REGULATORY T CELLS

IN HUMAN UVEITIS

By

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ABSTRACT

Regulatory T cells (Treg) are critical for the maintenance of tolerance to self and control of inflammation. Defects in Treg have been reported for a number of autoimmune and inflammatory diseases. In this thesis I wished to determine whether there are quantitative and/ or qualitative defects of Treg in patients with idiopathic non infectious uveitis.

Using stringent gating procedures, an increased frequency of CD4+CD25highCD127low Treg was observed in the peripheral blood of acute anterior uveitis (AAU) patients but not those with chronic disease. Treg from both acute and chronic anterior uveitis patients expressed defective suppressive capacity *in vitro*.

I also observed an accumulation of memory Treg in the aqueous humor from AAU patients, expressing high levels of FoxP3 and CTLA-4. *In vitro* activated Treg up-regulated their FoxP3 expression to levels as seen in the eye, suggesting that the aqueous humor Treg might be recently activated. Using an *in vitro* model for analysing Treg function, I observed that exposure to uveitis aqueous humor did not affect the suppressive ability of Treg.

In summary, Treg with a potent regulatory phenotype accumulate in the aqueous humor of acute anterior uveitis patients, whereas the peripheral Treg population from both chronic and acute patients express a defective function.

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ABBREVIATIONS

AAU	Acute anterior uveitis
ACAID	Anterior Chamber Associated Immune Deviation
AMP	Adenosine mono-phosphate
APC	Antigen Presenting Cell
APC Cy7	Allophycocyanin-Cyanine 7
APU	Acute pan uveitis
AqH	Aqueous humour
ATP	Adenosine tri-phosphate
BSA	Bovine Serum Albumin
CAU	Chronic anterior uveitis
CD	Cluster of Differentiation
CFA	Complete Freund's adjuvant
CFSE	Carboxy Fluorescein diacetate Succinimidyl Ester
CGRP	Calcitonin Gene Related Peptide
CNS	Central Nervous System
CRP	Complement regulatory protein
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic T Lymphocyte associate Antigen 4
DC	Dendritic cell
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
EAU	Experimental Autoimmune Uveitis
EDTA	Ethylene Diamine Tetra Acetic acid

EdU	5-ethynyl-2'deoxyuridine
EIU	Endotoxin induced uveitis
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead box Protein 3
FS	Forward scatter
GITR	Glucocorticoid-induced tumor necrosis factor receptor
HIFCS	Heat Inactivated Foetal Calf Serum
HLA	Human Leukocyte Antigen
ICAM	Intercellular Cell Adhesion Molecule
IDO	Indoleamine 2, 3 Dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRBP	Interphotoreceptor Retinoid-Binding Protein
iTreg	induced Treg
ITS	Insulin/Transferin/sodium selenate mix
LAG 3	Lymphocyte Activation Gene 3
LFA1	Lymphocyte Function associated Antigen 1
LPS	Lipopolysaccharide
MACS	Magnetic Assisted Cell Selection
MCP	Monocyte Chemotactic Protein
MG	Myasthenia Gravis
MHC	Major Histocompatibility complex

MIF	Macrophage migration inhibitory factor
MIP	Macrophage Inflammatory Protein
MS	Multiple Sclerosis
NFAT	Nuclear factor of activated T-cells
NK	Natural Killer
NS	Not significant
nT reg	natural regulatory T cell
PB	Pacific blue
PBMC	peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PECAM	Platelet Endothelial Cell Adhesion Molecule
PHA	Phytohaemagglutinin
PI	Propidium idodide
RA	Rheumatoid Arthritis
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Rosswell Park Memorial Institute medium
SD	Standard deviation
SLE	Systemic lupus erythematosus
SS	Side scatter
STAT	Signal Transducer and Activators of Transcription
Treg	T regulatory cell

T1D	Type 1 diabetes
Tconv	conventional T cell
TCR	T Cell Receptor
TGF-β	Tumour Growth Factor- β
Th	T helper cells
TNF	Tumour Necrosis Factor
TNFR	Tumour Necrosis Factor Receptor
Tr1	T regulatory cell type 1
TRAIL	TNF Related Apoptosis Inducing Ligand
VIP	Vasoactive Intestinal peptide
α-MSH	α-Melanocyte Stimulating Hormone

TABLE OF CONTENTS

LIST OF FIGURES

1 GENERAL INTRODUCTION

2 MATERIALS AND METHODS

3 REGULATORY T CELLS IN THE PERIPHERAL BLOOD OF CHRONIC NON INFECTIOUS UVEITIS PATIENTS

5 REGULATORY T CELLS IN TH E AQUEOUS HUMOR OF ACUTE UVEITIS PATIENTS

LIST OF TABLES

1 GENERAL INTRODUCTION

1.1 Inflammation

The human body is under constant threat by environmental pathogens that could potentially cause harm. Most of the time, epidermal and mucosal barriers prevent the entry of these pathogens into the body. However in some instances some potential pathogens may gain entry into the system, following which it must be recognized as "foreign", contained and then eliminated from the body. Inflammation is the body's defence mechanism to tissue damage caused by injury, infection or irritation. Acute inflammation lasts for a short period where leukocytes accumulate at the site and remove the pathogen and repair the tissue. Chronic inflammation, on the other hand is a prolonged and dysregulated response with active inflammation causing tissue destruction (reviewed by R. Medzhitov (Medzhitov, 2008). Chronic infection with bacteria, fungi, virus or parasites is one of the major causes of chronic inflammation. Bacterial products and toxins such as endotoxin, or LPS of Gram-negative bacteria can act as exogenous mediators of inflammation (Martich *et al*., 1993). Persistent inflammation is also associated with many chronic human conditions and diseases such as autoimmune diseases, cancer, etc. The primary objective of inflammation is to localize and remove the cause of damage and repair the surrounding tissue. Specialized immune cells in the bloodstream carry out these necessary responses to invading pathogen.

The pathogen and/or its secreted antigens are ingested by immature dendritic cells in the infected tissue. Dendritic cells function as phagocytic cells and transport the

antigens to the draining lymph node via the lymphatics. Similar to dendritic cells, macrophages and B cells can also act as Antigen presenting cells (APC). In the draining lymph node, these antigens are presented to the naive recirculating lymphocytes. Upon recognizing its specific antigen on an activated APC, the lymphocytes stop immigrating and start to proliferate and generate effector cells. The antigen specific effector cells then return to the circulation via the lymphatics and migrate to the infected site.

The inflammatory response involves three major stages: (1) Dilation of capillaries to increase blood flow- small blood vessels in the damaged area dilate (vasodilation), increasing blood flow into the area. (2) Increased vascular permeability - the walls of the blood vessels become more permeable allowing plasma proteins to escape the blood stream. (3) Leukocyte transmigration through endothelium and accumulation at the site of injury- this is the main cellular event in inflammation and it takes place in 4 steps.

(i) *Capture/ Tethering*- This is the first contact of a leukocyte with an activated endothelium. Capture occurs after margination, which allows leukocytes to move out of the centre of blood vessel and interact with the vascular endothelium. P-selectin and E-selectin on the endothelial surface and L-selectin on the leukocytes are the major adhesion molecules that initiate the capture of leukocytes and their rolling along the endothelial surface (Ley and Tedder, 1995).

(ii) *Rolling*- The reversible binding of leukocytes to the endothelium is mediated by the interaction of selectins with their glycosylated ligand P-selectin glycoprotein

ligand 1 (PSGL1) which is expressed on the leukocytes (Janeway *et al* (2005) *Immunobiology: the immune system in health and disease*. 6th ed). The selectin- ligand interaction cannot anchor the cells against the shear force of blood flow and hence they roll along the endothelium continuously making and breaking contact.

(iii) *Activation and firm adhesion*- During inflammation, activated endothelial cells produce chemokines and other chemo attractants which mediate the interaction of integrins (LFA-1, Mac-1 etc) on the leukocytes to adhesion molecules like ICAM-1 induced on the endothelium. Tight binding between these molecules arrests the rolling of leukocytes and cause adhesion of leukocytes to the endothelium (Ley *et al*., 2007).

(iv) *Transmigration*- Transmigration through venular walls is the final step in the process of leukocyte emigration into inflamed tissues. This takes place under the influence of chemokines. The leukocyte integrins, LFA-1 and Mac-1 are required for the migration of leukocytes towards chemoattractants. Leukocytes penetrate the basement membrane with the help of a matrix metalloproteinase enzyme which is expressed at the cell surface. The leukocytes then migrate along a concentration gradient of chemokines, secreted by the cells at the site of inflammation (Janeway *et al* (2005) *Immunobiology: the immune system in health and disease*. 6th ed)

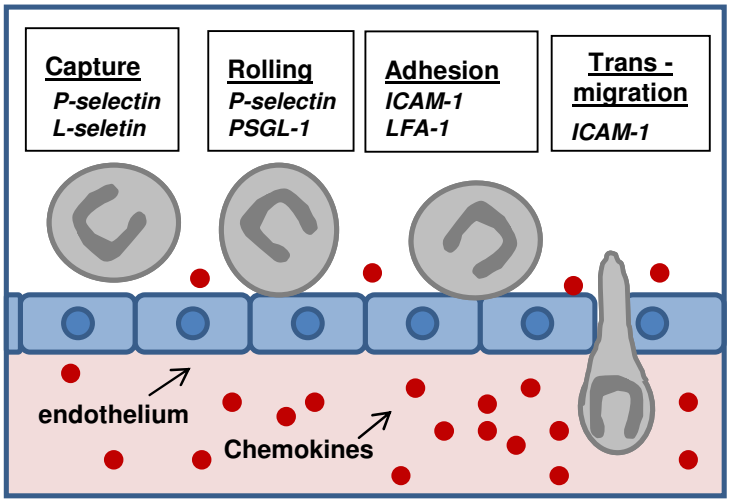

Fig: 1.1 Leukocyte migration to the target tissue

Leukocytes are captured by the endothelium followed by their rolling, adhesion and transmigration towards a chemokine gradient. Selectins, integrins and adhesion molecules mediate this process.

1.1.1 Inflammatory cytokine release

Extravasation of leukocytes into the tissue is regulated by the background cytokine environment produced by the inflammatory response. Cytokines are intercellular signalling peptides, which are major determinants of the cellular infiltrate, the state of cellular activation, and the systemic responses to inflammation. Most of them are multifunctional and can elicit their effects locally or systemically in an autocrine or paracrine manner (Feghali and Wright, 1997).

Cytokines released in the initial immune response induce vasodilation and facilitate intermolecular binding. Once the leukocytes enter the tissue, they release additional pro or anti inflammatory cytokines which then mediate or regulate inflammation. The main inflammatory cytokines and their functions are detailed in Table: 1.1.

Cytokine	Source	Effect
IL-1β	T cells, B cells, macrophage, fibroblast	T cell proliferation, activates vascular endothelium, IL-6 and TNF production by macrophages
IFN-γ	T cells, NK cells	Increases antigen presentation and activates macrophage, promotes adhesion and leukocyte migration
TNF-α	Macrophages, lymphocytes, endothelial cells, fibroblasts	Induces apoptotic cell death, chemoattractant for neutrophils, stimulates macrophage phagocytosis
IL-6	Macrophages, T cells, endothelial cells	Activates B cells and plasma cells, T cell activation and proliferation, haematopoietic stem cell differentiation
IL-2	T cells	T and B cell proliferation and differentiation, augments neutrophil and macrophage function
IL-4	T cells, macrophages, mast cells	T and B cell proliferation, differentiation of Th2 cells, B cell class switching to IgE, upregulate MHC class II expression
IL-8	Macrophages, endothelial cells, lymphocytes, neutrophils	Neutrophil activation and chemotaxis
IL-10	T cells, B cells, macrophages	Inhibits pro-inflammatory cytokines, downregulates MHC class II, induces regulatory cytokines,
TGF-β	Platelets, fibroblasts, monocytes	Suppress B and T cell proliferation, inhibits NK activity, chemotactic for macrophages, induction and/or development of regulatory T cells

Table: 1.1 Inflammatory cytokines and their functions

The main cytokines produced by immune cells during inflammation along with their source and major functions detailed in this table (Feghali and Wright, 1997)

1.2 T lymphocytes

T cells are one of the main cellular mediators of inflammation (and one of the main focuses of this thesis). Lymphoid progenitor cells arise in the bone marrow and migrate to the thymus. These cells lack expression of CD4 and CD8, and are termed double-negative (DN). Within the thymus they receive a (Notch) signal that instructs them to commit to T cell lineage. These cells give rise to a either αβ or γδ TCR expressing cells (Robey and Fowlkes, 1994). The αβ T cells develop through different stages in which both CD4 and CD8 are expressed (double positive thymocytes).

