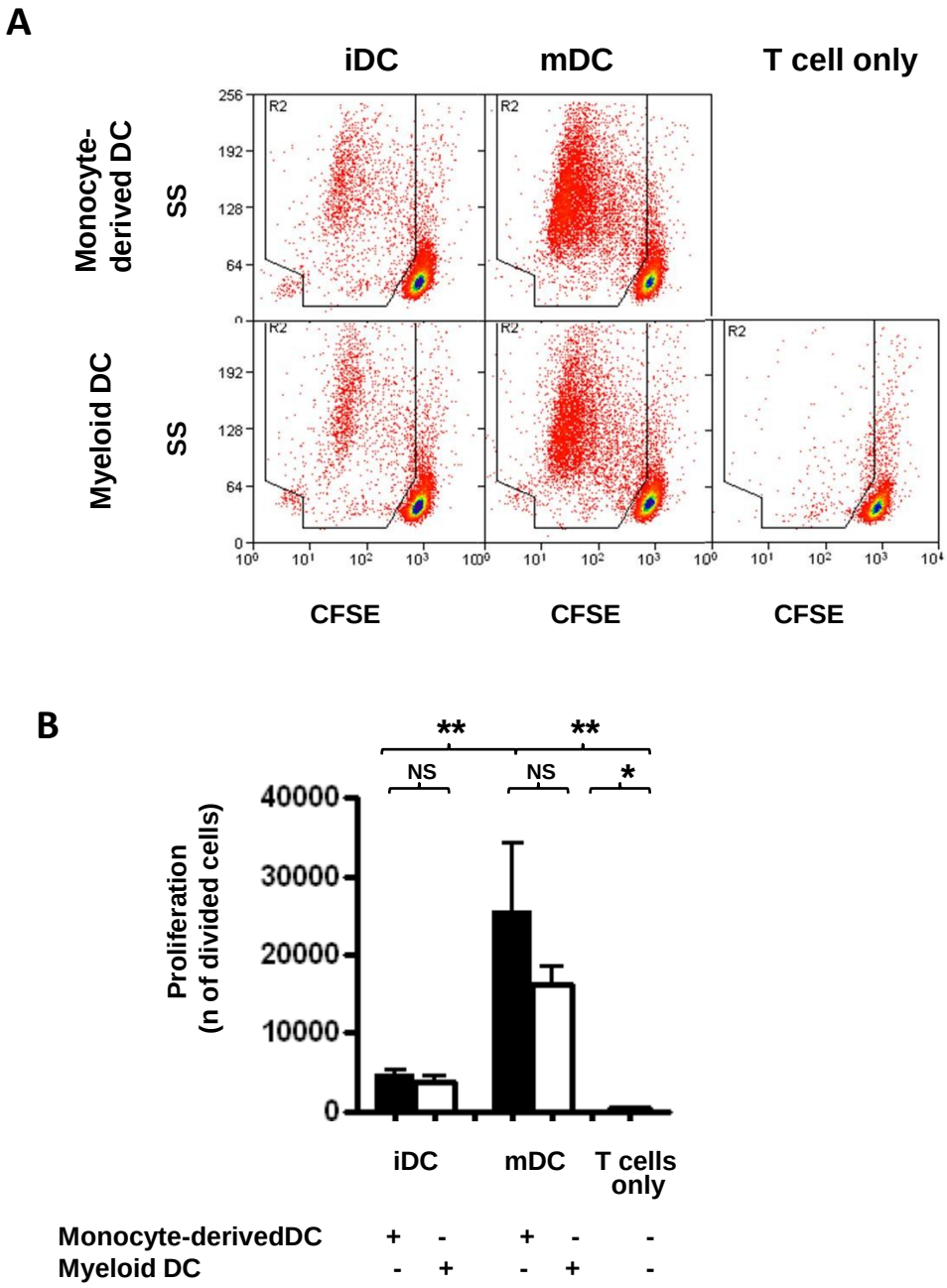



**Figure 3.15 Myeloid DC treated with inflammatory cytokines induce higher levels of proliferation of naive CD4+ T cells, indicative of DC maturation**
CFSE labelled naive CD4+ T cells were cultured for 4 days in the presence or absence of monocyte-derived DC or bead-purified myeloid DC (either untreated (iDC) or pre-treated with inflammatory cytokines (mDC)). (A) Flow cytometric detection of CFSE levels (B) number of divided cells with mean ± SD of triplicate cultures shown. (A) and (B) are representative of three separate experiments. One-way ANOVA with Bonferroni post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; NS = not significant

# CHAPTER 4
# Dendritic cell function in the ocular microenvironment: the suppressive role of non-inflammatory aqueous humour

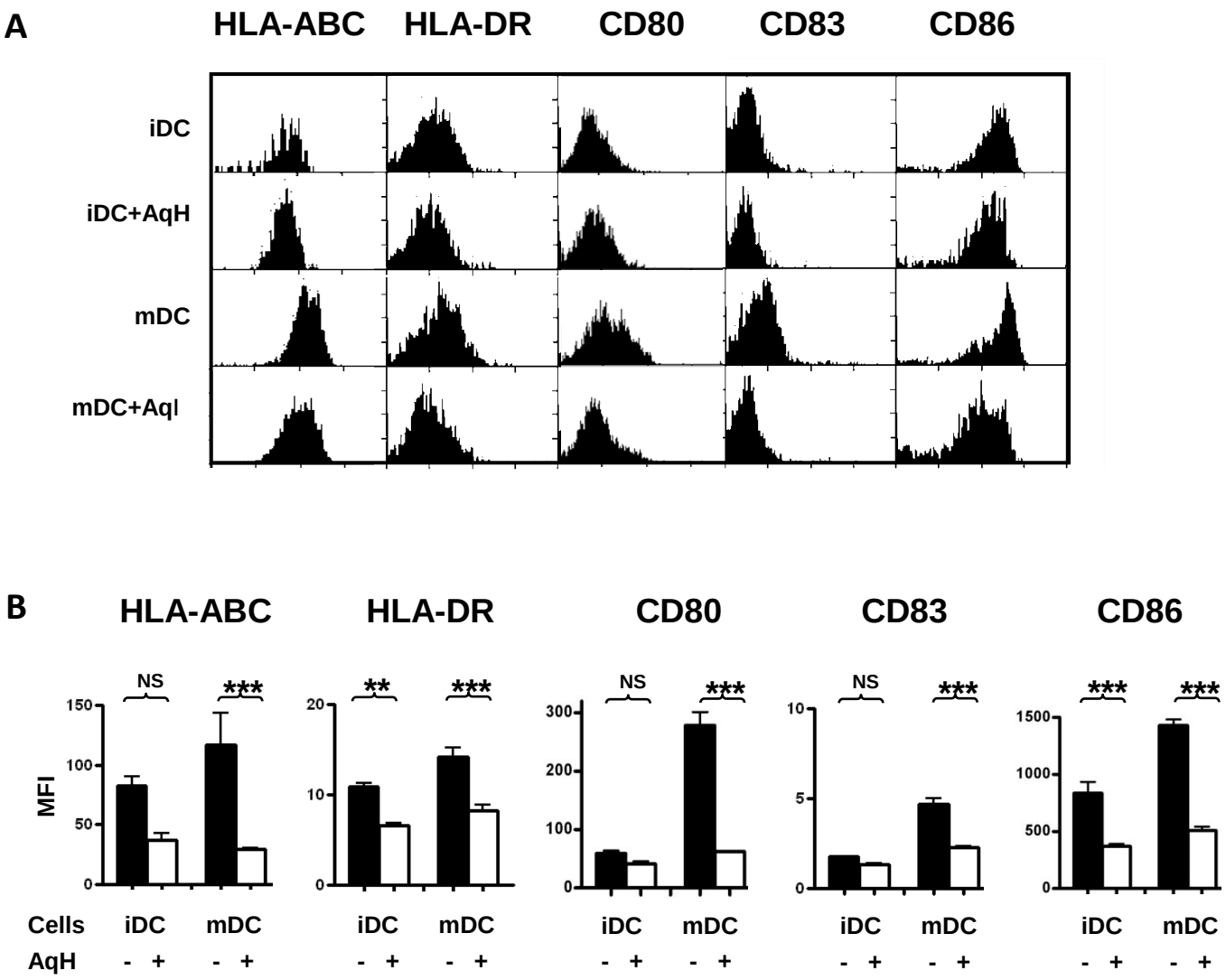



**Figure 4.1 Non-inflammatory AqH reduces expression of MHC and CD86 on immature monocyte-derived dendritic cells and prevents upregulation of MHC and costimulatory molecules in response to inflammatory cytokines.**
Monocyte-derived DC were cultured for 48h in the presence or absence of inflammatory cytokines (IL-1β, IL-6, TNFα and $PGE_2$) with or without 50% AqH. (A) Flow cytometry histograms and (B) MFI of HLA-ABC, HLA-DR, CD80, CD83 and CD86 for mean ± SD of triplicate cultures; representative of six separate experiments.
One-way ANOVA with Bonferroni post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

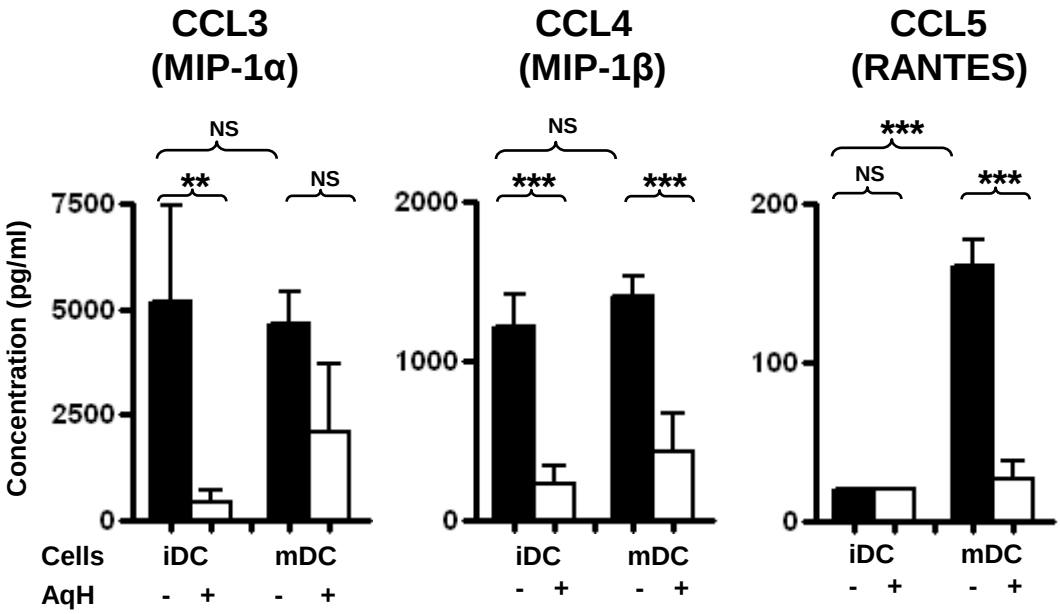



**Figure 4.2 Non-inflammatory AqH reduces production of selected chemokines by monocyte-derived dendritic cells.**
Monocyte-derived DC were cultured for 48h in the presence or absence of inflammatory cytokines (IL-1β, IL-6, TNFα and $PGE_2$) with or without 50% AqH. Supernatants were harvested after 48h and analysed by multiplex-bead immunoassay. Mean ± SD concentrations from triplicate cultures; representative of four separate experiments.
One-way ANOVA with Bonferroni multiple group comparison for differences in cytokines; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