Most double positive (DP) thymocytes express T cell receptors which interact poorly with the available self-antigen, complexed with MHC molecules on cortical epithelial cells, so that the intracellular signals that are required to sustain viability are not generated. These cells undergo rapid apoptosis (reviewed by Germain R.N (Germain, 2002). Double positive cells that can bind with antigen-MHC complex with adequate affinity and produce only a weak signal undergo positive selection and go on to mature. Thymocytes also undergo negative selection where those cells capable of responding to self antigens and generate a strong signal undergo rapid apoptosis. About 2% of Thymocytes escape this dual screening and mature as single positive T cells (CD4+CD8- or CD4-CD8+) and are exported from thymus to form the peripheral T cell repertoire. It has been suggested that a TCR affinity/signal strength that is between those required for the positive and negative selection for conventional T cells give rise to natural regulatory T cells (nTreg) (Workman *et al*., 2009)

Following activation by APC, naive CD4+ T cells differentiate into effector T cells. Different subsets of effector T cells have been identified over the years mainly based

on their cytokine production and their functional differentiation is mediated by lineage-specific transcription factors. The features of main T cell subsets are described below.

1.2.1 T helper 1 (Th1) cells

Th1 cells play an important role in the defence against intracellular pathogens including bacteria, parasites, yeasts and viruses and mediate delayed type hypersensitivity (DTH) responses. The hallmark cytokines of Th1 cells include IFN-γ and lymphotoxin, which can activate microbicidal activity as well as cytokine production in macrophages (Mosmann and Coffman, 1989). Th1 cells also produce TNF-α and IL-2. The principle TH1 cytokine, IFN-γ, could activate macrophage enhancing their microbicidal activity. They express transcription factor T bet which determine the lineage commitment of Th1 cells and control IFN-γ expression (Abbas *et al*., 1996; Szabo *et al*., 2000). During Th1 differentiation, the engagement of IFN-γ with its receptor activates STAT1 and T-bet. T-bet in turn, increases the production of IFN-γ which leads to the activation of IL-12 receptor signalling subunit, IL-12Rβ2, through STAT4, is then essential for the maintenance of Th1 responses (Afkarian *et al*., 2002).

1.2.2 T helper 2 (Th2) cells

The main cytokines produced by Th2 cells are IL-4, IL-5 and IL-13. They can activate mast cells and eosinophils, and are involved in the defence against helminths and other extracellular parasites. These cells also mediate allergic and atopic manifestations (O'Garra and Arai, 2000). One of the signature cytokines of

Th2, IL-4 induces the production of IgE by B cells and IL-5 activates eosinophills. Transcription factor GATA-3 has been shown to be required for the Th2 cytokine gene expression in CD4+ T cells (Zheng and Flavell, 1997). For Th2 cells, signalling through IL-4 receptor activates STAT6, which together with GATA-3 increases IL-4 production and Th2 commitment (Kaplan *et al*., 1996).

1.2.3 T helper 17 (Th17) cells

Th17 cells produce cytokines such as IL-17A, IL-17F, and IL-22. TGF-β along with IL-6 could mediate the differentiation of Th17 cells from naïve T cells (Veldhoen *et al*., 2006). Analogous to STAT4 and STAT1 in Th1 and STAT6 in Th2 differentiation, Th17 differentiation is mediated by STAT3 (Harris *et al*., 2007). The orphan nuclear receptor RORγt act as the key transcription factor that specifies Th17 lineage (Ivanov *et al*., 2006). Th17 cells also express another related nuclear receptor, RORα, which could also dictate lineage specificity in Th17 cells (Yang *et al*., 2008b). IL-17 has been shown to induce the production of pro-inflammatory cytokines such as IL-6, TNF-α and IL-8 and has been implicated in various autoimmune diseases.

1.2.4 T follicular helper (Tfh) cells

Tfh cells are a distinct subset of CD4+ helper T cells that produce IL-21 and regulate the development of antigen-specific B cell immunity. They express CXCR5 and localize to B cell follicle where they support immunoglobulin production by B cells (Breitfeld *et al*., 2000). The transcriptional factor Bcl-6 has been associated with Tfh cell lineage commitment and the expression of Bcl-6 is controlled by IL-6 and IL-21 (Nurieva *et al*., 2009).

1.2.5 T regulatory (Treg) cells

Treg cells are a distinct subset of T cells that can either be produced from thymus as mature differentiated cells or can be induced in the periphery following activation of naïve T cells by APC. The main cytokines involved in the peripheral induction of Treg include TGF-β and IL-10. The phenotypic characteristics and functions of different Treg subsets are described in the next section.

The development and differentiation of different T cells subsets are summarised in Fig: 1.2.

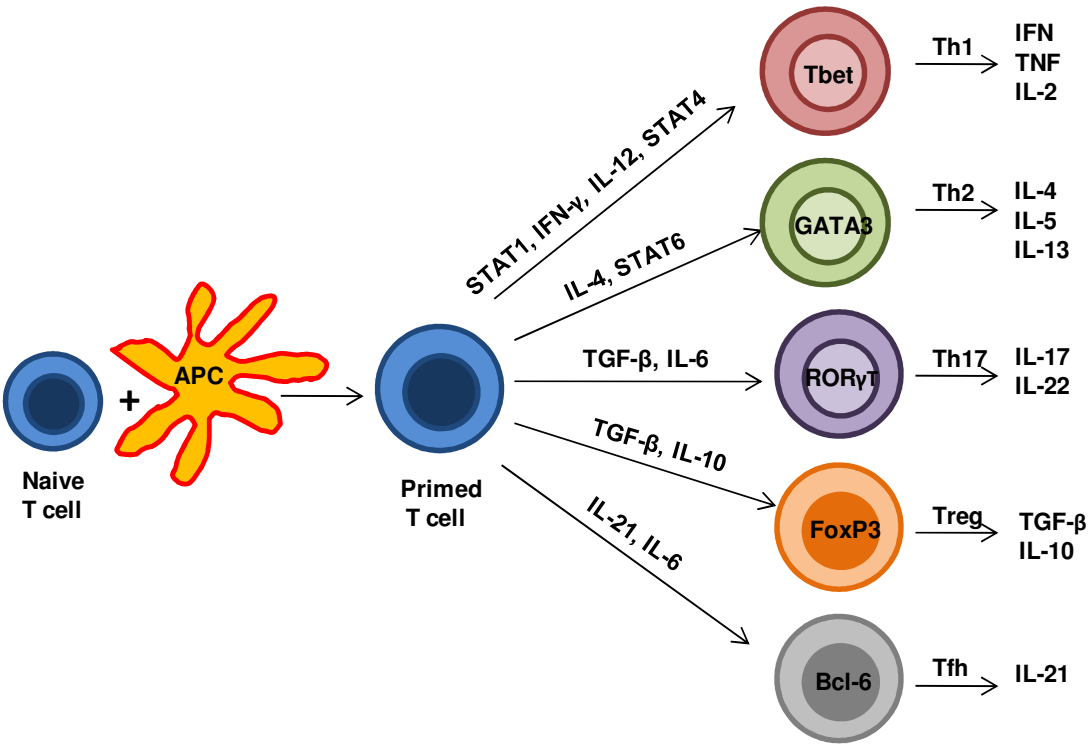

Fig: 1.2 T cell differentiation

Naïve T cells primed by APC are differentiated into Th1, Th2, TH17, Treg and Tfh subsets in the presence of particular cytokines. The lineage commitment for each subset is also determined by the transcription factor specifically expressed by them.

1.3 Regulatory T cells

Adaptive immunity requires a tightly controlled equilibrium between maintaining peripheral immune self-tolerance while at the same time preserving the potential to generate protective life-long immunity to a variety of pathogens. In order to control these two drastically different immunological outcomes, several mechanisms exist, such as apoptosis of immature self-reactive lymphocytes upon exposure to self-antigen or activation-induced cell death of mature effector cells. Also there is evidence that a subpopulation of T cells, called regulatory T cells (Treg), actively suppresses pathological and physiological immune responses, thereby contributing to the maintenance of immunological self-tolerance and immune homeostasis (Miyara and Sakaguchi, 2007)

Naturally occurring regulatory T cells were first reported by Sakaguchi *et al* in 1995 as a small group of T cells with a particular cell surface phenotype (CD4+ with co expression of IL-2 Receptor alpha chain) that help to maintain self tolerance (Sakaguchi *et al*., 1995). When T cells depleted of CD25+ cells were adoptively transferred, the recipient animal developed multi-organ autoimmunity. Reconstitution of CD4+CD25+ cells within a limited period after transfer of CD4+CD25- cells prevented these autoimmune developments in a dose-dependent fashion. These regulatory CD25+ cells represent about 1-5% of total CD4+ T cells in humans and mice (Sakaguchi *et al*., 1995).

1.3.1 Development of regulatory T cells

Developmentally regulatory T cells can be classified into two groups: natural and induced Treg. Natural regulatory T cells (nTreg) constitutively express high levels of IL-2 receptor α chain (CD25). They are produced during the normal process of T cell maturation in the thymus, resulting in an endogenous population of antigen specific Treg cells which survive as a long-lived population in the periphery poised to prevent pathological autoimmune reactions. They are anergic in cultures and this anergy can be broken by exogenous IL-2. Adaptive/induced Treg develop as a consequence of activation of mature T cells under particular conditions of sub-optimal antigen exposure and/or co-stimulation. They include the IL10 secreting Tr1 cells, TGF-β secreting TH3 cells and FoxP3+ induced Treg.

1.3.1.1 Natural regulatory T cells (nTreg)

Similar to all other T cells, nTreg originate from progenitor cells in bone marrow and undergo lineage commitment in the thymus. nTreg represent about 5-10% of peripheral CD4+ T cell population (Sakaguchi, 2004). Thymectomy in mice on day 3 after birth, but not adult thymectomy, resulted in autoimmunity (Sakaguchi *et al*., 1995). This suggests that Treg cells are produced relatively late during neonatal development. However, subsequent examination of mice thymectomized at day 3 has shown reduced but considerable numbers of CD25+CD4+ thymocytes and peripheral T cells that were fully functional (Dujardin *et al*., 2004). Expression of transcription factor FoxP3 has been shown to be crucial for the development of nTreg. The role of FoxP3 in the development and function of Treg is discussed later in this chapter. Most of the Foxp3+ thymocytes are found in the thymic medulla, indicating that the

differentiation of developing T cells to express Foxp3 occurs in the thymic medulla (Ohkura and Sakaguchi, 2010).

Conventional T cells (Tconv) undergo selection in the thymus based on the strength of signal they receive from thymic APC which present self-peptides. High affinity/avidity interactions between the TCR and MHC: peptide complexes cause "strong" signals and results in negative selection. No or very low signal causes thymocyte death by neglect. Positive selection of thymocytes that will survive and populate the periphery occurs when intermediate to weak signals are delivered via engagement of TCR (Workman *et al*., 2009). In transgenic mice expressing TCR from thymic-derived Treg in a RAG1-/- background, almost all the CD4+T (both FoxP3+ and FoxP3-) cells were deleted indicating that this TCR was specific for an endogenous self-peptide in the thymus (DiPaolo and Shevach, 2009). However, it has been suggested that nTreg are positively selected on a TCR affinity/signal strength that is between those required for the positive and negative selection for conventional T cells (Kronenberg and Rudensky, 2005).

Many co-stimulatory signals have been implicated in the development and lineage commitment of nTreg including: CD28 ligation by CD80/CD86 (Salomon *et al*., 2000; Tai *et al*., 2005), IL-2R (Burchill *et al*., 2007), TGF-β and thymic stromal-derived lymphopoietin receptor (TSLPR) (Watanabe *et al*., 2005). The role of CD28–B7 interactions in Treg development was suggested by the observation of decreased numbers of peripheral CD25+ Treg cells in CD28- or B7.1/B7.2-deficient non-obese diabetic (NOD) mice, with spontaneous exacerbation of diabetes (Salomon *et al*., 2000). Studies by Tai *et al* showed that a lack of CD28 led to reduced production of

IL-2 by Foxp3^{-} thymocytes and peripheral T cells which was insufficient to induce upregulation of CD25 and to support Treg development. They also demonstrated that co-stimulatory signals transduced into developing thymocytes through the Lck binding motif in the CD28 cytosolic tail initiated Foxp3 expression and initiated the Treg cell differentiation program (Tai *et al*., 2005).

As mentioned earlier, nTreg constitutively express IL-2R chain CD25, but are unable to secrete IL-2 themselves. The significant role of IL-2 in Treg development is evident from the work of Malek *et al* where they showed that IL-2Rβ deficient mice lacked functional Treg and developed lethal autoimmunity. This was prevented by thymic transgenic expression of wild type IL-2Rβ or adoptive transfer of CD4+CD25+ regulatory T cells (Malek *et al*., 2002). IL-2 was also shown to be important for the peripheral maintenance and homeostasis of Treg cells. Later it was shown by Burchill *et al* that IL-2Rβ dependent activation of STAT-5 which binds specifically to the FoxP3 promoter was required for the development of natural regulatory T cells (Burchill *et al*., 2007).

In addition to IL-2, signalling via TGF-β also plays a critical role in the development and maintenance of Treg. Conditional deletion of transforming growth factor-β receptor 1 (TβR1) in T cells greatly reduced the numbers of CD4+CD25+FoxP3+ thymocytes in young mice between days 3-5. But this reduction was temporary as the numbers of thymic FoxP3+ Treg recovered within a few days due to the increased production of IL-2. However genetic ablation of IL-2 in TβR1 mutant mice resulted in complete absence of FoxP3+ Treg from thymus and periphery suggesting that TGF-β is a key upstream mediator of FoxP3 expression and Treg development(Liu et

al., 2008b). Also TGF-β signalling protected thymocytes from negative selection and inhibited nTreg cell apoptosis (Ouyang *et al*., 2010).

A group of epithelial cells expressed in the thymic medulla called the Hassall's corpuscles were suggested to play a role in the differentiation and development of human nTreg. Thymic stromal lymphopoietin (TSLP) expressed by human Hassal's corpuscles activates a subpopulation of dendritic cells in the thymic medulla to express CD80 and CD86. It has been suggested that ligation of MHC II and CD80/86 by CD4+ thymocytes induces differentiation of CD4+ thymocytes into Foxp3+ nTreg (Watanabe *et al*., 2005).

1.3.1.2 Induced/adaptive Treg (iTreg)

The peripheral pool of Treg cells not only include those differentiated in the thymus but also include Treg cells generated extra-thymically through the 'conversion' of naïve T cells, also known as induced Treg (iTreg).

Two main subsets of iTreg have been described, based upon the cytokines they produce and that cause their induction: type 1 regulatory T cells (Tr1), which are induced by IL-10 (Vieira *et al*., 2004), and T helper 3 (Th3) cells, which are induced by TGF-β (Weiner, 2001). Both subsets exert their suppressive activity through secretion of the same cytokines that are responsible for their induction, IL-10 and/or TGF-β, respectively. While TGF-β and IL-10 are the primary cytokines involved in iTreg formation, it has also been demonstrated that IL-4 and IL-13 can also induce the development of Foxp3+ Treg from Foxp3- naïve T cells independently of TGF-β and IL-10 (Skapenko *et al*., 2005). IL-4-induced CD25+ Treg, phenotypically and

functionally resemble naturally occurring Treg in that they are anergic to mitogenic stimulation, inhibit the proliferation of autologous responder T cells, express high levels of the FoxP3 and the surface receptors glucocorticoid-induced TNFR family-related protein (GITR) and CTLA-4, and inhibit effector T cells in a contact-dependent, but cytokine-independent, manner (Skapenko *et al*., 2005). The main features of the subsets of iTreg cells are described below.

1.3.1.2.1 Type 1 regulatory cells (Tr1 cells)

Activating both human and murine CD4+T cells in the presence of interleukin (IL)-10 has been shown to produce CD4+ T-cell clones with low proliferative capacity, producing high levels of IL-10, low levels of IL-2 and no IL-4. These antigen-specific T-cell clones, termed Tr1 cells could suppress the proliferation of CD4+T cells in response to antigen, and prevent colitis induced in SCID mice by pathogenic CD4+CD45RBhigh splenic T cells (Groux *et al*., 1997). Colitis could be induced in C.B-17 SCID mice by transfer of CD45RBhiCD4+ T cells from normal BALBc mice even at very low numbers. However the transfer of CD45RBlowCD4+ population did not cause disease and more importantly when injected together with the CD45RBhi population, prevented disease induction (Powrie *et al*., 1993). Systemic administration of rIL-10 also prevented the development of colitis in this model (Powrie *et al*., 1994). Groux *et al* later showed that transfer of OVA-specific Tr1 cells into SCID mice prevented IBD induced by theCD45RBhi splenic T cells. Not surprisingly, the Tr1 cells had to be activated *in vivo* to be effective by feeding the mice with OVA (Groux *et al*., 1997).

Following TCR mediated activation, Tr1 cells produce high levels of IL-10, TGF-β and IL-5, low amounts of IL-2 and IFN-γ and no IL-4 (Bacchetta *et al*., 2002). The autocrine production of IL-10 accounts for their low proliferative capacity, as the addition of anti 1L-10 monoclonal antibody partially restores this response (Bacchetta *et al*., 1994). However exogenous IL-15 and to a lesser extend IL-2 induce and support the proliferation of Tr1 cells even in the absence of TCR stimulation (Bacchetta *et al*., 2002). Human Tr1 cells in the resting phase express a vast repertoire of chemokine receptors including Th1 associated CXCR3 and CCR5 and Th2 associated CCR3, CCR4 and CCR8 (Sebastiani *et al*., 2001). FoxP3, which is constitutively expressed by natural Treg cells, is not expressed by Tr1 cells. But upon activation, it can be activated to levels as seen in activated CD4+CD25- T cells. Despite the absence of FoxP3 expression, Tr1 cells suppress the *in vitro* proliferation of conventional T cells with similar efficiency to that of natural Treg (Vieira *et al*., 2004).

Tr1 cells regulate immune responses through secretion of the immunosuppressive cytokines IL-10 and TGF-β, and they suppress both naïve and memory T cell responses *in vivo* and *in vitro* (Bacchetta *et al*., 1994; Bacchetta *et al*., 2002). Antigen specific Tr1 cells require TCR mediated activation to exert their suppressive function. However, once activated they can mediate bystander suppressive activity against other antigens and this bystander suppression is likely to be mediated by local secretion of IL-10 and TGF-β (Groux, 2003) which act on both APC and T cells. IL-10 downregulates the expression of co-stimulatory molecules and pro-inflammatory cytokine production by APC and inhibits TNF-α and IL-2 production by T cells. TGF-β also plays a role in downregulating the function of APC and T cells.

1.3.1.2.2 Th3 cells

Th3 cells were first identified by Chen *et al* as a group of T cell clones which secreted TGF-β and IL-10 in culture after following a protocol of feeding SJL mice with large doses of myelin basic protein (MBP). Cytokine secretion was dependent on antigen specific stimulation *in vitro* with the fed antigen and the cells preferentially secreted TGF-β as well as other Th2 cytokines and suppressed EAE induced by either MBP or proteolipid protein (PLP) (Chen *et al*., 1994). Zheng *et al* later reported that TGF-β could induce activated CD4+CD25- T cells to become Th3 suppressor cells (Zheng *et al*., 2002). Although the precursors of these TGF-β producing CD4+ cells were CD25 negative, they upregulated CD25 expression following stimulation with TGF-β. Moreover, these cells displayed suppressive characteristics, preventing IgG production in cultures conatining fresh CD4+ cells and B cells in a TGF-β dependant manner (Zheng *et al*., 2002).

In humans, it has been shown that activated self reactive T cells have a Th3/Tr1 like cytokine profile (Kitani *et al*., 2000). TGF-β secreting Th3 cells have been observed in multiple sclerosis patients following oral administration of myelin proteins (Fukaura *et al*., 1996). Andersson *et al* reported that TGF-β mediated bystander immune suppression could be associated with remission of chronic idiopathic thrombocytopenic purpura (Andersson *et al*., 2000).

1.3.1.2.3 FoxP3+ induced Treg

The peripheral pool of regulatory T cells comprises of FoxP3- Tr1 and Th3 cells as well as FoxP3+ induced Treg. It has become evident in recent years that FoxP3+ Treg could be generated outside the thymus under a variety of conditions. Peripheral

conversion of naïve CD4+ T cells into FoxP3+ T cells was identified when polyclonal CD4+CD25- naïve T cells adoptively transferred to lymphopenic mice gave rise to CD25+FoxP3+ T cells with regulatory function (Curotto de Lafaille *et al*., 2004). The production and function of these iTreg was IL-2 dependent (Furtado *et al*., 2002; Curotto de Lafaille *et al*., 2004). Chen *et al* in 2003 reported that addition of TGF-β to TCR stimulated naïve CD4+ T cells induced transcription of FoxP3, converting them into anergic regulatory T cells which could suppress CD4+ T cells activation and Th1/Th2 cytokine production *in vitro* (Chen *et al*., 2003). These FoxP3+ iTreg also suppressed immune responses *in vivo* in an experimental asthma model (Chen *et al*., 2003). In TGF-β mediated *in vitro* induction of iTreg, IL-6 hampered the differentiation of naïve T cells to FoxP3+ T cells (Bettelli *et al*., 2006). Interestingly, TGF-β stimulation in the presence of IL-6 facilitates Th17 differentiation in mice (Bettelli *et al*., 2006).

IL-2 also plays an important role in the generation and/or homeostasis of iTreg cells. Studies using antibody neutralization and experiments with IL-2-deficient mice have revealed that IL-2 is required for TGF-β to induce naive CD4+ T cells to become CD25+FoxP3+, and develop into regulatory T cells (Zheng *et al*., 2007a). Another important factor required for the development of iTreg cells is CTLA-4 costimulation. Zheng *et al* in 2007 reported that TGF-β could not induce naïve CD4+ T cells from CTLA-$4^{(-/-)}$ mice to express normal levels of FoxP3 or develop suppressor activity (Zheng *et al*., 2006). It was shown using time course studies that CTLA-4 ligation of CD80 shortly after T cell activation was required for TGF-β to induce naïve CD4+CD25- cells to express FoxP3 and develop suppressor activity (Zheng *et al*.,

2006). In humans, activated CD4+CD25- T cells expressed FoxP3 and acquired regulatory functions (Walker *et al*., 2003b).

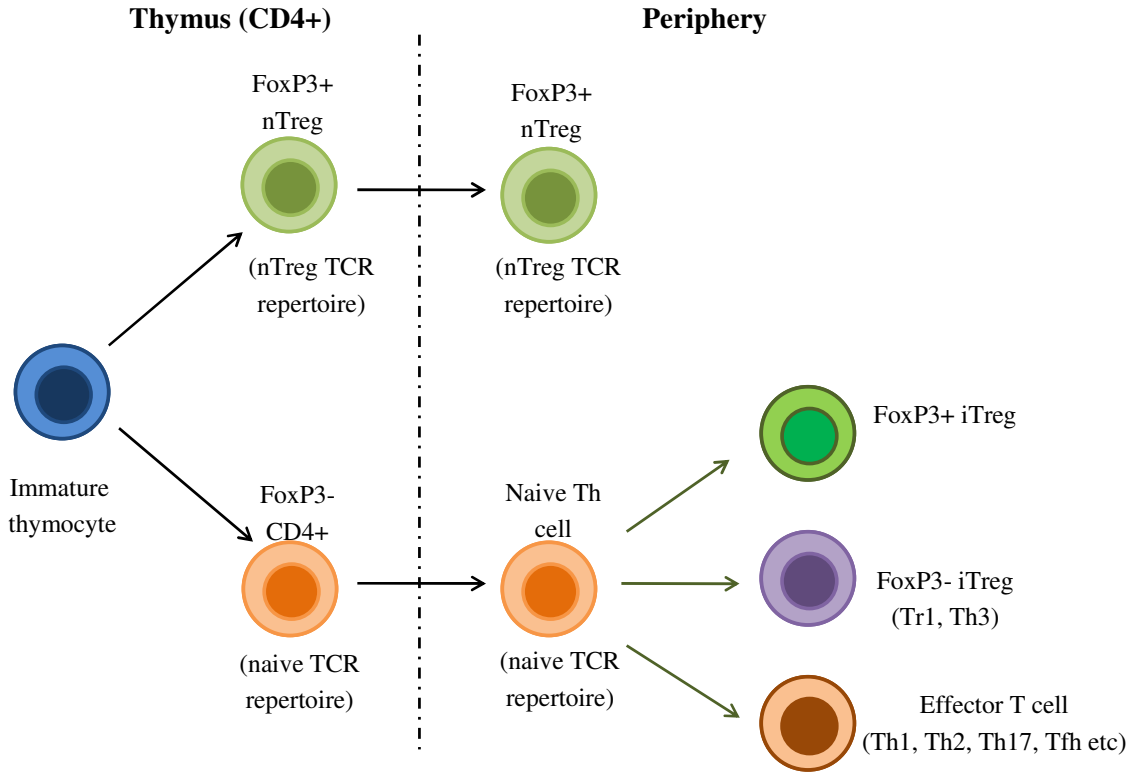

Fig: 1.3 Regulatory T cell development

In the thymus, immature thymocyte that express high avidity TCR against self peptides/MHC class II presented by thymic epithelium differentiates into nTreg cells. Cells that express low avidity TCRs are positively selected as FoxP3- conventional T-helper (Th) cells. Thymus derived Treg cells enter peripheral circulation and express a distinct nTreg TCR repertoire. Th cells with a naïve TCR repertoire differentiate in the periphery into T-helper cell subsets such as Th1, Th2 and Th17. Extrathymically generated Treg cells, eg: FoxP3- Tr1 and Th3 cells as well as FoxP3+ iTreg cells can be generated under various tolerogenic and/or inflammatory conditions.