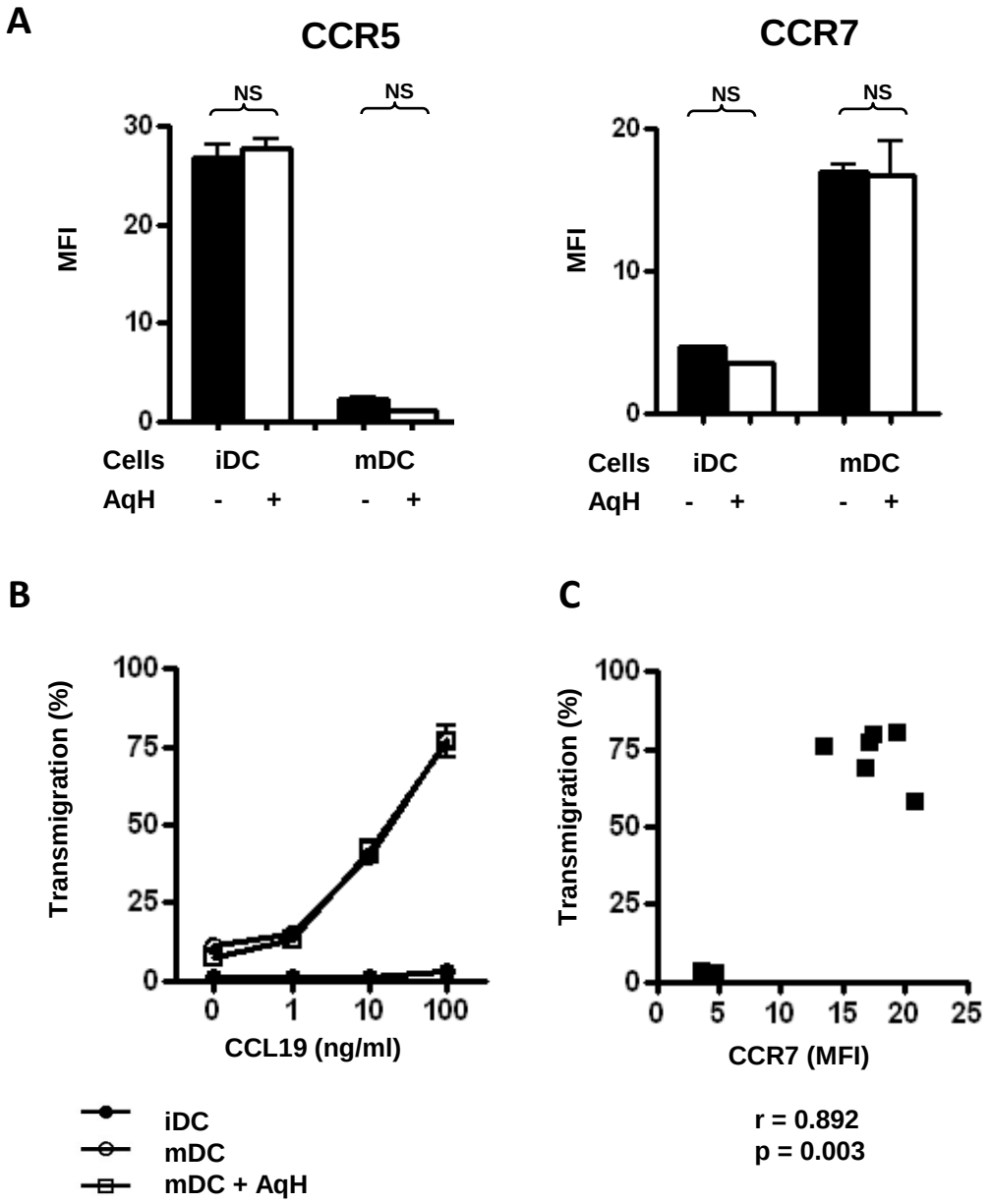



**Figure 4.3 Non-inflammatory AqH does not alter chemokine receptor expression and function in response to inflammatory cytokines**
Monocyte-derived DC were cultured for 48h in the presence or absence of inflammatory cytokines (IL-1β, IL-6, TNFα and $PGE_2$ to generate mature mDC vs immature iDC) with or without 50% AqH. (A) MFI of chemokine receptor levels analysed by flow cytometry, (B) migration to CCL19 assessed in a 96-well transmigration chapter and (C) correlation of CCR7 expression and ability to transmigrate to CCL19; representative of (A) four or (B) two separate experiments.
One-way ANOVA with Bonferroni post-hoc test for selected columns (A) and Pearson r with p value shown (B); NS = not significant.

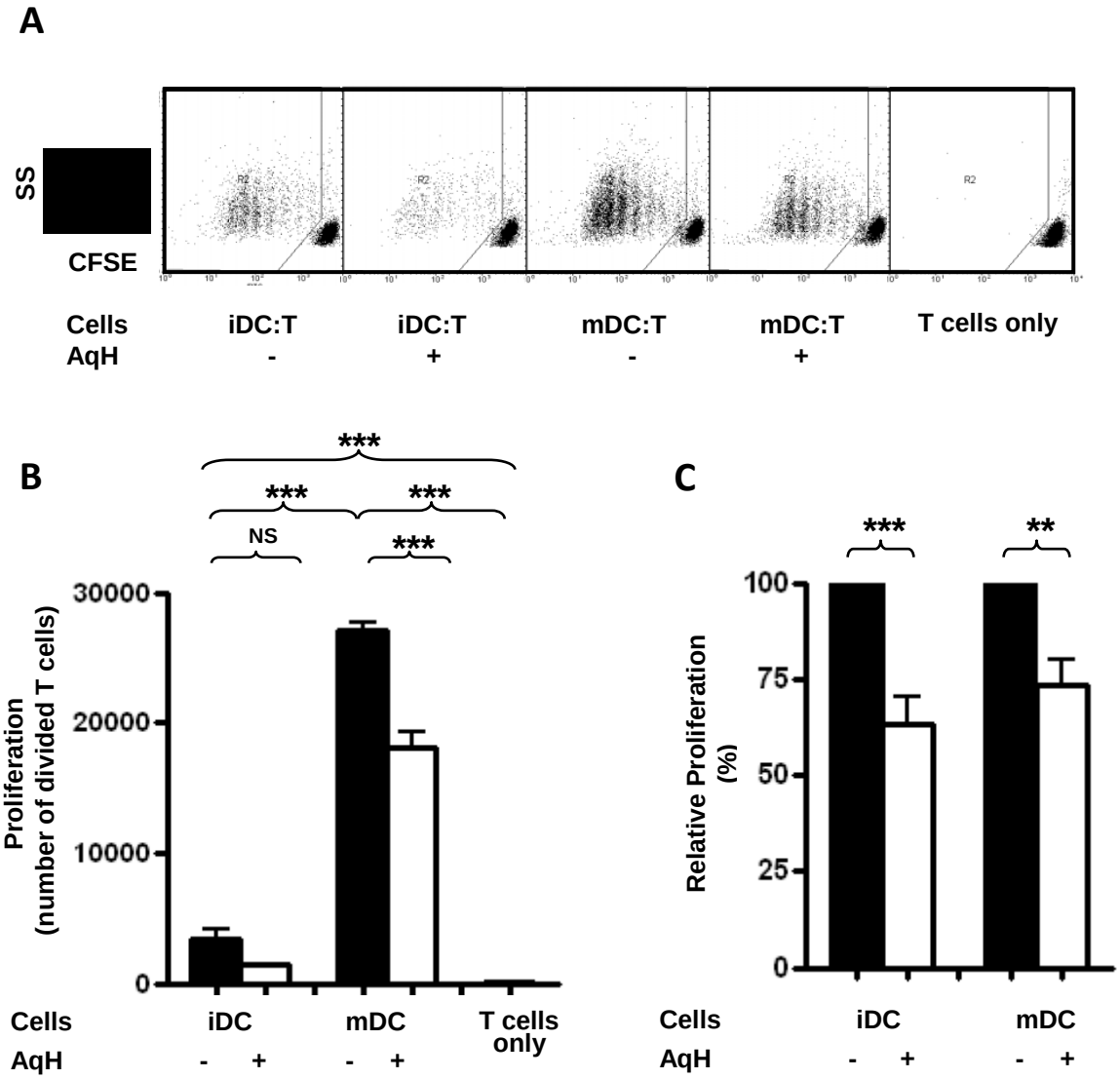



**Figure 4.4 Non-inflammatory AqH inhibits the capacity of dendritic cells to induce proliferation of allogeneic naïve CD4+ T cells.**

CFSE-labelled allogeneic naïve CD4+ T cells were cultured for 4 days with immature or mature monocyte-derived DC which had been pre-treated with medium or 50% AqH. (A) CFSE vs side scatter (SS) for live cells and (B) number of divided cells for mean ± SD of triplicate cultures; representative of six separate experiments. (C) Relative proliferation induced by AqH treatment for iDC and mDC; mean ± SEM of six experiments.

One-way ANOVA with Bonferroni multiple group comparison; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

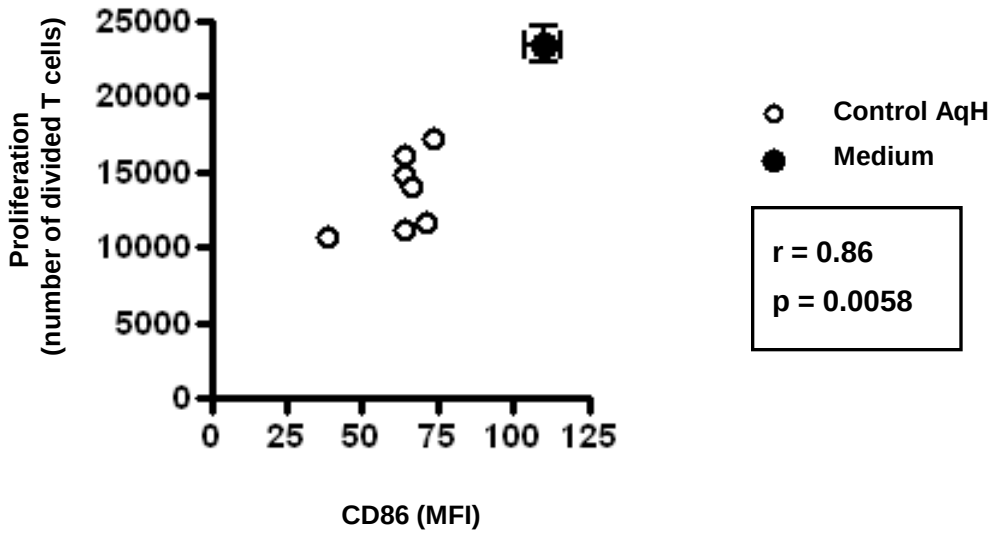



**Figure 4.5 Reduction in dendritic cell CD86 expression by non-inflammatory AqH correlates with inhibition of their capacity to induce proliferation of allogeneic naïve CD4+ T cells.**

Monocyte-derived DC were cultured in the presence or absence of 50% non-inflammatory AqH as described previously. CD86 expression (MFI) by DC vs number of divided T cells shown for immature DC and AqH treated immature DC; representative of six separate experiments.

Pearson r = 0.860, p = 0.006. Normality of distribution demonstrated by Kolmogorov-Smirnov test.