1.3.2 Phenotypic characteristics of regulatory T cells

Over the years various surface and intracellular markers have been reported to be associated with Treg. Regulatory T cells come in many forms, including those that express the CD4 or CD8 membrane glycoprotein. Later research suggests that Treg are defined by expression of the forkhead family transcription factor FoxP3. The large

majority of FoxP3-expressing Treg are found within the major histocompatibility complex (MHC) class II restricted CD4+ helper T cell population and express high levels of the interleukin-2 receptor alpha chain (CD25). This population constitutes to 5-10% of total CD4+ T cells in humans.

CD25 was first identified as a Treg marker when CD25 depleted CD4+ T cell suspensions prepared from BALB/c nu/+ mice lymph nodes and spleens inoculated into BALB/c athymic nude (nu/nu) mice led to spontaneous development of autoimmune diseases with some mice also developing graft-vs.-host-like wasting disease. Reconstitution of CD4+CD25+ cells within a limited period after transfer of CD4+CD25- cells prevented these autoimmune developments in a dose-dependent fashion (Sakaguchi *et al*., 1995). However, human CD4+ T cells exhibit a continuous and primarily low expression of CD25 in which 2-4% express high levels of CD25, while up to 30% express low levels of CD25. Although the entire population of CD4+CD25+ T cells expressing both low and high CD25 levels exhibit regulatory function in the mouse, only the CD4+CD25high population exhibit a similarly strong regulatory function in humans (Baecher-Allan *et al*., 2001).

It was later observed that nTreg expressed very low levels of IL-7 receptor alpha (CD127) whereas other T cells expressed higher levels of this marker (Banham, 2006). Liu *et al* combined gene expression microarray, flow cytometry, and functional assays in their study and observed that IL-7Rα (CD127) was down-regulated on all human T cells after activation. In contrast with the reported re-expression of CD127 on the majority of effector and memory T cells, FoxP3+ T cells remained CD127low. They also demonstrated that FoxP3 interacted with the CD127 promoter and that

CD127 biomarker could be used to selectively enrich human Treg cells for *in vitro* functional studies (Liu *et al*., 2006).

1.3.2.1 FoxP3 (Forkhead/winged helix transcription factor)

FoxP3 (47 kDa) is a member of the forkhead/winged helix family of transcriptional regulators. Members of the Fox family are both transcriptional activators and transcriptional repressors. The forkhead (FKH) domain at the C terminus is critical for nuclear localisation and DNA binding. FoxP3 has also got an N terminus transcriptional repressor domain, followed by a C2H2 zinc finger domain and a leucine zipper domain both of which may mediate DNA binding and dimerization (Li *et al*., 2006a).

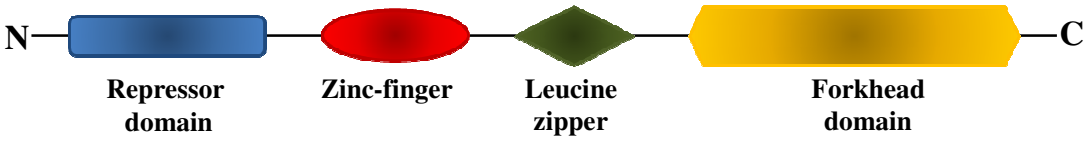

Fig: 1.4 Structure of FoxP3

Schematic representation of FoxP3 showing the locations of the various domains within the molecule

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Mutations in FoxP3 gene cause both the human X-linked fatal autoimmune disease, 'immune dysregulation polyendocrinopathy, enteropathy X-linked syndrome' (IPEX) (Bacchetta *et al*., 2006; Bennett *et al*., 2001) and an analogous X-linked pathology in scurfy(*sf*) mutant mice (Brunkow *et al*., 2001). Both human IPEX and the *sf* mouse were characterised by defective Treg function, due to FoxP3 malfunction. The striking similarities between FoxP3 mutations and depletion of CD4+CD25+ Treg led several groups to investigate the relationship of this gene to Treg development and function.

Using RT-PCR, Hori *et al* showed that FoxP3 was predominantly expressed in CD4+CD25+ Treg cells and that transduction of FoxP3 could convert naive T cells to a regulatory T cell phenotype functionally similar to nTreg (Hori *et al*., 2003). In humans, FoxP3 is also highly expressed in CD4+CD25high T cells with suppressor function (Ziegler, 2006). However it is also expressed transiently by activated conventional T cells and confer regulatory capacity on them transiently (Pillai *et al*., 2007; Wang *et al*., 2007). Similar to as seen in mice, FoxP3 gene transfer confers suppressor function upon naïve human CD4+ T cells (Yagi *et al*., 2004).

Using genome wide analysis and chromatin immunoprecipitation, Zheng *et al* identified FoxP3 binding regions of ~700 genes and observed that these genes were involved in TCR signalling, cell communication and transcriptional regulation (Zheng *et al*., 2007b). FoxP3 mediates distinct transcriptional programmes in thymus and in periphery, acting as both transcriptional activator and repressor. Wu *et al* proposed that FoxP3 controlled Treg gene expression by interacting with the transcription factor NFAT1 (Nuclear factor of activated T cells) resulting in antagonism or synergism of NFAT mediated transcription, depending on the promoter (Wu *et al*., 2006). FoxP3 was also shown to regulate transcription through direct chromatin remodelling (Chen *et al*., 2006). In this study Chen *et al* showed that FoxP3 induced histone deacetylation at the IL-2 and IFN-γ loci in Tcells, inhibiting chromatin remodelling and opposing gene transcription. Conversely, FoxP3 increased histone acetylation at the GITR, CD25 and CTLA-4 loci (Chen *et al*., 2006). In addition to transcriptional regulation of protein coding regions, FoxP3 regulates the expression of non-coding RNA as well. FoxP3 binds to and upregulates regulatory MiR-155 microRNA, which can alter gene expression (Zheng *et al*., 2007b). In humans, TCR activation has been

shown to lead to the binding of NFAT and Activator protein 1 (AP1) to the FoxP3 promoter (Mantel *et al*., 2006). A FoxP3 enhancer containing CpG island motif has been identified and transcription factors Smad3 and NFAT were shown to be required for the activity of this enhancer and essential for histone deacetylation in the enhancer region to induce FoxP3 expression (Tone *et al*., 2008). Tone *et al* proposed that during thymic Treg development, TCR signalling through self antigen activates NFAT and Smad3 may be induced by signalling through TGF-β receptor in some of these cells to induce FoxP3, indicating a role TGF-β in the development of nTreg (Tone *et al*., 2008).

Recently several groups have observed that epigenetic mechanisms play a crucial role in controlling the expression of FoxP3 locus. The FoxP3 promoter, which is located 6 kb upstream of the translational start site can be activated following TCR stimulation through binding of NFAT and AP1 (Mantel *et al*., 2006). An evolutionarily conserved CpG domain termed Treg cell specific demethylated region (TSDR) has been identified in both humans and mice which is fully demethylated in Treg cells and methylated in Tconv cells (Baron *et al*., 2007; Floess *et al*., 2007). Importantly, activated Tconv cells and TGF-β treated cells that expressed FoxP3, displayed no FoxP3 demethylation, whereas *ex vivo* expanded Treg, remained demethylated even after extensive *in vitro* stimulation and expansion (Baron *et al*., 2007; Floess *et al*., 2007). Furthermore, the TSDR in CD4+CD25+ Treg showed stronger association with acetylated histones compared to CD4+CD25- conventional T cells, indicating that FoxP3 promoter is more accessible in Treg cells (Baron *et al*., 2007; Floess *et al*., 2007).

The other important markers for Treg which are required for their suppressive function include CTLA-4, CD39 and LAG-1 which will be discussed later in the chapter. In addition to the markers mentioned above, other surface markers reported to be associated with Treg include GITR (glucocorticoid induced TNFR related protein) (Shimizu *et al*., 2002), CD62L (Lange *et al*., 2011), CD103 (Allakhverdi *et al*., 2006) etc. However, all these molecules are also expressed by naïve CD4+CD25- T cells upon activation, thereby hampering the discrimination between regulatory and activated conventional T cells.

1.3.3 Regulatory T cells and immune suppression

Treg have been shown to suppress the proliferation and cytokine production by a wide variety of immune cells. Treg require activation through their TCR to exert suppression, but once activated they can inhibit T cells irrespective of their antigen specificity. Several mechanisms have been proposed for Treg mediated suppression which includes (i) cell contact mediated mechanism (ii) cytokine mediated mechanism and (iii) cytotoxicity.

1.3.3.1 Cell contact mediated mechanism

Cell contact dependent mechanisms of suppression are best illustrated by the finding that Treg suppression was abolished when the Treg and responder cells were separated by a semi permeable membrane (Takahashi *et al*., 1998). Several accessory molecules expressed on Treg including membrane bound TGF-β, CTLA-4, LAG-3 etc. and co-stimulatory molecules like CD80 and CD86 on APC contribute to this mechanism. Stimulated Treg express high and persistent levels of TGF-β on their cell

surface. Membrane bound TGF-β has been shown to mediate the cell contact dependant suppression mechanism of Treg (Fig:1.6) and this suppression was abolished by anti TGF-β antibody (Nakamura *et al*., 2001).

1.3.3.1.1 Role of CTLA-4

CTLA-4 (CD152) is a CD28 homologue which is constitutively expressed by regulatory T cells (Takahashi *et al*., 2000; Sansom and Walker, 2006) and play a crucial role in Treg mediated suppression. CTLA-4 is not generally expressed by resting conventional T cells but is induced upon T cell stimulation and the cell surface levels of CTLA-4 remain limited due to the rapid endocytosis. The CTLA-4 gene consists of 4 exons and is located on chromosome 2 in humans and on chromosome 1 in mice. Exon 1 contains the leader peptide sequence and exon 2 the ligand binding site, both of which are extracellular. Exon 3 encodes the transmembrane region and exon 4, the cytoplasmic tail (Valk *et al*., 2008).

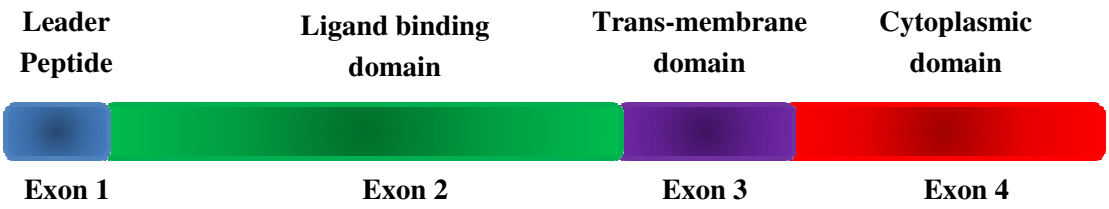


Fig: 1.5 Structure of CTLA-4

Schematic representation of CTLA-4 structure representing each exon and their cellular locations.

CTLA-4 transfection into resting human T cells made the T cells suppressive in function, even in the absence of FoxP3 expression, whereas transfection of FoxP3 alone did not confer suppressive function, indicating that the acquisition of

suppressive function by activated conventional T cells requires the expression of CTLA-4 (Zheng *et al*., 2008).

There are various mechanisms by which CTLA-4 exerts its role in Treg mediated suppression. CTLA-4 binds to CD80 and CD86 on APC and down modulates the expression of these B7 molecules (Oderup *et al*., 2006), hampering the activation and antigen presentation function of APC (Misra *et al*., 2004). This may also transduce a co-stimulatory signal to Treg to exert suppression (Fig: 1.6A). CTLA-4 on Treg could also bind directly to B7 molecules on conventional T cells and exert suppression (Paust *et al*., 2004) (Fig:1.6C). Ligation of CTLA-4 on Treg with CD80 and/or CD86 on DCs could also induce the production of the enzyme indolamine 2, 3 dioxygenase (IDO) in DCs (Fig: 1.6A). IDO catalyzes the conversion of tryptophan to kynurenine and other metabolites which has immunosuppressive effect on the local DC environment (Fallarino *et al*., 2003). It has also been shown that engagement of CTLA-4 induces the production of immunosuppressive cytokine, TGF-β by T cells (Chen *et al*., 1998). CTLA-4 also up regulates the expression of Lymphocyte function associated antigen 1 (LFA-1) thereby augmenting the physical interaction between Treg and APCs (Schneider *et al*., 2005). It was also shown *in vitro* cultures, that Treg preferentially aggregate around DCs and actively inhibit their maturation in a CTLA-4 and LFA-1 dependant manner (Onishi *et al*., 2008).

Using both human and mouse Tcells, Qureshi et al recently showed that following TCR engagement, CTLA-4 can capture its ligands (CD80 and CD86) from APCs by a process called trans-endocytosis. These costimulatory ligands are then degraded

inside CTLA-4–expressing cells, resulting in impaired costimulation via CD28 (Qureshi et al., 2011).