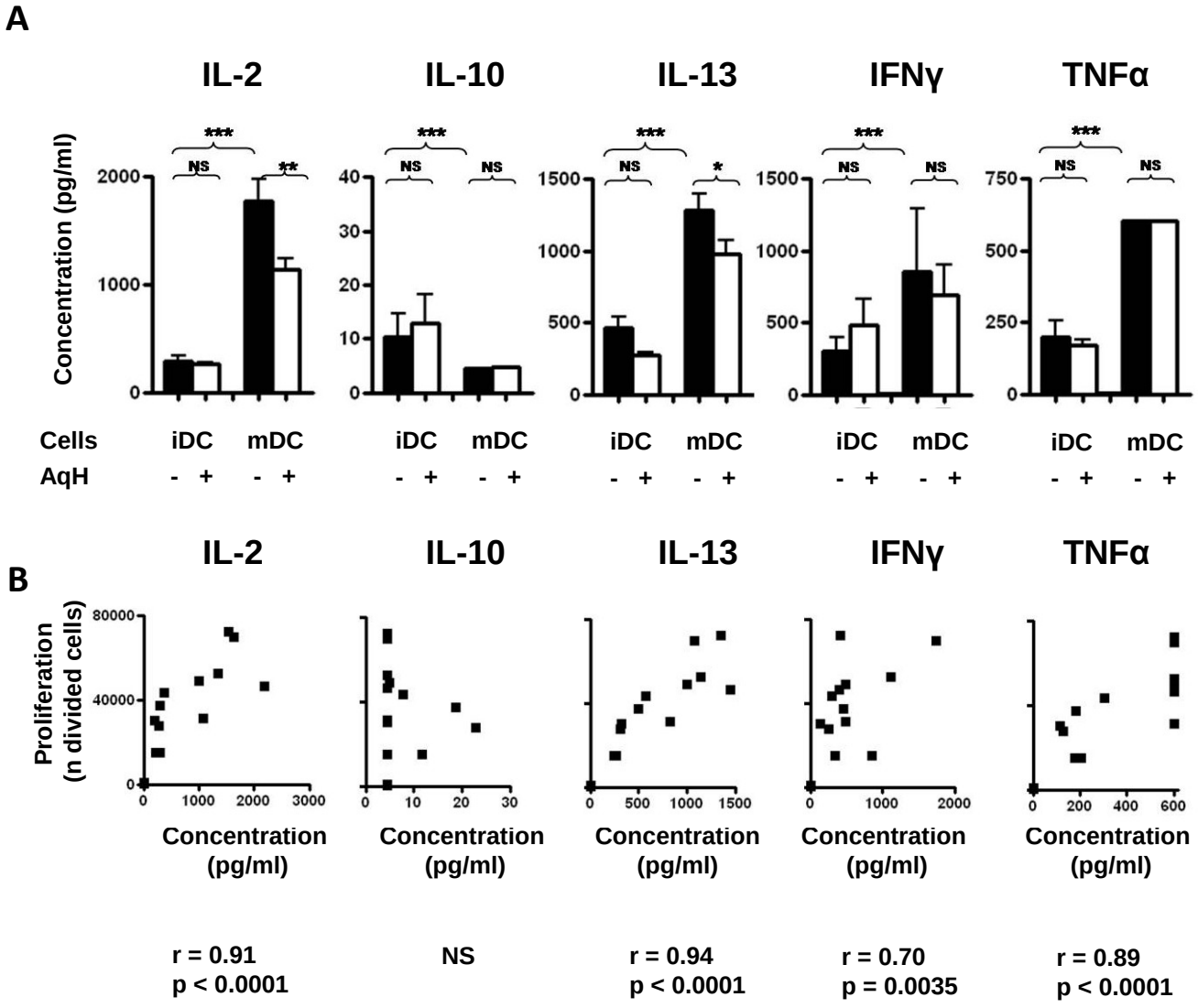



**Figure 4.6 CD4+ naïve T cells stimulated by human AqH treated dendritic cells (DC) show generalised reduction in cytokine production correlating with reduced T cell proliferation and with no significant skewing of T cell phenotype.** Multiplex-bead immunoassay of supernatants of DC:T cell co-cultures harvested after 4d of culture showing (A) cytokine concentrations (mean ± SD of triplicate values) and (B) correlation of cytokine levels with T cell proliferation. Representative of four separate experiments.
One-way ANOVA with Bonferroni post-hoc test for selected columns shown (parametric distribution within groups); Spearman r for correlation (since non-parametric when groups combined); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

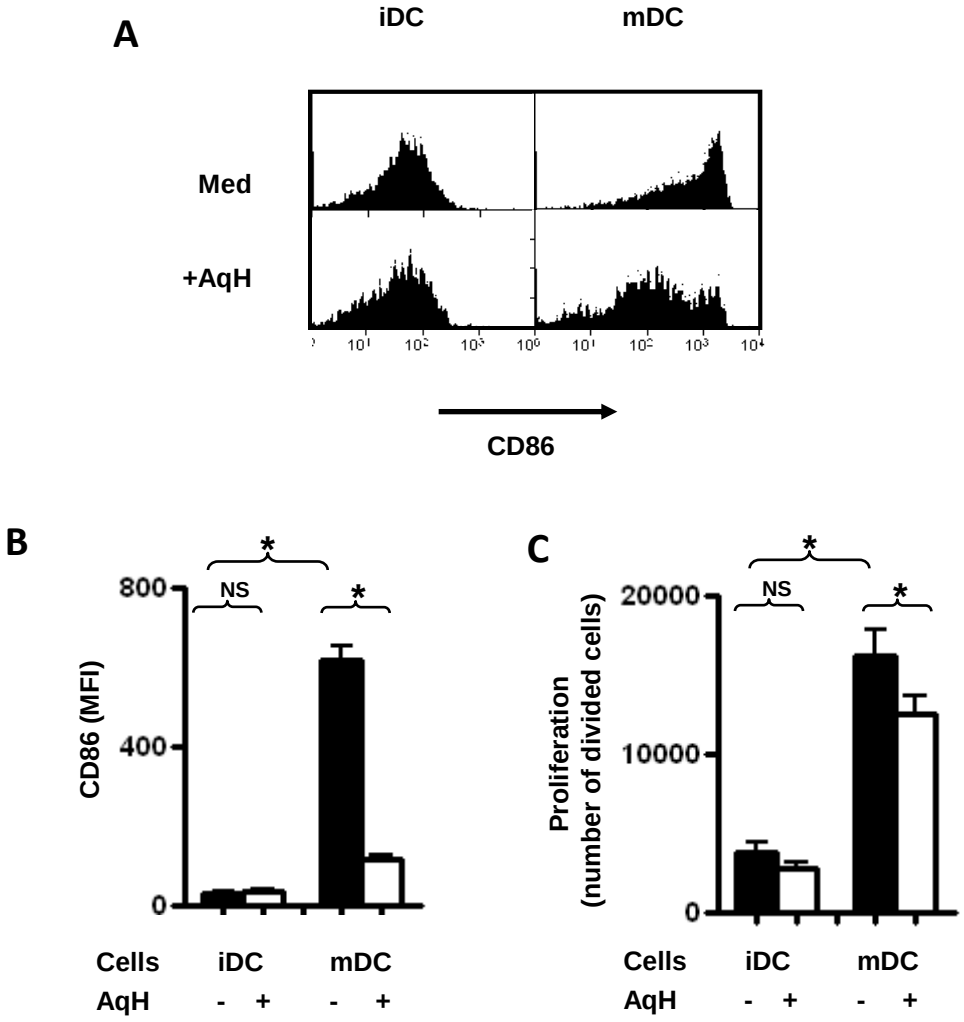



**Figure 4.7 Non-inflammatory AqH treatment of myeloid dendritic cells causes downregulation of CD86 and reduced capacity to induce naïve CD4+ T cell proliferation under inflammatory conditions**

Bead-purified myeloid dendritic cells were cultured in medium (iDC) or inflammatory cytokines (mDC) with or without the addition of non-inflammatory human AqH. Myeloid DC expression of CD86 with (A) representative flow cytometric histograms and (B) MFI, and (C) induction of naïve CD4+ T cell proliferation; Mean ± SD for triplicate wells; representative of three separate experiments.

One-way ANOVA with Bonferroni post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

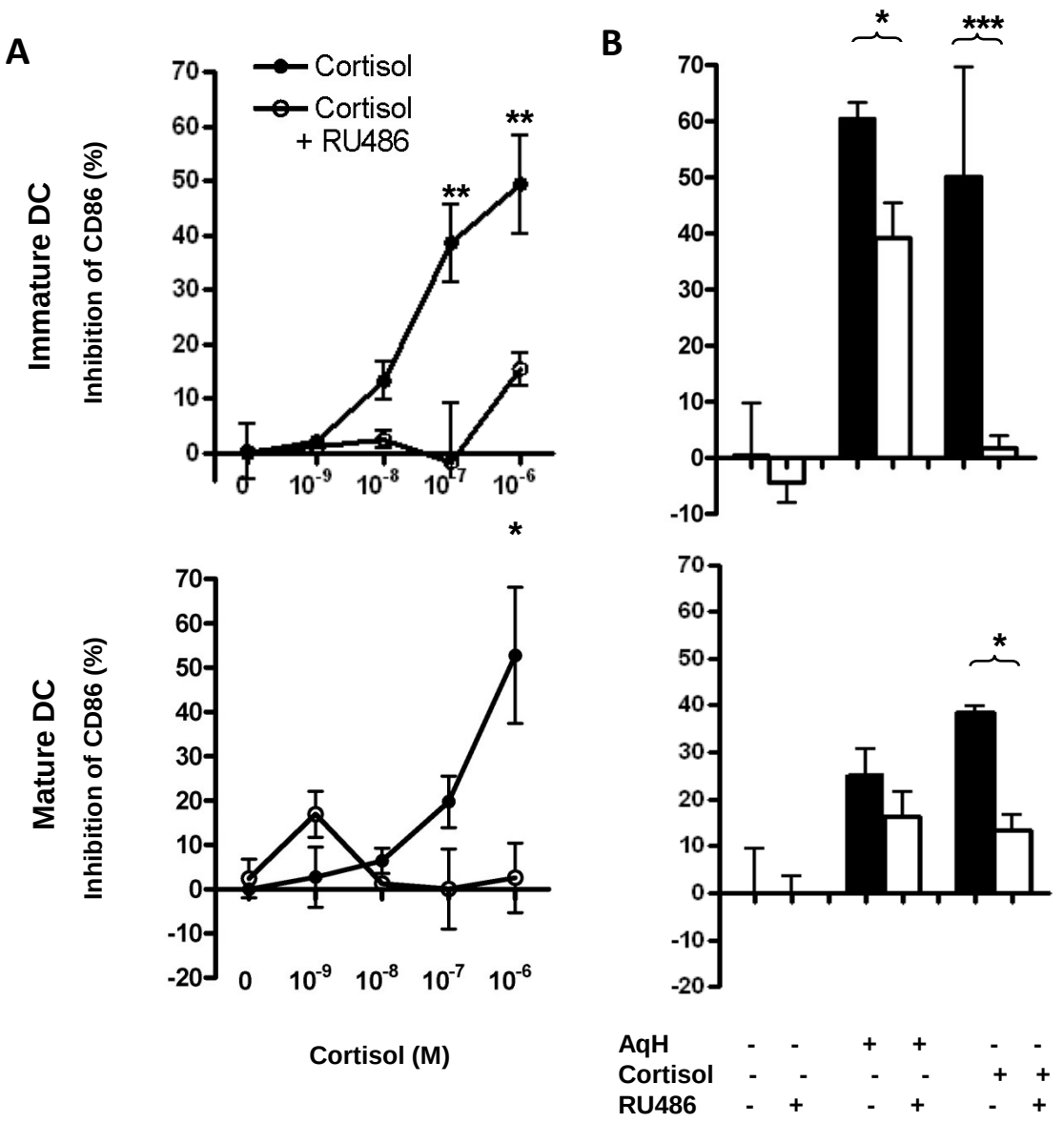



**Figure 4.8 Cortisol causes dose-dependent reduction in CD86 expression by dendritic cells, and contributes to CD86 inhibition by AqH**

(A) Monocyte-derived DC were cultured in the presence of 10-9 – 10-6M cortisol with or without the glucocorticoid receptor antagonist, RU486. Mean percentage inhibition of CD86 ± SEM for three separate experiments shown. (B) DC were cultured in the presence or absence of 50% non-inflammatory AqH with or without RU486. Cortisol (10-7M) was used as a positive control. Mean percentage inhibition of CD86 (± SD) for triplicate cultures; representative of three separate experiments shown.