1.3.3.1.2 Role of CD39 and CD73

In the immune system, extracellular ATP acts as an indicator of tissue destruction. CD39 is the cell surface-located prototypic member of the ectonucleoside tripohosphate diphosphorylase (E-NTDPase) family, which controls the pool of extra cellular nucleoside triphosphates (NTP) (Borsellino *et al*., 2007). Biological activity of CD39 is a consequence (at least partly) of the regulated phosphohydrolytic activity on extra cellular nucleotides (Dwyer *et al*., 2007). However in humans, CD39 is expressed only by a subset of Treg with a memory phenotype (Borsellino *et al*., 2007).

Bopp *et al* showed that cyclic adenosine monophosphate (cAMP) is a key component in Treg mediated suppression (Bopp *et al*., 2007). They showed that Treg harboured high levels of cAMP and could transfer the cAMP into activated target cells via gap junctions. Increased levels of cAMP in the target cells inhibited their proliferation and IL-2 synthesis (Bopp *et al*., 2007). In parallel, Treg cells also induce local generation of adenosine through ectonucleotidases CD39 and CD73 expressed on their surface (Borsellino *et al*., 2007; Kobie *et al*., 2006). CD39, an ectoenzyme converts ATP to AMP, which is in turn rapidly degraded to adenosine by soluble or membrane bound CD73 (Deaglio *et al*., 2007). Binding of adenosine to the adenosine A2A receptor on target cells increases their cAMP levels (Huang *et al*., 1997) and suppress them (Fig:1.6E).

1.3.3.1.3 Role of LAG-3

Lymphocyte activation gene 3 (LAG-3/CD223), a CD4 associated adhesion molecule is highly expressed on activated Treg and plays an important role in Treg-APC interaction (Huang *et al*., 2004). Huang *et al* observed that LAG-3 was differentially expressed on murine Treg cells and was required for the maximal regulatory function (Huang *et al*., 2004). Ectopic expression of LAG-3 was shown to confer regulatory activity in CD4+ T cells (Huang *et al*., 2004). LAG-3 binds MHC class II molecules on dendritic cells with very high affinity and induces an inhibitory signal that suppresses DC maturation and immunostimulatory capacity (Fig: 1.6B).

1.3.3.2 Cytokine mediated mechanism

Although cell contact is required for *in vitro* Treg mediated suppression, several studies indicate that inhibitory cytokines like IL-10 and TGF-β are required for suppression *in vivo* or for conditioning a suppressive milieu (Fig:1.7). Treg isolated from IL-10 knockout mice failed to prevent colitis and homeostatic proliferation of CD4+ T cells from Rag deficient mice (Annacker *et al*., 2001). Blockade of IL-10 receptor and neutralization of TGF-β also abolished Treg mediated inhibition of the disease (Annacker *et al*., 2001; Asseman *et al*., 1999). It has also been shown that tumor microenvironment promotes the generation of Tr1 cells which mediate IL-10 dependant immune suppression in a cell contact independent manner (Bergmann *et al*., 2007). TGF-β is required for the function and homeostasis of Treg. T cells that expressed a dominant negative TGF-β receptor type II and therefore could not respond to TGF-β, escaped control by Treg cells *in vivo* in a mouse IBD model (Fahlen *et al*., 2005). Co-stimulation with TCR and TGF-β has also been shown to induce FoxP3 expression in naïve T cells and convert them into regulatory T cells

(Chen *et al*., 2003). Also, TGF-β produced by Treg has been implicated in anti tumour immunity in head and neck carcinoma (Vignali *et al*., 2008).

Recently, a new inhibitory cytokine IL-35, a new member of the heterodimeric IL-12 cytokine family has been shown to be preferentially expressed on Treg and required for their maximal suppressive capacity (Collison *et al*., 2007). The constitutive expression of CD25 by Treg gives them competitive advantage for the consumption of IL-2 over naïve T cells, which express CD25 only after TCR stimulation and therefore starve actively dividing conventional T cells (de la Rosa et al., 2004) (Fig:1.7). In trans-well system where Treg and conventional T cells are separated, TGF-β induces suppression in IL-2 depriving conditions (Wang *et al*., 2010).

1.3.3.3 Cytotoxicity

Cytolytic activity has been invoked as another possible mechanism of suppression by regulatory T cells. Activated human Treg cells have been shown to express granzyme A and granzyme B and kill target cells in a perforin dependant manner and this cytotoxicity was dependant on CD18 adhesive interaction and independent of Fas/FasL (Grossman *et al*., 2004a; Grossman *et al*., 2004b). Also, granzyme B deficient mouse Treg cells had reduced suppressive activity *in vitro* and this granzyme B dependant suppressive activity was perforin independent (Gondek *et al*., 2005) (Fig:1.8A). Human Treg have been shown to express Fas and FasL in cocultures containing Treg and responder T cells and activated Treg induced Fas mediated apoptosis in autologous CD8+ cells (Strauss *et al*., 2009) (Fig:1.8B). Activated Treg cells could also induce apoptosis of conventional T cells through a tumour necrosis factor related apoptosis inducing ligand- death receptor 5 (TRAIL-DR5) pathway

(Ren *et al*., 2007) (Fig:1.8C). Also galectin-1, which can induce cell cycle arrest and/or apoptosis in T cells has been shown to be up regulated by Treg upon activation and galectin-1 deficient Treg have reduced regulatory function *in vitro* (Garin *et al*., 2007) (Fig:1.8D).

Thus, various *in vivo* and *in vitro* studies suggest that several molecules and multiple mechanisms may operate in Treg mediated suppression. One question that arises is how such multiple modes of suppression interact with each other in maintaining immune homeostasis. It is possible that a particular mechanism may play a dominant role under a particular condition, with different mechanisms operating in various situations, depending on the environment in which Treg act. It is possible that in lymphoid tissue, Treg may act mainly on DCs, forming stable contacts with them, inhibiting stable interactions between DCs and naive T cells (Onishi *et al*., 2008) and thereby preventing effective priming. Here, they may act via cell contact mediated mechanisms involving CTLA-4 and/or membrane bound TGF-β. Under inflammatory conditions, primed Treg cells could migrate to non lymphoid tissues. Within the inflamed tissues, Treg may exert their suppressive function mainly by the release of anti-inflammatory cytokines such as TGF- β and IL-10, which could inhibit effector T cells as well as limit the recruitment of other inflammatory cells types such as neutrophils, monocytes and eosinophils. Treg may also exert their cytotoxic effect on inflammatory cells under inflammatory conditions. However, the most likely scenario is that multiple mechanisms of suppression may operate simultaneously and synergistically *in vivo* and that dysfunction of any one of them may not be sufficient to seriously impair Treg function.

CELL CONTACT MEDIATED MECHANISM

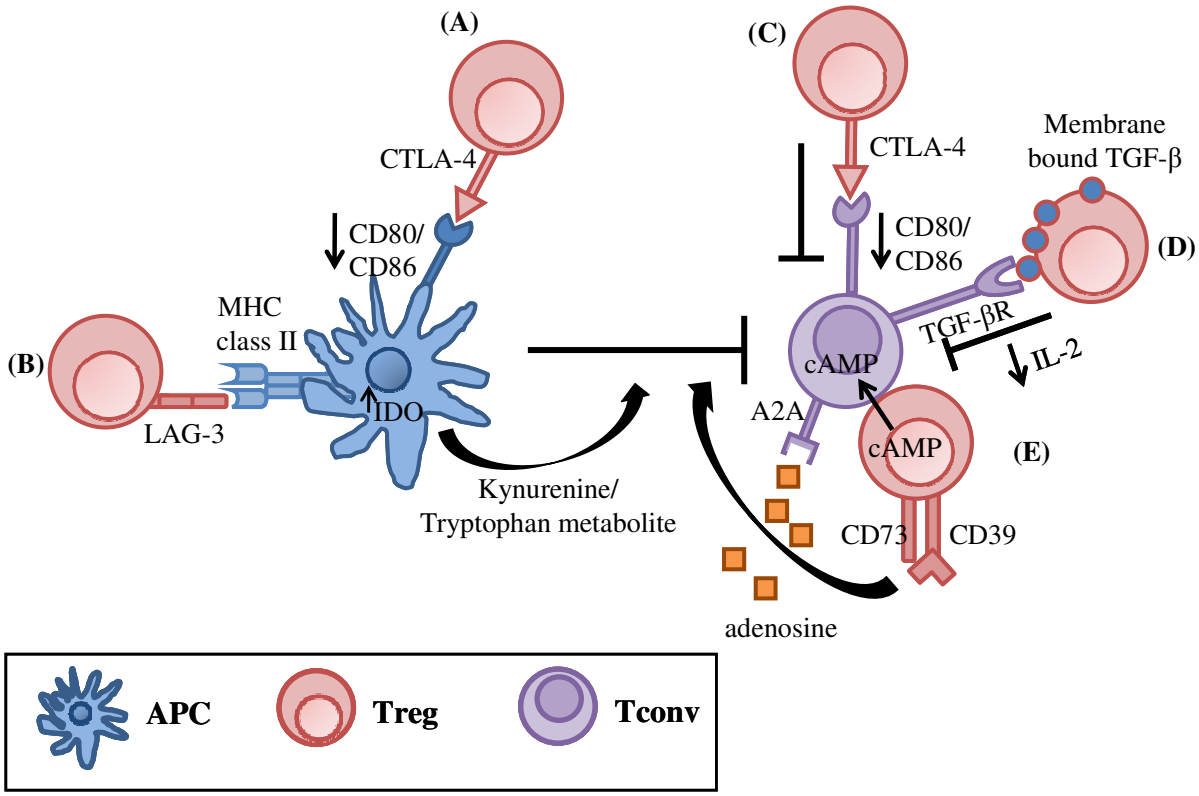


Fig: 1.6 Cell contact mediated suppression

(A) CTLA-4 down modulates the expression of CD80 and CD86 on APC and hampers the activation and antigen presentation of APCs. Ligation of CTLA-4 also induces production of indolamine 2, 3 dioxygenase (IDO) in DCs, and converts tryptophan to kynurenine which has immunosuppressive effect on the local DC environment. (B) LAG-3 on Treg binds to MHC class II on APC and inhibits stable contact between APC and Tconv. (C) CTLA-4 can bind directly to B7 molecules on Tconv and exert suppression. (D) Membrane bound TGF-β on Treg acts on Tconv cells and inhibits their proliferation and cytokine production. (E) Treg harbour high levels of cAMP and can transfer cAMP into activated target cells via gap junctions. CD39 and CD73 expressed on Treg can also induce local generation of adenosine which can bind to A2A receptor on target cells and increase the intracellular cAMP level, which in turn is inhibitory in function.

CYTOKINE MEDIATED MECHANISM

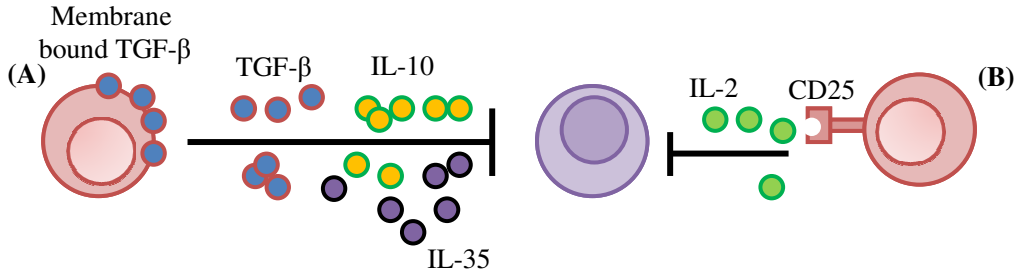


Fig: 1. 7 Cytokine mediated suppression

(A) Treg can mediate suppression via membrane bound and/or soluble TGF-β and other soluble cytokines like IL-10 and IL-35. (B) Due to the constitutive expression of CD25 by Treg, they can compete with naïve T cells, which express CD25 only after TCR stimulation, for the consumption of IL-2, and therefore starve actively dividing Tconv cells.

CYTOLYTIC MECHANISM

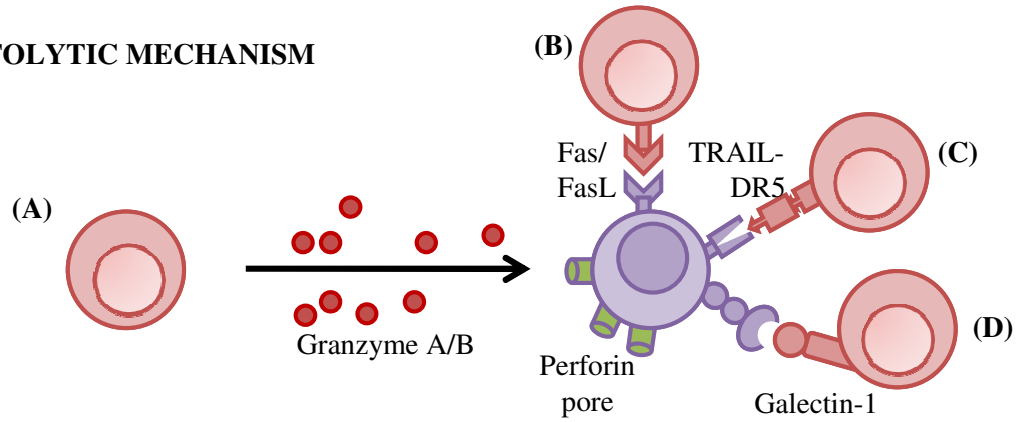


Fig: 1.8 Cytotoxic mechanism of suppression

(A) Activated Treg express granzyme A and granzyme B which can kill target cells in a perforin dependant manner. (B) Treg also express Fas/FasL and can mediate Fas induced apoptosis of target cells. (C) Treg can also induce apoptosis of target cells via TNF related apoptosis inducing ligand- death receptor 5 (TRAIL-DR5) and (D) galectin.