One-way ANOVA with Bonferroni post-hoc test for selected concentrations/ columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

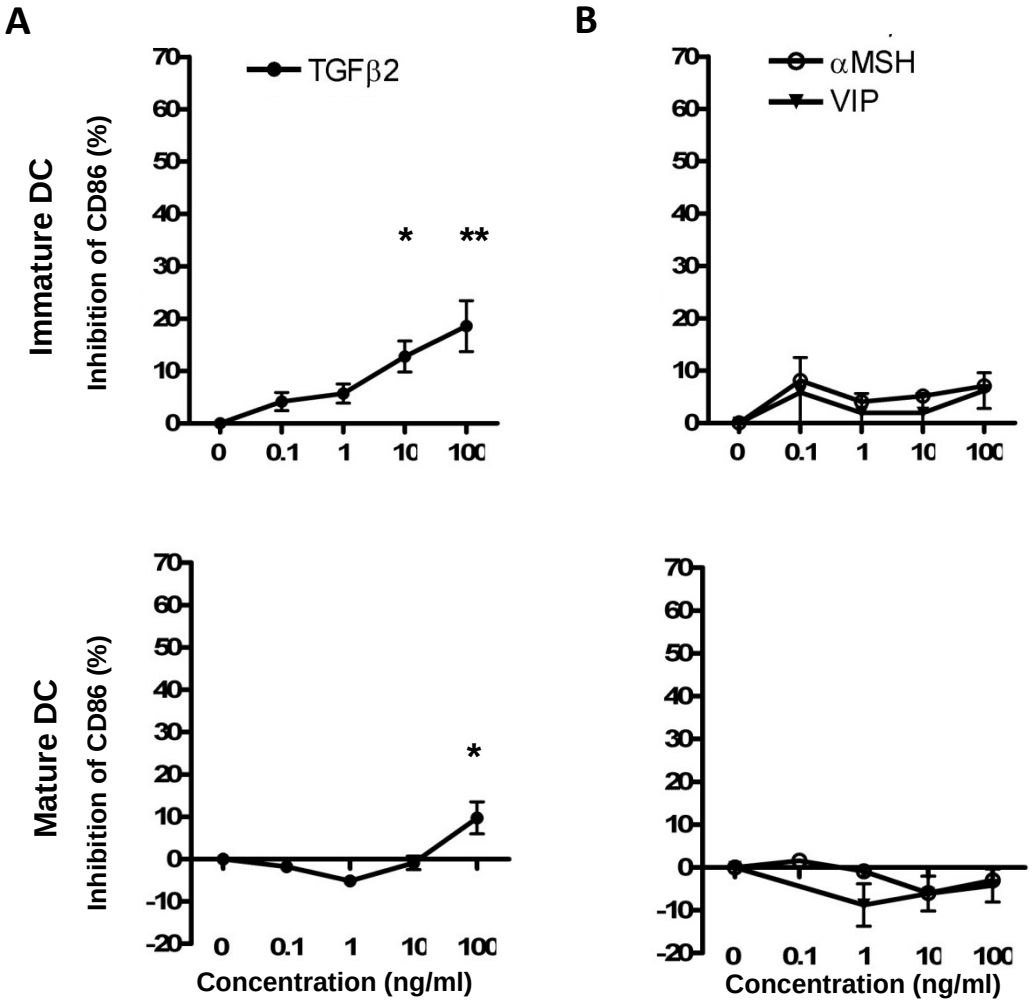



**Figure 4.9 TGFβ2 but not αMSH or VIP cause dose-dependent reduction in CD86 expression for immature and mature DC.**

(A) Monocyte-derived DC were cultured in the presence of (A) 0.1 – 100ng/ml TGFβ2 or (B) 0.1-100ng/ml αMSH or VIP. Mean percentage inhibition of CD86 ± SEM for three separate experiments shown.

One-way ANOVA with Bonferroni post-hoc test for selected concentrations shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

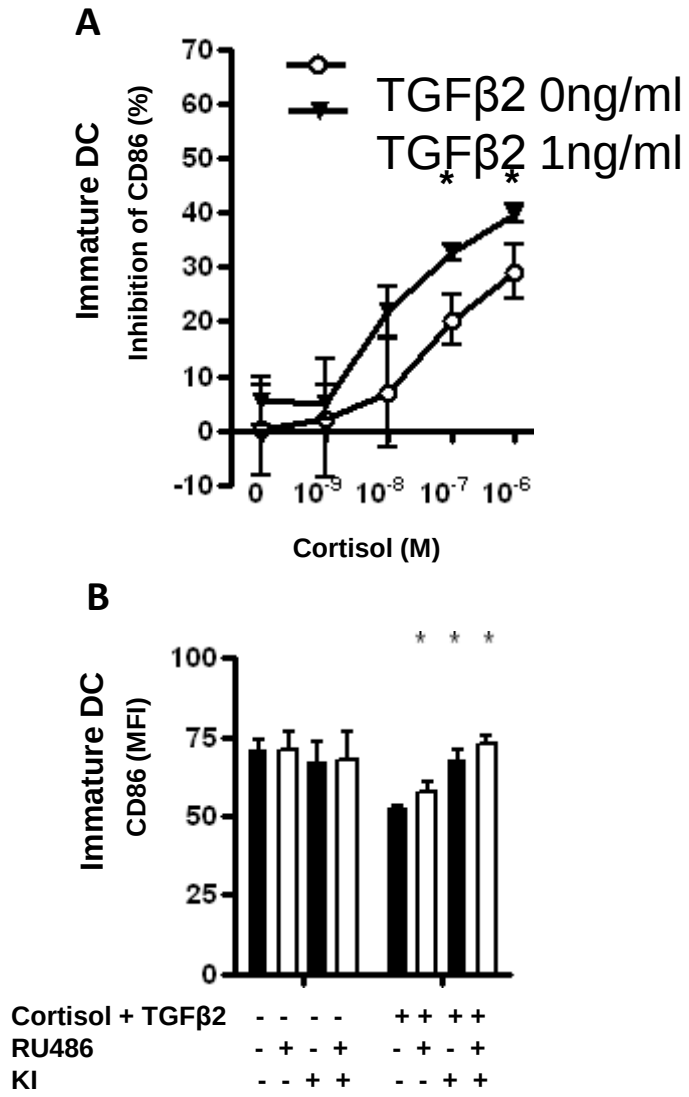



**Figure 4.10 Cortisol and TGFβ2 have additive inhibitory effects on dendritic cell expression of CD86.**

(A) Monocyte-derived DC were cultured in the presence of 10-9 – 10-6M cortisol with or without the addition of 1 or 10 ng/ml TGFβ2. Mean percentage inhibition of CD86 ± SD for triplicate cultures; representative of three separate experiments. One-way ANOVA with Bonferroni post-hoc test comparing cortisol treatment with or without TGFβ2 at multiple cortisol concentrations; * $p < 0.05$; otherwise not significant.

(B) Monocyte-derived DC were cultured in the presence or absence of cortisol (10-7M) and TGFβ2 (10 ng/ml) with or without the glucocorticoid inhibitor RU486 and/or the ALK kinase inhibitor SB431542 (which inhibits TGFβ function). One-way ANOVA with Bonferroni post-hoc test comparing the effects of RU486 and SB431542 with baseline inhibition by cortisol and TGFβ2; * $p < 0.05$; otherwise not significant.

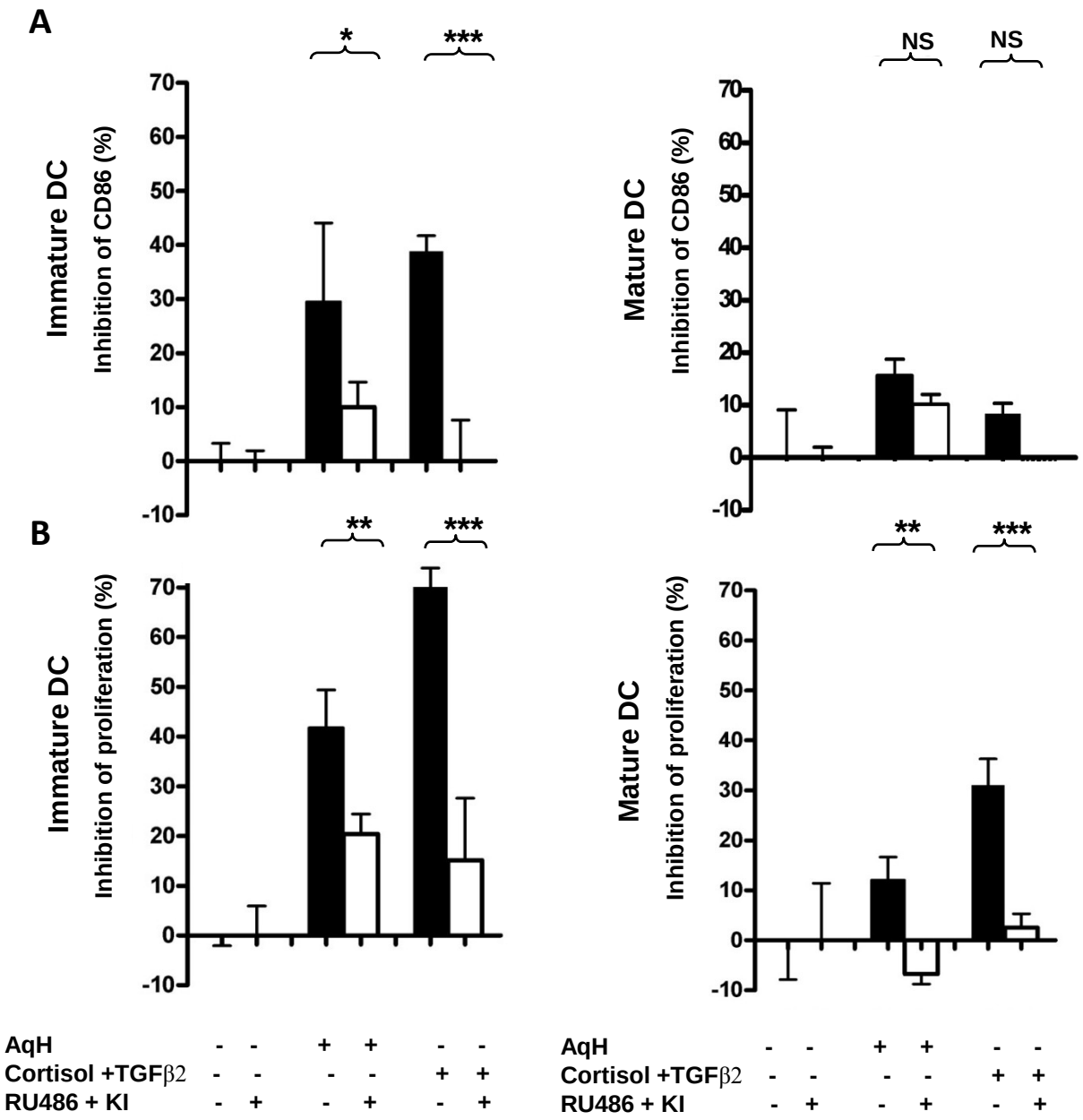



**Figure 4.11 Cortisol and TGFβ2 jointly contribute to CD86 inhibition by non-inflammatory AqH**

Monocyte-derived DC were cultured in the presence or absence of 50% non-inflammatory AqH with or without the glucocorticoid inhibitor RU486 and the ALK kinase inhibitor SB431542. Cortisol (10-7M) and TGFβ2 (10ng/ml) were used as a positive control. (A) Mean percentage inhibition of CD86 ± SD and (B) mean percentage inhibition of induction of proliferation of allogeneic naïve CD4+ T cells ± SD for triplicate cultures; representative of three separate experiments. One-way ANOVA with Bonferroni post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

# CHAPTER 5
# Dendritic cell function in uveitis: sustained suppression of dendritic cell function despite IFNγ mediated upregulation of MHC