1.4 Immune privilege

Immune privilege is an evolutionary adaptation that provides vulnerable tissues incapable of regeneration with immune protection while avoiding loss of vital functions such as vision, reproduction etc (Niederkorn, 2006). Immune privileged sites (eg: anterior chamber of the eye, brain, testis, pregnant uterus) are sites where foreign tissue grafts enjoy extended, often indefinite, survival, whereas similar grafts placed at conventional body sites (skin, beneath kidney capsule) are rapidly rejected. Similarly, grafts prepared from these immune privileged tissues (testis, cornea, brain) experience extended survival when implanted at conventional body sites, whereas grafts prepared from conventional tissues (skin, heart, kidney) are rapidly rejected.

Immune privilege was first identified by Medawar who recognized that tissue grafts placed in the anterior chamber of the eye survived for an extended period of time (MEDAWAR, 1948). He attributed this to the absence of lymphatic drainage from the brain and the eye resulting in sequestration of antigen. Later works revealed that it is the product of various anatomical, physiological and immunoregulatory mechanisms that prevent destructive inflammation taking place in the immune privileged sites.

The main immune privileged sites in human body are central nervous system (CNS), testis, maternal-fetal interface and eye. The best characterised immune privileged sites are located in the eye. Integrity of the precise and delicate microanatomy of the eye is essential for the maintenance of physiological function. The visual axis of the eye that focuses light images on the retina is extremely delicate and intolerant to distortion that follows inflammation. Moreover, certain cellular components of the eye are incapable of regeneration. . Even a minor deviation in the anatomic integrity of the visual axis or

the loss of vital ocular cells can result in impaired vision. Inflammation, if it occurs within the eye, is a profound threat to vision. Many mechanisms have been suggested to contribute to the maintenance of immune privilege in the eye as well as in other immune privileged sites, both anatomical and immunological.

1.4.1 Anatomical mechanisms

The blood-brain barrier (BBB) provides both anatomical and physiological protection for the central nervous system (CNS) and regulates the entry of many substances and blood borne cells into the nervous tissue (Pachter *et al*., 2003). The initial site of antigen encounter for most unactivated, naive, antigen-inexperienced T cells is in the secondary lymphoid tissues. However, the CNS lacks a traditional lymphatic system which is thought to play a part in maintaining immune privilege (Carson *et al*., 2006).

The blood testis barrier consists of highly specialized tight junctions (zonula occludens) between neighbouring sertoli cells capable of restricting the passage of larger hydrophilic molecules, particularly proteins through the intercellular spaces. In the maternal-fetal interface, fetal trophoblast cells are the specific cell layer that protects the embryo from those components of the maternal immune system dedicated to destroying foreign tissues (Niederkorn and Wang, 2005).

The posterior chamber of the eye is separated from the immune system by blood-retinal barrier composed of the retinal pigment epithelium (RPE) and the retinal vascular endothelial cells. Vascular endothelial barriers also limit diffusion of molecules from the blood into the anterior chamber. The blood-ocular barrier is very selective and excludes molecules even as small as 376 Da, which is the size of sodium

fluorescein used routinely in the clinic to assess the integrity of the blood-retinal barrier (http://www.mrcophth.com/ffainterpretation/ffaprinciples.html). Although the external surface of the eye and the sub-conjunctival space is drained to the regional (preauricular) lymph nodes, the interior of the non-inflamed eye is believed to have no direct lymphatic drainage.

1.4.2 Expression of FasL

FasL is a type II membrane protein belonging to tumour necrosis factor (TNF) family and is expressed primarily on activated T cells, some tumour cells and immune privileged sites. The residential brain cells (neurons, astrocytes, oligodendrocytes, microglia and the vascular endothelium) express FasL constitutively and endow the CNS with the immune suppression to limit potentially adverse inflammatory responses (Choi and Benveniste, 2004). . The sertoli cells of testis have also been shown to express FasL constitutively (Bellgrau *et al*., 1995). Fetal cytotrophoblasts and maternal decidual cells of the placenta express FasL which is involved in protecting the fetus from lysis by activated immune cells that express Fas receptor. Another pro apoptotic molecule, TRAIL (TNF related apoptosis inducing ligand) which is expressed on many fetal tissues that express FasL is also involved in maintaining the immune privilege status of the maternal fetal interface (Niederkorn and Wang, 2005).

FasL is expressed abundantly in a number of strategic locations throughout the eye and is placed at or near areas that comprise the blood-ocular barrier, as well as locations where ocular tissues can interact with inflammatory cells (Ferguson and Griffith, 2007). FasL system induces apoptosis of any activated cells that may enter

the eye thereby contributing to immune privilege. FasL induced cell death in the eye can also lead to tolerance and blocks the growth of blood vessels that can damage the eye and impair vision (Ferguson and Griffith, 2007). TRAIL, another pro-apoptotic molecule is also expressed on many ocular tissues and is believed to play a part in ocular immune privilege (Ferguson and Griffith, 2007).

1.4.3 Expression of non-classical MHC molecules

Although all nucleated cells in the body express MHC class Ia molecules, they are absent from or weakly expressed by cells at the immune privilege sites which helps them evade lysis by cytotoxic T lymphocytes (CTL). The absence of MHC class Ia molecules on the trophoblast cells renders the embryo invisible to allospecific CTL and thus contributes to immune privilege at the maternal fetal interface (Niederkorn and Wang, 2005). However, failure to express MHC class I molecules can arouse the attention of natural killer (NK) cells which can lyse MHC class I negative cells. This is compensated by the expression of non classical MHC class Ib molecules such as HLA-G and HLA-E which has the capacity to engage NK cell inhibitory receptor (Rouas-Freiss *et al*., 1997; Lee *et al*., 1998). Trophoblast cells also produce a soluble form of HLA-G that triggers apoptosis of activated CD8+ T cells (Fournel *et al*., 2000). In CNS, the expression of MHC class I has been shown to be low or absent (Joly *et al*., 1991). However, non classical MHC class Ib molecules are expressed in the CNS (Lidman *et al*., 1999).

Similar to maternal fetal interface and CNS, corneal endothelial cells and many of the cellular elements of the retina have absent or weak expression of MHC class Ia (Abi-Hanna *et al*., 1988) and often express non classical MHC class Ib molecules such as

HLA-G (Le Discorde *et al*., 2003). Furthermore, HLA-G also inhibits the trans-endothelial migration of NK cells, shifts the cytokine balance towards Th2 dominance and suppresses CD4+ T cell proliferation (Carosella *et al*., 2001).

1.4.4 Immunosuppressive microenvironment

Immunosuppressive factors like transforming growth factor-β (TGF-β), vasoactive intestinal peptide (VIP), alpha-melanocyte stimulating hormone (α-MSH) and macrophage migration inhibitory factor (MIF) have been shown to be produced by the cells in immune privileged sites such as CNS, testis, maternal fetal interface and the eye (Reinke and Fabry, 2006; Niederkorn, 2006; Fijak and Meinhardt, 2006). Astrocytes which are the most abundant glial cells in the brain have the capacity to directly inhibit T helper cell activity *in situ* by upregulating the expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) (Gimsa *et al*., 2004). Complement regulatory proteins (CRPs) are essential for the protection of mammalian cells from damage caused by complement activation and CRPs like decay-accelerating factor (CD55) and membrane cofactor protein (CD46) on the mouse trophoblast are crucial for sustaining pregnancy (Niederkorn, 2006).

The immunomodulatory microenvironment in the eye has been extensively studied. Aqueous humour (AqH) is a clear colourless plasma-like fluid produced by the ciliary body at around 2.5µl/min (in human). Its functions include providing support to the anterior segment of the eye, nutrition to the avascular lens and cornea, light transmission and immunoregulation. AqH has been most extensively analyzed and contains biologically relevant concentrations of various immunomodulatory neuropeptides, cytokines, growth factors and soluble cell-surface receptors (Streilein,

1999; Chowdhury *et al*., 2010). AqH inhibits T-cell activation and differentiation *in vitro* and the activity was shown to be neither species specific nor directly cytotoxic to cells (Kaiser *et al*., 1989). Moreover, normal AqH also inhibits innate immune effector cells (Streilein and Stein-Streilein, 2000). Some of the immunomodulatory molecules in the AqH are explained below.

1.4.4.1 Transforming growth factor- β (TGF-β)

TGF-β was the first significant immunomodulatory molecule to be identified in AqH (Cousins *et al*., 1991). TGF-β exists in three isoforms, TGF-β1-3. Although mRNA for all three isoforms may be found in ocular cells, TGF-β2 is the only isoform that is expressed at sufficient levels to be recovered from healthy aqueous humour (Cousins *et al*., 1991; Pasquale *et al*., 1993). In healthy aqueous humour, the concentration of TGF-β2 is between 1 and 10ng/ml, the vast majority being in its latent form (Jampel *et al*., 1990).

APC treated with TGF-β2 lose their ability to activate Th1 cells and induce delayed type hypersensitivity (DTH). These APC are impaired in their IL-12 production and have lower expression of various accessory molecules (Takeuchi *et al*., 1997; Tsunawaki *et al*., 1988). TGF-β2 treatment of macrophages also reduces their production of inflammatory cytokines and their ability to generate reactive oxygen species (Tsunawaki *et al*., 1988).

TGF-β present in AqH has also been shown to be capable of modulating T cell proliferation directly. In an IL-2 dependent lymphocyte proliferation assay, Cousins *et al* showed that TGF-β can inhibit lymphocyte proliferation in a dose dependant

manner and also that the AqH inhibition of proliferation was completely reversed by the addition of anti-TGF-β antisera (Cousins *et al*., 1991).

TGF-β has been shown to inhibit proliferation of naive T cells by inhibiting IL-2 production via Smad3 (McKarns *et al*., 2004) and inhibit the differentiation of Th1 and Th2 subtypes via T-bet/Stat-4 and GATA-3/NF-AT respectively (reviewed by Li *et al*) (Li *et al*., 2006b). Significantly, TGF-β also induces a regulatory phenotype in CD4+CD25- T cells through FoxP3 induction and down-regulation of Smad-7 (Fantini *et al*., 2004). TGF-β2 in the AqH acts in synergy with α-MSH to induce the activation of regulatory T cells (Treg) which can suppress delayed type hypersensitivity (DTH) (Namba *et al*., 2002; Nishida and Taylor, 1999).

1.4.4.2 α- Melanocyte stimulating hormone (α-MSH)

The neuropeptide alpha-melanocyte stimulating hormone (α-MSH) is a 13 amino acid long (1.6 kDa) cytokine which is a proteolytic cleavage product of pro-opiomelanocortin hormone (POMC). It is produced by a number of cell types including pituitary cells, neurons, macrophages and keratinocytes. α-MSH is constitutively expressed in the healthy eye with aqueous humour levels of around 20pM (Taylor *et al*., 1992).

AqH as well as α-MSH (at its ocular physiological concentration) can suppress the IFN-γ production by antigen stimulated primed T cells (Taylor *et al*., 1992). In a mouse model of experimental autoimmune uveitis (EAU), when alpha-MSH was injected i.v. into mice at the time of peak retinal inflammation, the severity of EAU was significantly suppressed (Taylor *et al*., 2000). Taylor *et al* showed that treatment

of primed T cells with α-MSH converted them into TGF-β1 producing CD4+ CD25+ regulatory T cells (Treg) with no IFN-γ or IL-10 production. When transferred into cultures of activated T cells, these Treg cells suppressed the production of IFN-γ by activated T cells (Taylor and Namba, 2001). The ability of α-MSH to induce Treg cells is enhanced by TGF-β2 in AqH (Namba *et al*., 2002). In a model of local adoptive transfer of delayed type hypersensitivity (DTH) in which lymph node cells from immunized C57BL/6 mouse were reintroduced after incubation with mycobacterium tuberculosis antigen, the suppressive effects of AqH were found to be partially reversed by removal of the α-MSH fraction (Taylor *et al*., 1994a).

α-MSH has also been shown to suppress activated macrophages, reducing their expression of pro-inflammatory cytokines, reactive oxygen intermediate and nitric oxide, whilst increasing their production of IL-10 (Star *et al*., 1995).

1.4.4.3 Vasoactive intestinal peptide (VIP)

VIP is a 3.3 kDa, neuropeptide which is 28 amino acid long and is produced by neurons. Various peptide forms of VIP are also being produced by polymorponuclear leukocytes and mast cells (Taylor *et al*., 1994b). It is present in the normal AqH at 12 $\pm$ 1 nM. VIP, at equivalent ocular immunoreactive concentration, inhibits both antigen stimulated lymph node cell proliferation and IFN-γ production (Taylor *et al*., 1994b). Absorption of VIP from the low molecular weight fraction of AqH reversed the suppressive activity of AqH on IFN-γ production, but did not affect the suppressive activity of AqH on the proliferation of antigen stimulated lymph node cells (Taylor *et al*., 1994b). VIP has been shown to inhibit mitogen-driven lymphocyte proliferation, IL-2 production and function (Ottaway, 1987).

Delgado *et al* showed that VIP, along with pituitary adenylate cyclase-activating polypeptide (PACAP), contributed to the development of bone marrow-derived tolerogenic DCs *in vitro* and *in vivo*. The VIP/PACAP-generated DCs induced functional Treg *in vitro* and *in vivo* which could suppress primarily Th1 responses including delayed-type hypersensitivity, and transferred suppression to naive hosts (Delgado *et al*., 2005).

In addition to the above mentioned factors, there are various other factors in the AqH that help maintain the immune privilege status of the eye. Some of these other factors and their effects are summarised in Table: 1.2.

Factor	**Effect**
Somatostatin (SOM)	Induces production of α-MSH by primed T cells (Taylor and Yee, 2003); Inhibits IFN-γ production by activated T cells
Calcitonin gene-related peptide (CGRP)	Inhibits production of nitric oxide by activated macrophages (Taylor *et al*., 1998)
Macrophage migration inhibitory factor (MIF)	Inhibits NK cell activity (Apte *et al*., 1998)
Complement regulatory protein (CRP)	Inhibits complement cascade and protect against damage mediated by complement activation (Bora *et al*., 1993; Chowdhury *et al*., 2010).

Table: 1.2 Soluble factors in AqH that promote ocular immune privilege

Other immunosuppressive factors in AqH under non inflammatory conditions which help to maintain the immune privilege status of the eye.

1.4.5 Anterior chamber associated Immune deviation (ACAID)

ACAID is an animal model of tolerance induced experimentally by injection of antigen into the anterior chamber of the eye. Antigens arising from or placed in the anterior chamber of the eye elicits a deviant systemic immune response that includes primed CD8+ cytotoxic T cells and the generation of non complement fixing antibodies, but excludes CD4+ Th1 and Th2 cells, and B cells that secrete complement fixing antibodies (Streilein, 2003). Antigens, within the eye are captured by $F4/80^+$ APCs, which under the influence of ocular cytokines like TGF-β, express a unique cytokine profile in which IL-12 is downregulated and IL-10 is upregulated (Wilbanks and Streilein, 1991). The antigen bearing $F4/80^+$ APCs migrate to spleen and come to rest selectively in the marginal zone of the spleen. Here they secrete macrophage inflammatory protein-2 (MIP-2) which in turn recruits NKT cells to the site (Faunce *et al*., 2001). Together these APCs and NKT cells recruit other $F4/80^+$ APCs, marginal zone B cells and γδ T cells to form a multicellular cluster and induce a microenvironment rich in TGF-β, thrombospondin and IL-10 (Faunce and Stein-Streilein, 2002; Streilein, 2003). Eventually $CD4^+$ and $CD8^+$ αβ T cells specific for the ocular antigen accumulate at the cluster (Faunce and Stein-Streilein, 2002). Later antigen specific regulatory T cells (Treg) that mediate ACAID emerge from these clusters (Wilbanks and Streilein, 1990). $CD4^+$ Treg cells suppress the initial activation and differentiation of naive T cells into Th1 effector cells whereas the $CD8^+$ Treg cells inhibit delayed type hypersensitivity (DTH) (Wilbanks and Streilein, 1990).

The eye derived antigen bearing $F4/80^+$ APCs can also migrate to thymus where they induce the generation of CD4- CD8- $NK1.1^+$ thymocytes that can migrate to spleen and induce the generation of splenic regulatory T cells (Wang *et al*., 1997).

The postulate in the ACAID paradigm that antigen injected into the anterior chamber of the eye is transported to the spleen by ocular APC via the blood, is mainly based on the observation that ACAID can be induced in a naive animal by the transfer of $F4/80^+$ ocular APCs originating from an animal that had previously received an intracameral injection of antigen (Wilbanks and Streilein, 1991). However, Camelo *et al.* later showed that fluorescent-labelled Ag (dextran, BSA) injected into the anterior chamber of Lewis rats was detected in the lymph nodes (LN) and spleen. The extensive distribution of Ag in lymph nodes together with the phenotype of Ag-bearing cells (mostly resembling resident macrophages which can internalize antigens) in the lymphoid organs, suggests that Ag leaves the eye predominantly in a soluble form and implies that other mechanisms of tolerance may contribute to ocular-specific immune responses (Camelo *et al*., 2006; Camelo *et al*., 2004). Using intravital microscopy to monitor the migration of ocular APCs, Dullforce *et al.* demonstrated that phagocytic APCs from anterior uveal tissue did not migrate from the eye to the draining lymph node and that the acquired immune responses are initiated in the lymph node by soluble antigen escaping the eye (Dullforce *et al*., 2004).

1.4.6 Regulatory T cells in the eye

One of the important mechanisms by which immune system controls unwanted and hazardous inflammation is via regulatory T cells (Treg). Ocular inflammation can also be inhibited by Treg induced within the eye that can limit the expression of antigen specific T cell mediated inflammation. There are several pathways by which Treg can be induced in the eye:

1.4.6.1 Induced by ACAID:

Two categories of Treg are generated during ACAID- CD4+ afferent Treg and CD8+ efferent Treg. Later studies by Keino *et al* showed that some of the CD4+ Treg of ACAID arise from CD25- precursors, and that the induction of ACAID is not dependent on the presence of natural CD4+CD25+ Treg as ACAID could be induced in mouse depleted of CD25+ T cells (Keino *et al*., 2006). Using an *in vitro* ACAID culture system, Skelsey *et al* showed that CD4+ T cells were required for the generation of ACAID suppressor cells and that these suppressor cells did not require direct cell-cell contact to produce their regulatory effect (Skelsey *et al*., 2003). The efferent $CD8^+$ Treg cells generated in ACAID has been shown to be antigen specific with increased FoxP3 expression and inhibited DTH (Stein-Streilein, 2008).

1.4.6.2 Induced by AqH

The immunosuppressive effect of AqH and its components were first reported by Kaiser *et al* who showed that normal AqH from rabbits and mice inhibited antigen/growth factor driven lymphocyte proliferation (Kaiser *et al*., 1989). Later it was demonstrated by Taylor *et al* that primed T cells activated in the presence of AqH were converted to TGF-β producing regulatory T cells (Treg) that could inhibit IFN-γ secretion by inflammatory cells (Taylor *et al*., 1997). Two major component of AqH, namely α-MSH and TGF-β were found to be responsible for the induction of these antigen specific Treg (Namba *et al*., 2002; Nishida and Taylor, 1999). T cells stimulated in the presence of α-MSH and cultured in the presence of TGF-β developed a regulatory phenotype and when transferred to EAU susceptible mice, these Treg reduced the severity and incidence of the disease (Namba *et al*., 2002). As the anterior chamber of the eye is an immune privileged site, it is possible that the T

cells entering the anterior chamber are immediately converted to regulatory T cells, thereby retaining the immune privileged status of the tissue.

1.4.6.3 Induced by pigment epithelial cells

The pigment epithelial (PE) cells of iris and ciliary body (I/CB) contribute to the integrity of blood-ocular barrier. Yoshida *et al* demonstrated that I/CB PE cells suppressed the proliferation and cytokine production by stimulated T cells through a direct cell-cell contact dependant mechanism (Yoshida *et al*., 2000b). In addition I/CB PE cells also induced the generation of TGF-β producing regulatory T cells that could suppress T cell proliferation and antigen specific DTH (Yoshida *et al*., 2000a). Later Sugita *et al* demonstrated that iris pigment epithelial (PE) cells constitutively expressed B7 co-stimulatory molecule which could engage CTLA-4 expressed on T cells and inhibited their proliferation. CD8+ Treg induced by the PE cells were able to engage CTLA-4^{+} bystander T cells and inhibited their proliferation through a TGF-β dependant manner (Sugita *et al*., 2006a; Sugita *et al*., 2006b).

1.4.6.4 Induced by endogenous antigens

Most of the ocular immune deviation studies relied on intraocular injection of antigen, a process in itself could cause trauma and induction of tolerance. Therefore Gregerson *et al* developed transgenic mice strains that expressed β-galactosidase (β-gal) on retinal pigment cell and observed that endogenous β-gal expression in the retina lead to depressed DTH response and reduced antigen specific proliferation of spleen cells to β-gal, a phenotype similar to ACAID (Gregerson and Dou, 2002). However the cytokine profile of splenic lymphocytes were different from that seen in ACAID (Gregerson and Dou, 2002). The same group also showed that the endogenous retinal

expression of β-gal induced the development of Treg cells, which when adoptively transferred to a non transgenic mice immunised with β-gal, suppressed DTH responses to β-gal (Gregerson *et al*., 2009). Thus the endogenous expression of antigen in the normal quiescent retina can lead to peripheral generation of Treg that can be attributed to retinal derived antigen. However, it is not clear from this study whether central tolerance play any role in this Treg generation.

Thymic expression of retina specific antigens (IRBP and retinal S antigen) was reported in both mouse and humans. It has been suggested that even a seemingly miniscule amount of tissue specific self antigen in the thymus can lead to central tolerance with the generation of antigen specific Treg (Takase *et al*., 2005; Avichezer *et al*., 2003).

Immune privilege of the eye is neither absolute nor permanent. Immune privilege can be lost by particular tissue grafts or by experimental manoeuvres or by pathological processes. Various autoimmune and inflammatory diseases affecting the eye may suggest that the many mechanisms of tolerance and immune privilege might have failed or circumvented in these situations.

1.5 Uveitis

Uveitis comprises of a group of diseases characterised by intraocular inflammation, which by its very existence challenges the paradigm of ocular immune privilege. In most of the patients, the disease resolves rapidly, but a significant number of patients develop persistent disease. This can cause damage to ocular tissues and cause visual impairment. It is not clear whether the development of uveitis reflects an inadequate

or failed immune privilege mechanisms or conversely whether the resolution of disease is due to a re-establishment of the normal immune privilege mechanisms.

Uvea is the "vascular tunic" of the eye comprising the iris, choroid coat and the ciliary body. Uveitis is an autoimmune disorder targeting the uvea (but in reality comprises a large group of diverse diseases affecting not only the uvea but also the retina, optic nerve and vitreous) (Bora and Kaplan, 2007; Forrester, 2007)

Uveitis predominantly affects people of working age, peaking in the 20 – 50 year age group (Durrani et al., 2004a). Although there are some variations in the prevalence of uveitis across the globe, it is the 5th commonest cause of visual loss accounting for about 10 – 15% of total blindness in the developed world (Durrani et al., 2004a; Durrani et al., 2004b). The annual incidences of uveitis is between 17 and 52.4 per 100,000 person-years and prevalence of between 38 and 370 per 100,000 population (Chang et al., 2005).

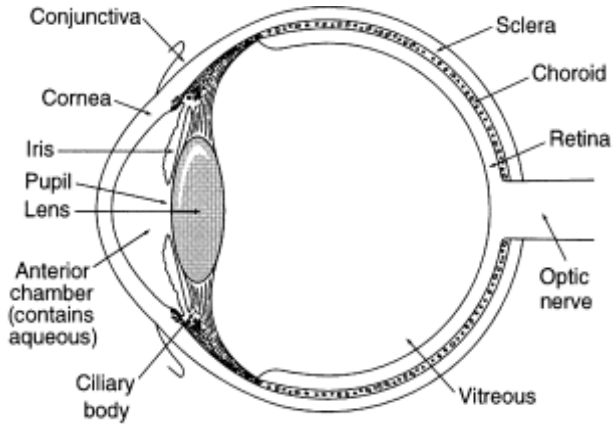


Fig: 1.9 Anatomy of ocular surface

Diagrammatic representation of eye in cross section: Uvea consists of iris, ciliary body and choroid layer (Smith *et al*., 1998).

Uveitis is etiologically classified either as infectious or non infectious. The predominant form of uveitis is thought to be non infectious. Many cases are often labelled as idiopathic, but in some it may be part of systemic disease process, such as sarcoidosis, multiple sclerosis, Behçhet's disease, Vogt-Koyanagi-Harada (VKH) disease or associated with the HLA-B27 positive group of diseases. Autoimmunity has been invoked in some of the cases, especially in idiopathic uveitis. Infectious agents, such as the herpes group of viruses, toxoplasma gondii, mycobacterium tuberculosis, and treponema pallidum are well-recognized causes for infectious uveitis (Bora and Kaplan, 2007; Forrester, 2007).