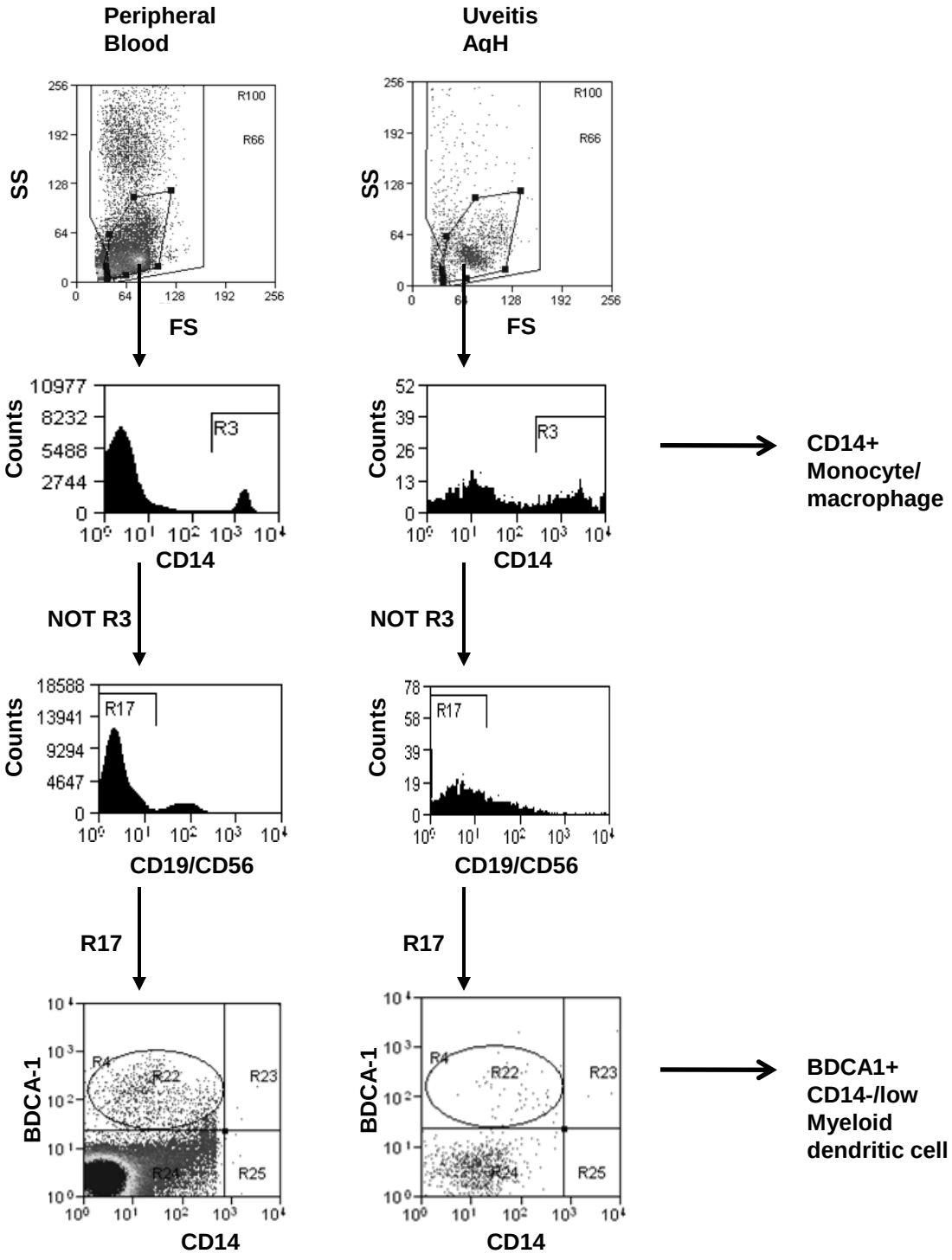



**Figure 5.1 BDCA1+ myeloid DC and CD14+ monocyte/macrophages can be identified in human aqueous humour during uveitis**

Myeloid DC were identified in the peripheral blood and aqueous humour of patients with active anterior uveitis. Gating strategy demonstrating identification of CD14+ monocyte/macrophage vs BDCA1+ myeloid DC (CD14-/low predominant subset).

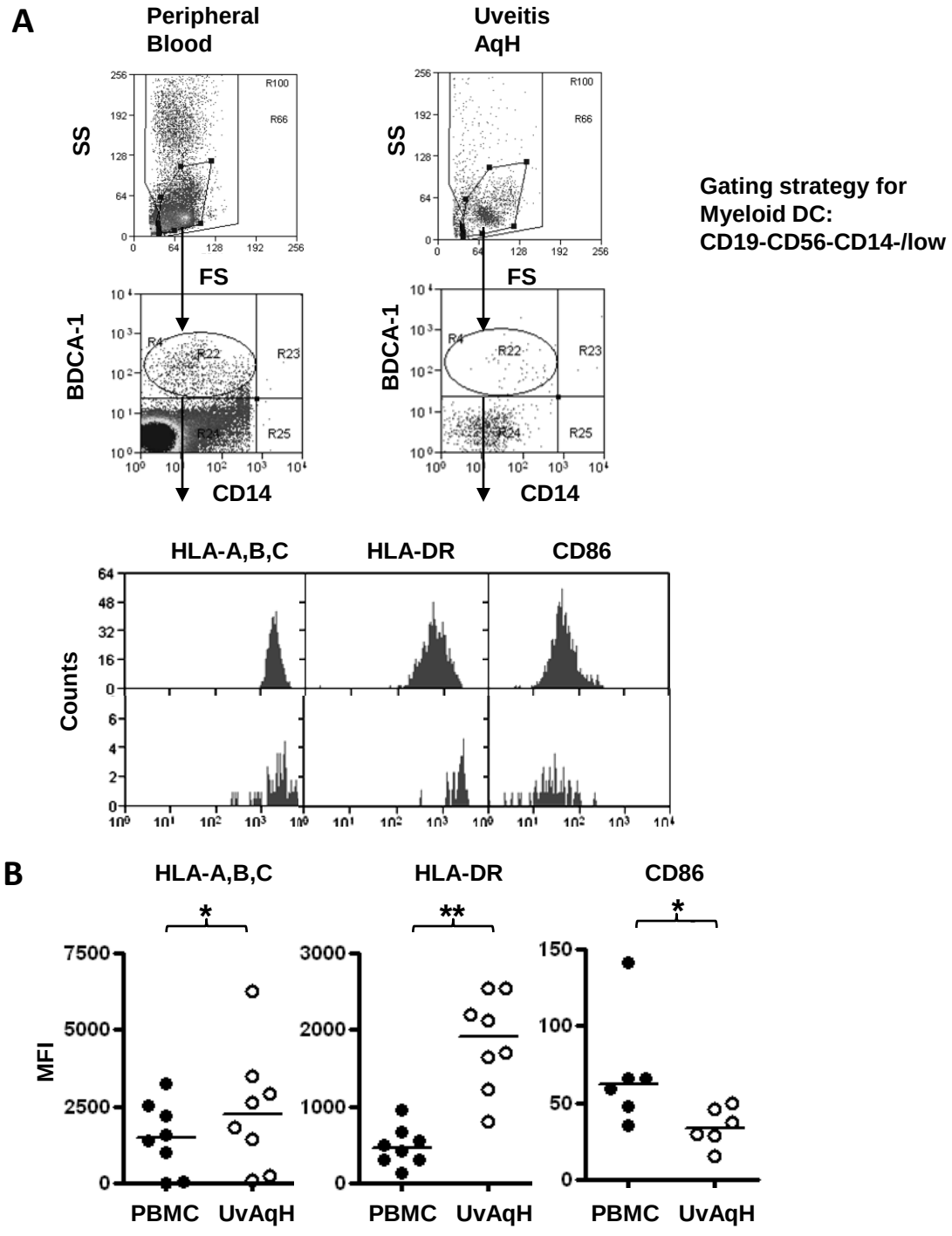



**Figure 5.2 Myeloid DC can be identified in AqH during acute anterior uveitis and have a distinct MHChi CD86lo phenotype**

Myeloid DC were identified in the peripheral blood and aqueous humour of patients with active anterior uveitis (A) Representative histograms and (B) MFI of MHC and CD86 from matched PBMC and AqH of six or more patients. Wilcoxon matched pairs analysis; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant

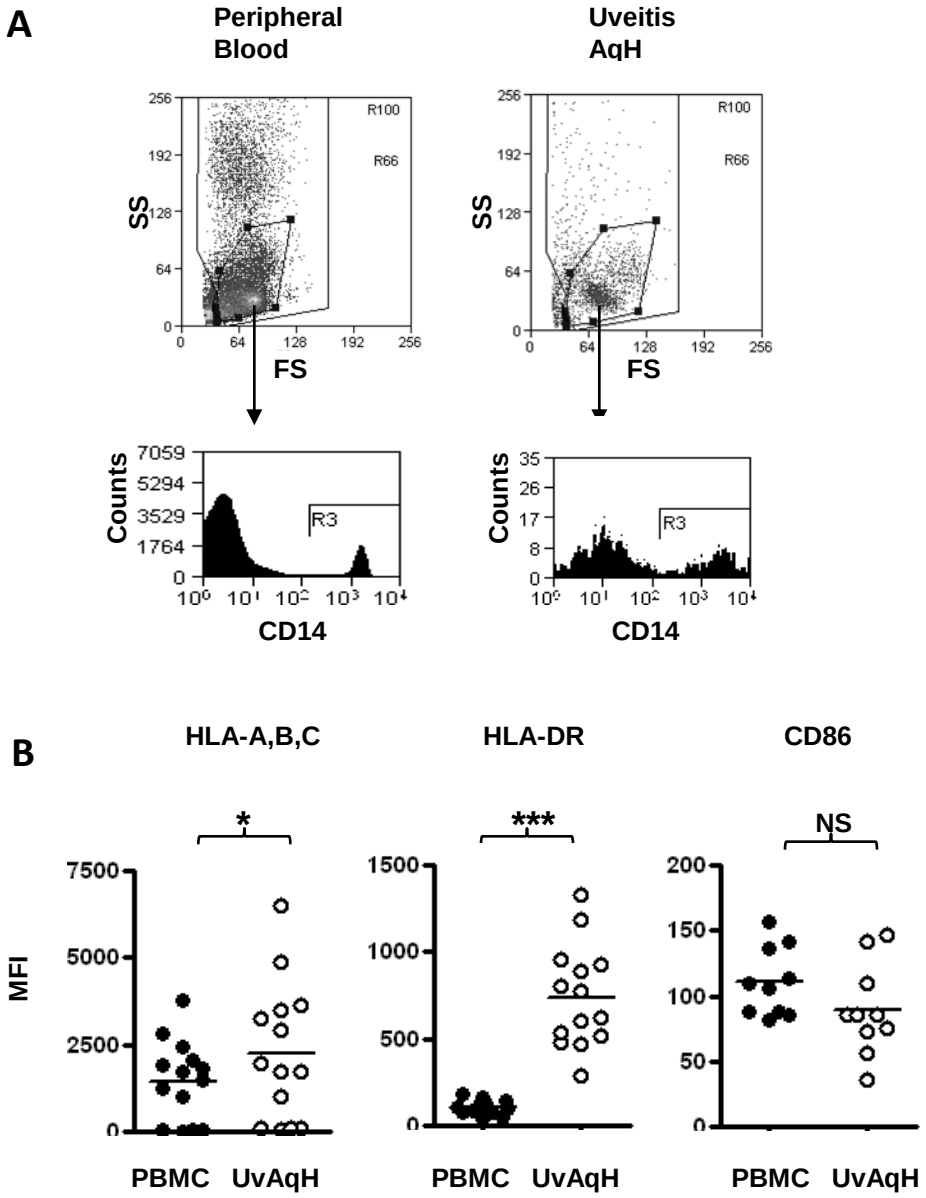



**Figure 5.3 CD14+ monocyte/macrophages identified in AqH are MHChi, but levels of CD86 are not affected**

CD14+ monocyte/macrophages were identified in the peripheral blood and aqueous humour of patients with active anterior uveitis (A) Representative histograms and (B) MFI of MHC and CD86 from matched PBMC and AqH of ten or more patients;

Wilcoxon matched pairs analysis;* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant

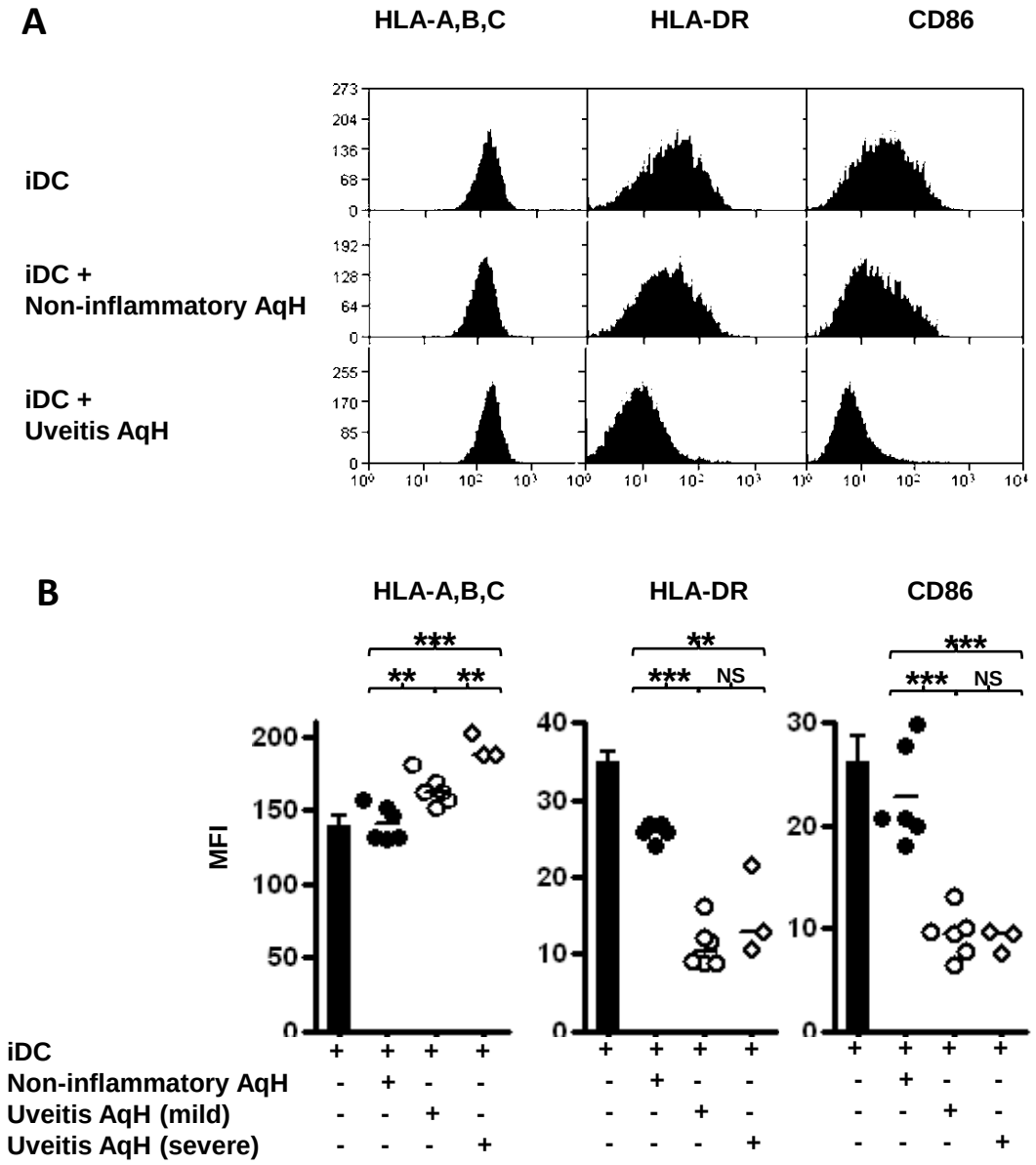



**Figure 5.4 Treatment of naïve monocyte-derived DC with uveitis AqH supernatant in vitro induces a similar MHCIhi CD86lo profile to that seen in UvAqH myeloid DC.**

Monocyte-derived DC were cultured for 48h in the presence or absence of 50% uveitic or non-inflammatory AqH. (A) Representative histograms and (B) MFI of MHC and CD86 for nine uveitis AqH and six non-inflammatory AqH; representative of three separate experiments; for medium alone the mean ± SD of MFI for triplicate cultures are given; mild = 1+/2+, severe 3+/4+ cells in the anterior chamber (see text).

Kruskal-Wallis with Dunn's post-hoc test for multiple comparisons; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant

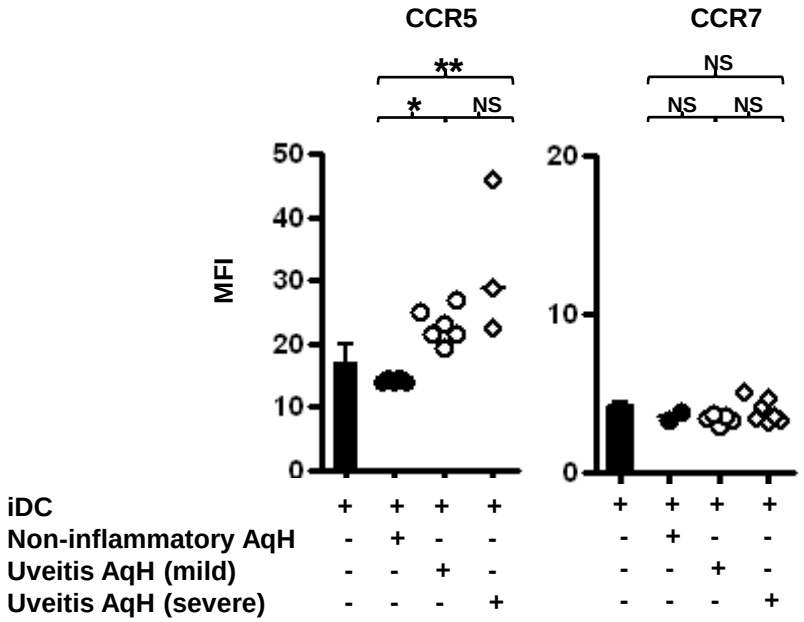



**Figure 5.5 Treatment of naïve monocyte-derived DC with uveitis AqH supernatant in vitro induces upregulation of CCR5 and no significant change in CCR7 expression.**

Monocyte-derived DC were cultured for 48h in the presence or absence of 50% uveitic or non-inflammatory AqH. (MFI for CCR5 and CCR7 for representative of three separate experiments; for medium alone the mean $\pm$ SD of MFI for triplicate cultures are given; mild = 1+/2+, severe 3+/4+ cells in the anterior chamber (see text).

Kruskal-Wallis with Dunn's post-hoc test for selected columns shown; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant

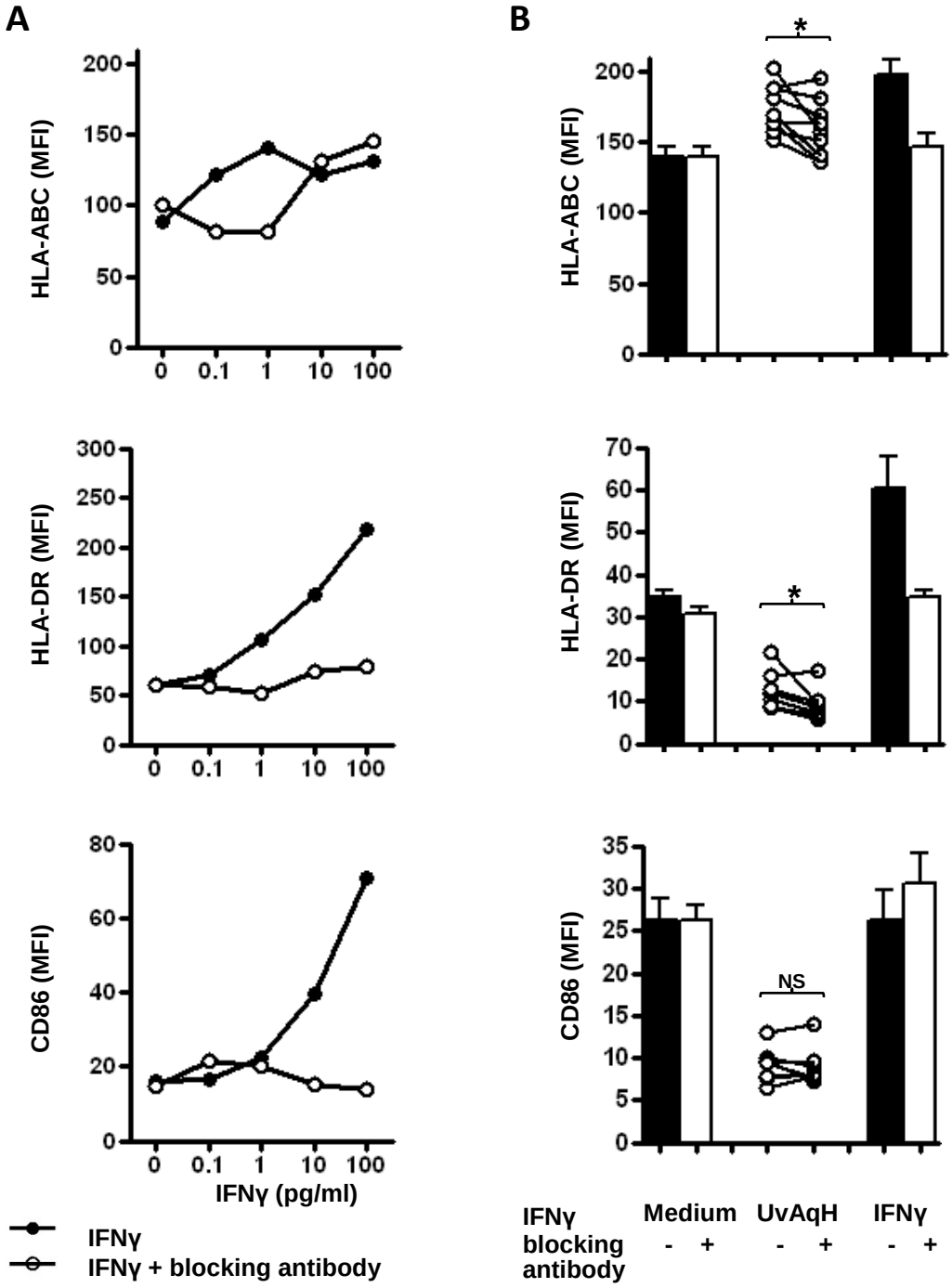



**Figure 5.6 Increasing IFNγ levels in AqH during uveitis promote upregulation of MHC correlating with disease severity, but is insufficient to overcome AqH-induced regulation of HLADR or CD86**

Monocyte-derived DC were cultured in the presence or absence of (A) 0.1-100ngml-1 IFNγ or (B) 50% uveitis AqH (UvAqH) with or without an IFNγ blocking antibody. (A) and (B) are each representative of three separate experiments; mean ± SD of MFI for triplicate cultures are given except for individual AqH samples.

Wilcoxon matched pairs analysis; * $p < 0.05$; NS = not significant.

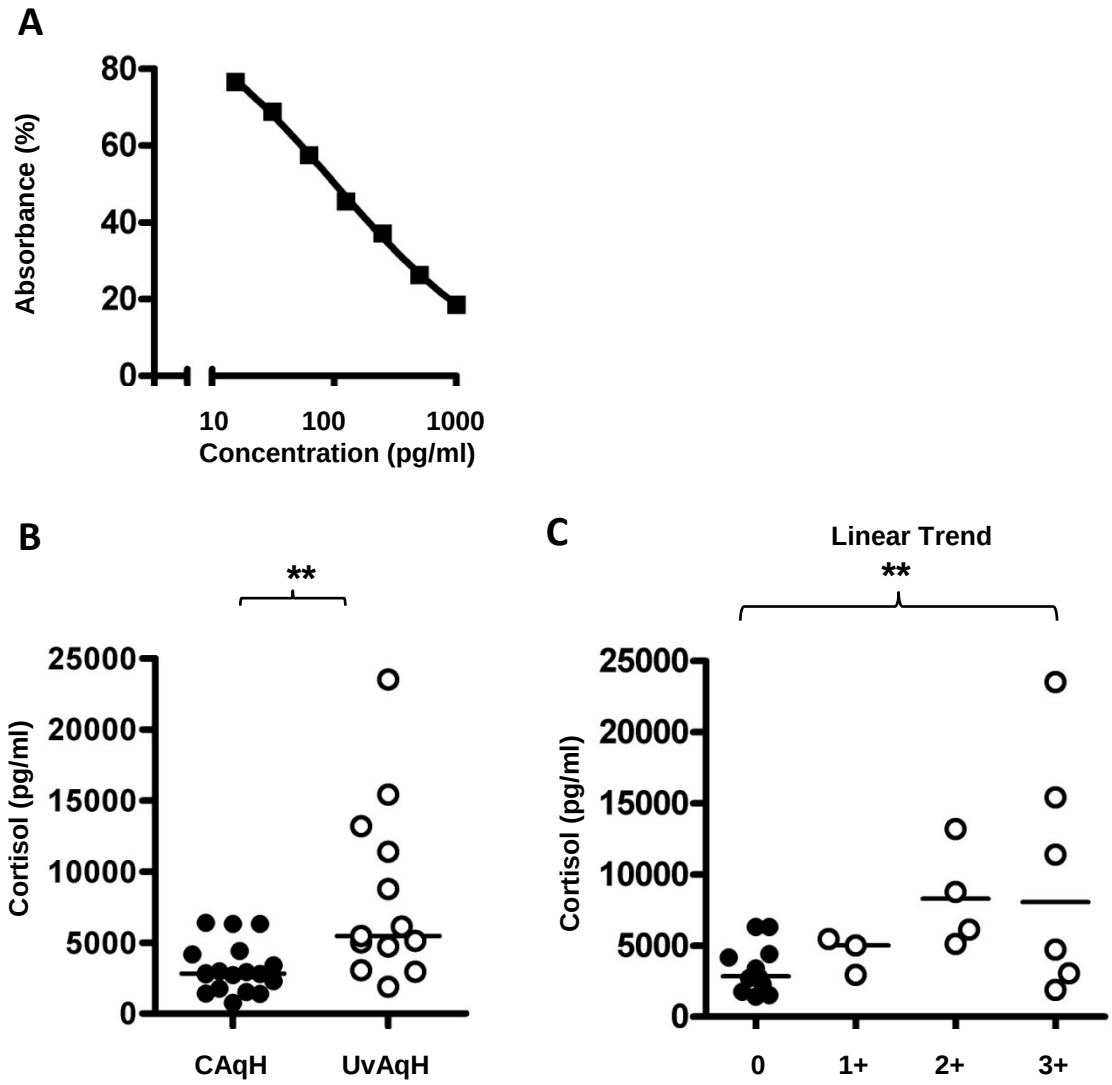



**Figure 5.7 Cortisol levels are elevated in uveitis AqH vs non-inflammatory AqH**

Cortisol levels in non-inflammatory (CAqH) and uveitic AqH (untreated; UvAqH) were measured by super-sensitive ELISA. (A) Standard curve (B) Cortisol levels in non-inflammatory AqH vs uveitis AqH and (C) Cortisol levels according to cellular activity of AqH sample.

Mann-Whitney U test (B) and Linear trend test for all four columns (C); ** $p < 0.01$

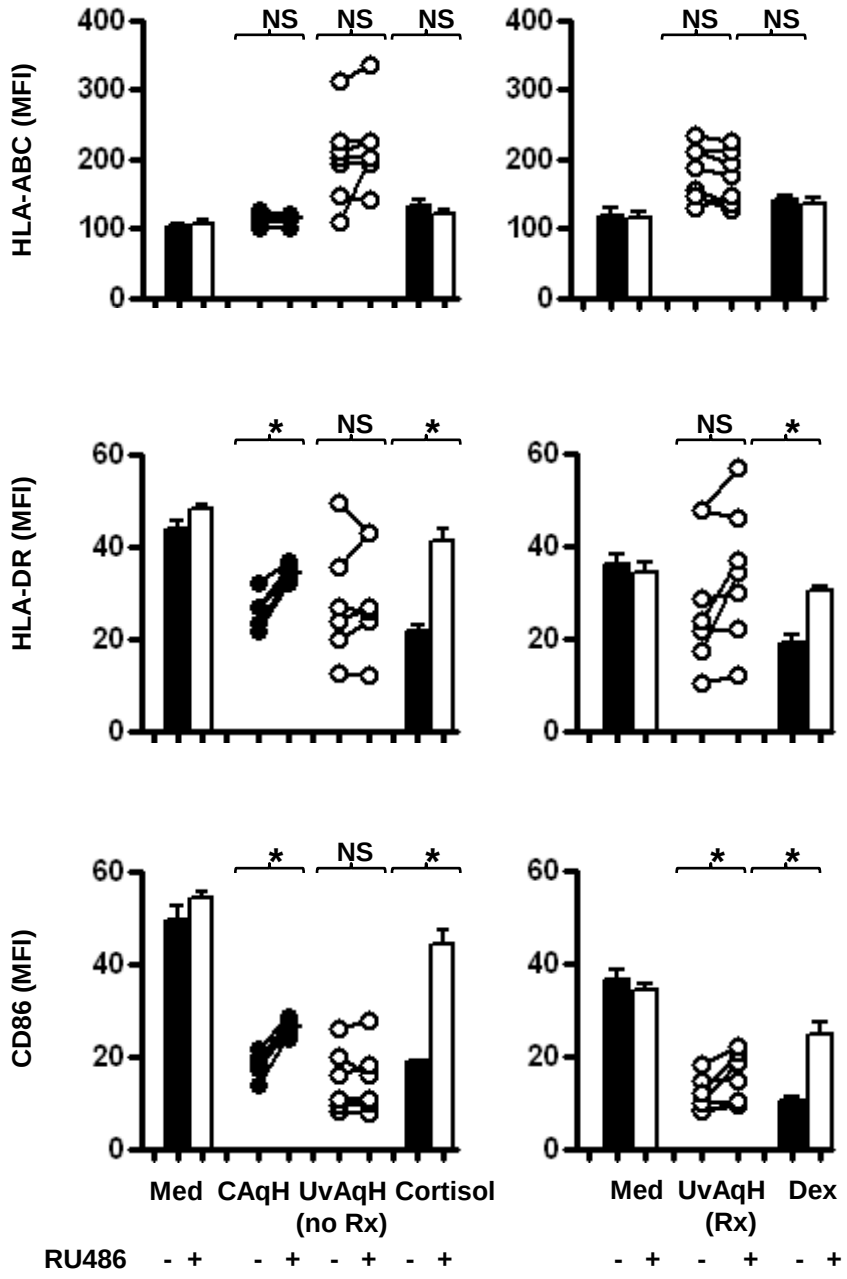



**Figure 5.8 In uveitis, AqH induced downregulation of CD86 expression on DC is no longer dependent on endogenous glucocorticoids.**
Monocyte-derived DC were cultured in the presence or absence of 50% non-inflammatory (CAqH) or uveitis AqH (UvAqH) with or without the glucocorticoid blocker, RU486; cortisol or dexamethasone (Dex) were used as positive controls. Mean ± SD of MFI for triplicate cultures are given except for individual AqH samples. Wilcoxon matched pairs analysis; * $p < 0.05$; NS = not significant.

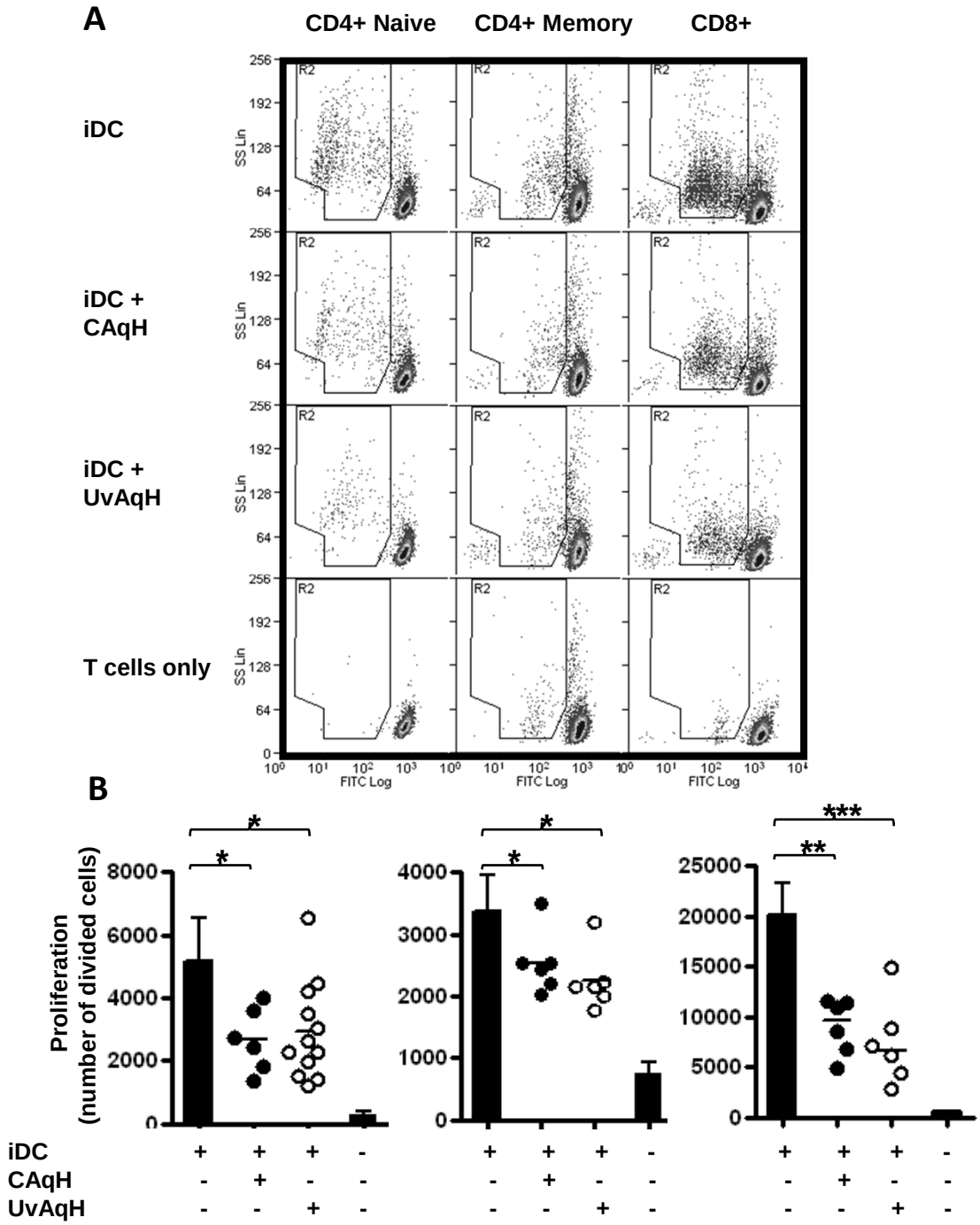



**Figure 5.9 Uveitis AqH inhibits DC capacity to induce T cell proliferation for CD4+ and CD8 + T cells**

CFSE-labelled allogeneic naïve CD4+ T cells were cultured for 4 days with monocyte-derived DC which had been pre-treated with medium or 50% AqH (non-inflammatory (CAqH) or uveitic (UvAqH)). (A) CFSE vs side scatter (SS) for live cells and (B) number of divided cells; representative of three separate experiments with the mean ± SD of triplicate cultures for medium alone. One-way ANOVA with Bonferroni post-hoc test for for multiple comparisons (normality of data demonstrated by Kolmogorov-Smirnov test); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS = not significant

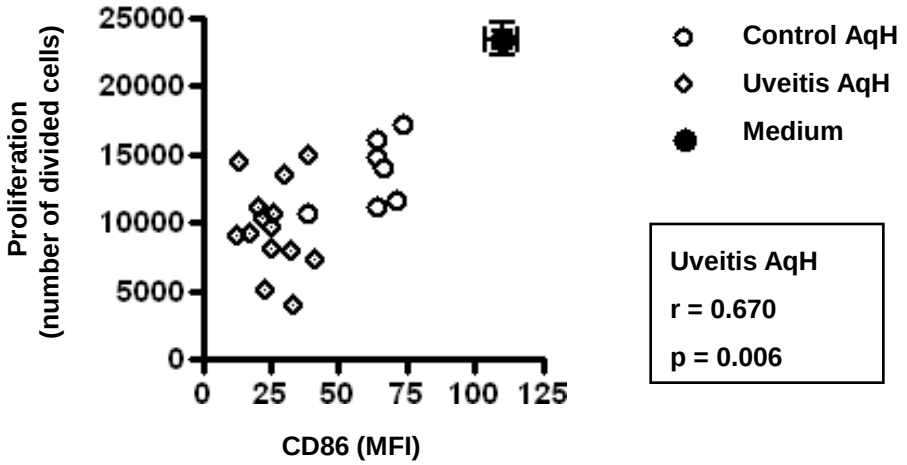



**Figure 5.10 Uveitis AqH inhibition of DC capacity to induce T cell proliferation for naïve CD4+ T cells correlates with downregulation of CD86**
CFSE-labelled allogeneic naïve CD4+ T cells were cultured with pre-treated monocyte-derived DC as described previously. Proliferation (number of divided cells) vs CD86 expression; representative of three separate experiments. Pearson r = 0.670, p = 0.006 for uveitis AqH-treated DC. Control AqH-treated DC shown for comparison (r = 0.860, p = 0.006; see also Figure 4.5). Normality of distribution demonstrated by Kolmogorov-Smirnov test**.**

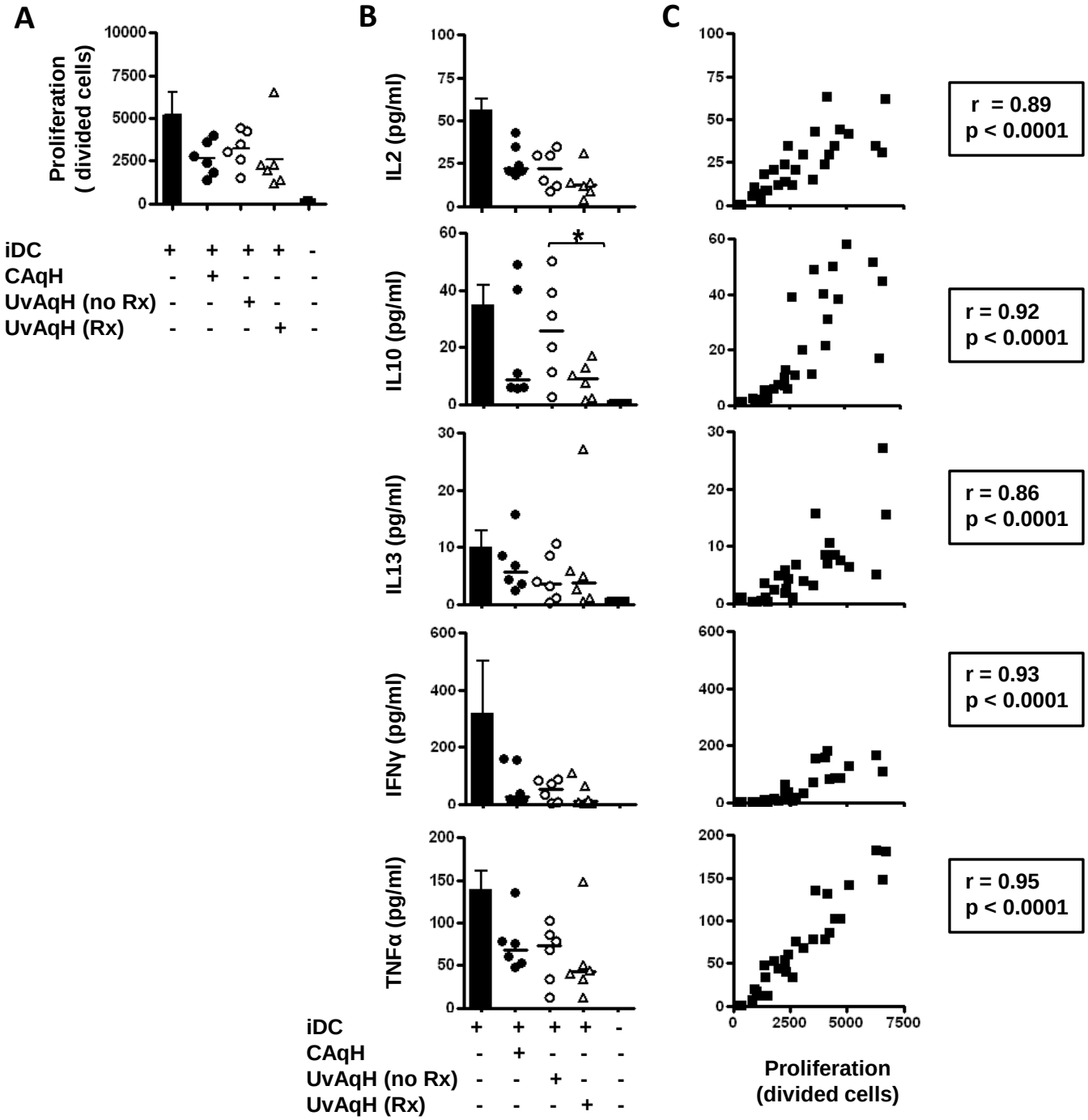



**Figure 5.11 Uveitis AqH is suppressive for DC function regardless of glucocorticoid treatment and without skewing T cell phenotype, but treatment does cause additional suppression of IL-10 production**

CFSE-labelled allogeneic naïve CD4+ T cells were cultured with pre-treated monocyte-derived DC as described previously. Supernatants were harvested after 4d and analysed by multiplex-bead immunoassay. (A) Proliferation (number of divided cells) or (B) cytokine concentration or (C) correlation of proliferation vs cytokine concentration shown; representative of three separate experiments with the mean ± SD of triplicate cultures for medium alone.

Kruskal-Wallis with Dunn's post-hoc test for multiple comparisons (B); * $p < 0.05$; all other differences non-significant. Spearman r for correlation (C).

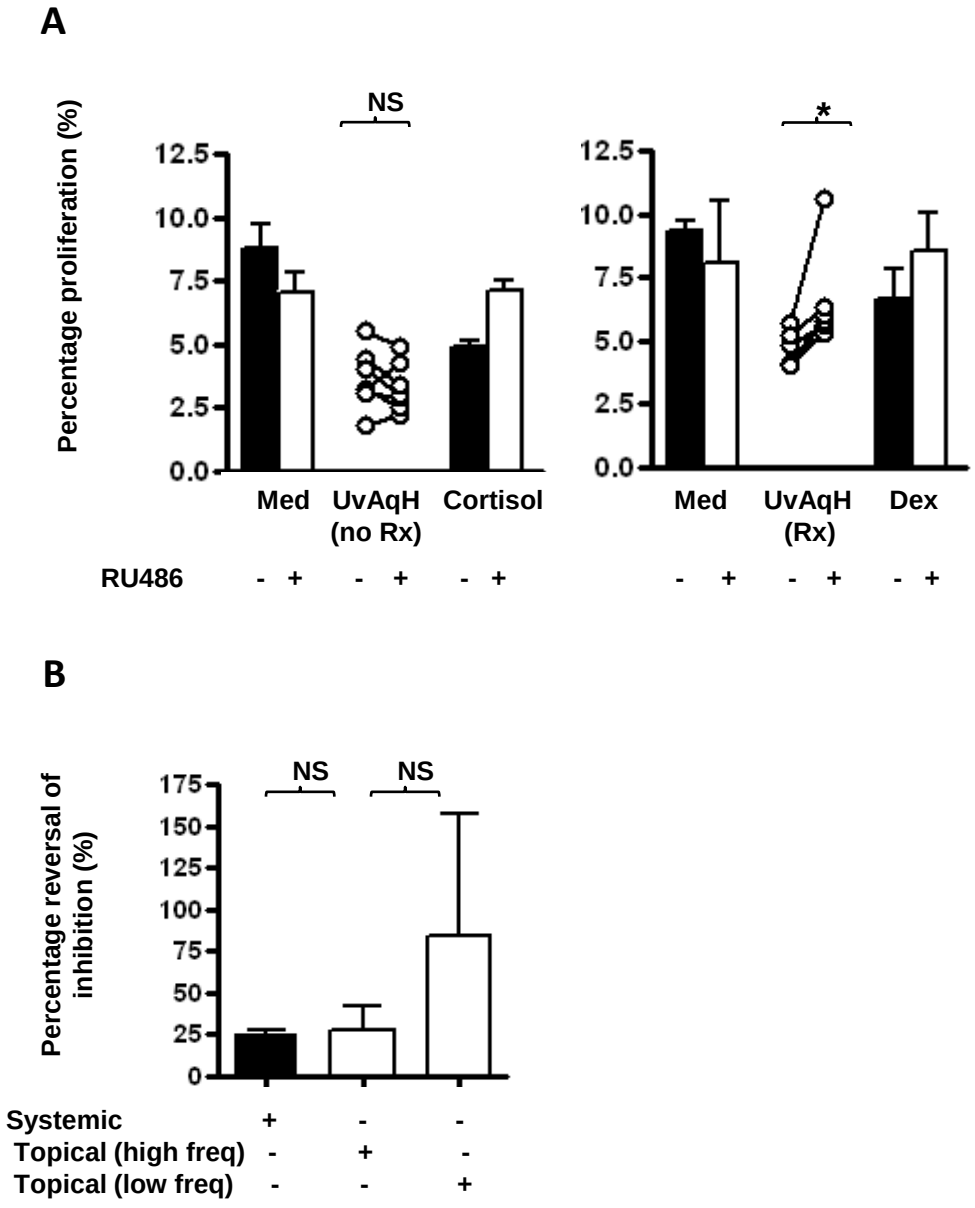



**Figure 5.12 In uveitis, AqH inhibition of DC function loses cortisol dependency which is restored in the presence of therapeutic glucocorticoids, but is not related to the intensity of therapy.**

CFSE-labelled allogeneic naïve CD4+ T cells were cultured for 4 days with monocyte-derived DC which had been pre-treated with medium or 50% AqH (non-inflammatory or uveitic) with or without the glucocorticoid blocker, RU486; cortisol or dexamethasone (Dex) were used as positive controls. (A) Mean ± SD of MFI for triplicate cultures are given except for individual AqH samples. (B) Reversal of inhibition by RU486 versus intensity of glucocorticoid therapy. (A) and (B) are representative of two separate experiments

Wilcoxon matched pairs analysis (A) and Kruskal-Wallis with Dunn's post-hoc test for multiple comparisons(B); * $p < 0.05$; NS = not significant.