The International Workshop on Standardization of Uveitis Nomenclature (SUN) separated uveitis anatomically by location of observed disease according to visible signs- anterior, posterior, intermediate and pan uveitis (Jabs *et al*., 2005) (Table 1.3). It can also be classified based on the disease course as acute, recurrent and chronic (Jabs *et al*., 2005) (Table 1.4).

The most common form of uveitis that accounts for about 75% of cases is anterior uveitis. Inflammation occurs in either iris or ciliary body with spillover of vitreous inflammatory cells into the space behind the lens. Sight threatening intra ocular inflammation that affects the posterior part of the eye involves peripheral retina and vitreous (intermediate uveitis) or the inflammation of the retina, retinal vessels and/or optic nerve with cellular infiltrates in the choroid and retina and edema at the macula (posterior uveitis) (Forrester, 2007).

The AqH in acute anterior uveitis contains many cells and severe flare (turbidity of AqH caused by increased protein levels and cells). In chronic cases, the number of cells is considerably smaller, but flare may be conspicuous. This is because the long-term inflammation may have caused structural alteration in the blood vessels leading to leakage of proteinaceous fluid and persistent flare.

The activity of anterior chamber inflammation is measured on the basis of the cells in the anterior chamber. The SUN working group standardized the grading of anterior chamber cells and flare to achieve better compatibility between data from different groups and different studies.

For anterior chamber cells, in a field size of 1x1-mm slit beam, the following grades were described: 0 (<1 cell), 0.5+ (1-5 cells), 1+ (6-15 cells), 2+ (16-25 cells), 3+ (26-50 cells), and 4+ (>50 cells). The presence of hypopyon (leukocyte exudate in the anterior chamber) was recorded separately (Jabs *et al*., 2005).

The grading for anterior chamber flare was standardized as follows: 0 (none), 1+ (faint), 2+ (moderate; iris and lens details clear), 3+ (marked; iris and lens details hazy), and 4+ (intense; fibrin or plastic aqueous) (Jabs *et al*., 2005).

Type	**Primary site of inflammation**	**Includes**
Anterior uveitis	Anterior chamber	Iritis, Iridocyclitis, Anterior cyclitis
Intermediate uveitis	Vitreous	Pars plantis, Posterior cyclitis, Hyalitis

Posterior uveitis	Retina or choroid	choroiditis, Chorioretinitis, Retinochoroiditis, retinitis, neuroretinitis
Pan uveitis	Anterior chamber, Vitreous and retina, or choroid	

Table: 1.3 Anatomical classification of uveitis (SUN 2005)

Classification of uveitis based on the location of the observed disease as anterior, pan, intermediate and posterior uveitis

Acute	Sudden onset + limited duration
Recurrent	Repeated episodes; inactive periods≥3 months without treatment
Chronic	Persistent; relapse in < 3 months after discontinuing treatment

Table: 1.4 Classification of uveitis based on disease course (SUN 2005)

Classification of uveitis based on the onset, duration and course of the disease

1.5.1 Causes of uveitis

In many cases the causes of uveitis is unknown (idiopathic). However sometimes it can be caused by one or more of the following reasons

1. Trauma.
2. Infection: common infectious causes include herpes simplex, varicella-zoster, cytomegalovirus and toxoplasmosis. Less common causes include histoplasmosis, lyme disease, syphilis, toxocariasis and tuberculosis.
3. Systemic autoimmune diseases such as ankylosing spondilosis, reiter's syndrome, rheumatoid arthritis, sarcoidosis, multiple sclerosis and inflammatory bowel disease (Gupta and Murray, 2006).

Uveitis can be a devastating sight-threatening condition. The main causes of visual loss are cystoid macular oedema and cataract. Other complications include secondary glaucoma, band keratopathy, vitreous opacities, optic neuropathy and retinal scars.

1.5.2 Uveitis prognosis

Usually uveitis resolves rapidly with treatment; but a significant number of patients may develop persistent disease, which may lead to visual impairment (Gupta and Murray, 2006). Some patients then go on to develop chronic disease, whose proportion is not currently known. Persistent inflammation may lead to permanent damage to the trabecular meshwork, glaucoma, cataracts, and macular oedema

Patients with acute anterior uveitis have the best visual outcome; whereas people with chronic anterior uveitis, posterior uveitis, and pan uveitis have a worse visual prognosis (Gutteridge and Hall, 2007). Permanent loss of vision may occur due to late detection, delayed treatment, poor control of inflammation and damage from recurrent attacks (Gutteridge and Hall, 2007).

1.5.3 Initiation of uveitis and HLA-B27

The process that leads to the initiation of uveitis is not yet known. In the case of acute anterior uveitis, the most common form is the one associated with HLA-B27 antigen. It accounts for about 32% of all anterior uveitis cases in western countries and between 6–13% of anterior uveitis cases in Asia. HLA-B27 uveitis has a male preponderance and it affects people between the ages of 20 and 40. The clinical

features of HLA-B27 associated uveitis include pain, photophobia, redness and blurred vision. Anterior segment inflammation may be severe with keratic precipitates, anterior chamber flares/cells/fibrin with or without hypopyon formation, posterior synechiae and vitreous cells. There is high tendency for recurrences and significant association with other HLA-B27 associated diseases (Chang et al., 2005; Linssen and Meenken, 1995). The ocular complications associated with HLA-B27 positive uveitis include cataract, hypertension, secondary glaucoma and the development of chronic anterior uveitis.

Several hypotheses have been proposed to explain the pathogenic link between the HLA-B27 and its associated diseases including molecular mimicry between the HLA-B27 and pathogen (Scofield et al., 1995), misfolding of HLA-B27 heavy chains (Mear et al., 1999), or neighbouring genes (such as MICA) existing in linkage disequilibrium with HLA-B27 (Goto et al., 1998). The association of recent infection with gram negative bacteria with the initiation of anterior uveitis has also been proposed (Saari et al., 1980).

1.5.4 Treatment of uveitis

Similar to most other inflammatory diseases, uveitis responds well to treatment with glucocorticoids (OKSALA, 1960; Dick et al., 1997). Topical treatment with synthetic glucocorticoids is usually sufficient in anterior uveitis. These typically include dexamethasone 0.1% or prednisolone 1%. In more severe forms of uveitis, systemic glucocorticoids are usually given (Dick et al., 1997; OKSALA, 1960). When systemic glucocorticoids at an acceptable dose are insufficient to control disease, alternative agents are used in addition or instead of glucocorticoids. These include anti-

metabolites such as azothiorpine or methotrexate, calcineurin inhibitors such as tacrolimus and ciclosporin, and TNF-α inhibitors such as infliximab, adalimumab and etanercept.

Experimental therapies that have shown promising results in animal models of uveitis are currently under investigation for use in human uveitis. These include tolerogenic DC vaccination as well as targeting IL-17 (Ke et al., 2009), LFA-1 (Ke et al., 2007) and ICAM-1 (Uchio et al., 1994).

1.5.5 Animal models of uveitis

Several animal models of uveitis that represent various aspects of ocular inflammation have been reported in the literature. Use of animal models has allowed for the identification of uveitic antigens and their epitopes and development of therapeutic strategies.

1.5.5.1 Experimental autoimmune uveitis (EAU)

EAU targets retinal antigens and serves as a model of autoimmune uveitis in humans. It is inducible with synthetic peptides derived from retinal auto-antigens in commonly available strains of rats and mice. EAU is traditionally induced by immunization with retinal antigens such as retinal soluble antigen (s-Ag) or interphotoreceptor retinoid-binding protein (IRBP) in complete Freund's adjuvant (CFA), but the disease can also be induced in unimmunised recipients by infusion of activated lymphocytes, cultured from immunized donors (Caspi *et al*., 2008). Traditionally regarded as a Th1 disease,

it has become increasingly apparent that Th17 cells may also play a critical role in uveitis immunopathology (Peng *et al*., 2007).

A humanised model of EAU has been developed in recent years where EAU is induced in mice that were deleted for the mouse class II molecules and made transgenic for the human HLA class II molecule (Pennesi *et al*., 2003). They showed that HLA-DR3 transgenic mice developed severe uveitis with S-Ag, to which wild type mice were resistant. *In vitro* proliferation of draining lymph node cells from HLA-DR3, -DQ6, and -DQ8 TG mice immunised with S-Ag, was blocked by specific monoclonal antibodies to human, but not to mouse MHC class II molecules (Pennesi *et al*., 2003).

1.5.5.2 Endotoxin induced uveitis

EIU is an animal model of acute anterior uveitis (AAU). It can be induced by local or systemic injection of endotoxin or LPS and is characterised by increased protein and cell content in the AqH and the inflammation reaches the peak after 24 hr post injection (Rosenbaum *et al*., 1980; Okumura and Mochizuki, 1988). Most cells entering the anterior chamber are polymorphonuclear leucocytes, but in addition there is a significant mononuclear cell component to the infiltrate as well. EIU is generally considered to be an inflammation of the anterior uvea. In humans, AAU occurs most commonly in young and middle-aged adults and the HLA B27-linked cases occur more commonly in males. Similarly EIU is more severe in male Lewis rats and its susceptibility is age dependent (Smith *et al*., 1998). However, posterior segment findings have also been reported in EIU which include choroiditis, vitritis, vitreous and retinal haemorrhage and inflammatory cell infiltration of the retina with

destruction of photoreceptor cells (Smith *et al*., 1998). Several factors expressed on the vascular endothelium, including E-selectin and P-selectin have also been shown to play an important role in the pathology of EIU (Whitcup *et al*., 1997).

1.5.5.3 Experimental melanin induced uveitis (EMIU)

EMIU is induced by immunisation of rats with ocular melanin. EMIU is another model of acute anterior uveitis which mimics AAU in clinical appearance, duration and the occurrence of spontaneous relapse (Broekhuyse *et al*., 1996). Leukocyte infiltration is first observed in the anterior uvea, but in severe cases, vitritis and choroiditis are also observed (Bora and Kaplan, 2007; Smith *et al*., 2008). Treatment with anti-CD4 antibody has been shown to abrogate the disease in fischer 334 rats (Smith *et al*., 1999).

1.6 Treg in uveitis

Regulatory T cells play an important role in the immune privileged status of eye. AqH treated T cells suppressed DTH response. It was shown that various AqH factors such as TGF-β and α-MSH could induce and promote regulatory T cell activity (Nishida and Taylor, 1999; Namba *et al*., 2002). Uveitis, characterized by intraocular inflammation represents a breach of immune privilege. Using a rat model of EAU, Ke *et al.* showed that ocular Treg could distinguish monophasic uveitis from recurrent autoimmune uveitis and that Treg cells derived from the eye during the recovery from monophasic EAU had stronger suppressor activity and were involved in the remission of intra-ocular inflammation whereas the suppressor function of these cells were weak in recurrent EAU (Ke *et al*., 2008) . Similarly, in a mouse model of EAU, Sun *et al*

observed that functionally active Treg cells were induced in EAU and may be involved in regression phase of the disease (Sun *et al*., 2010a). Yeh *et al.* evaluated FoxP3+ Treg from uveitis patients and observed that Treg were reduced in patients with active uveitis compared to inactive uveitis (Yeh *et al*., 2009).

Vogt-Koyanagi-Harada (VKH) syndrome is an organ specific autoimmune disease characterized by chronic bilateral granulomatous pan uveitis and involvement of the central nervous, auditory, and integumentory systems. It was demonstrated by Chen *et al.* that there was a significantly decreased frequency and diminished function of CD4+CD25high Treg cells from peripheral blood of VKH patients and this correlated with active uveitis in these patients (Chen *et al*., 2008).

1.7 Treg in other autoimmune and inflammatory diseases

Defects of Treg in autoimmune and inflammatory diseases have been studied extensively. Depletion of CD4+CD25+ Treg resulted in wide range of organ-specific and systemic autoimmune diseases in otherwise normal animals (Sakaguchi *et al*., 1995). In humans, imbalance in the number, phenotype and function of Treg have been implicated in various autoimmune diseases.

1.7.1 Rheumatoid arthritis (RA)

RA is a chronic inflammatory disorder that ultimately leads to the destruction of joint architecture affecting about 0.8% of the UK adult population. An increased frequency of Treg have been shown in inflamed joints of chronic rheumatoid patients (Cao *et al*.,

2004). These synovial Treg have regulatory function *in vitro* (Cao *et al*., 2004; Mottonen *et al*., 2005). However it has been shown that pro inflammatory cytokines like IL-17 and TNF-α which are present in large amounts in the rheumatoid synovium could abrogate Treg mediated suppression, which may well be the case *in vivo* (van Amelsfort *et al*., 2007). Treg from the inflamed synovium express higher levels of CTLA-4 and GITR and have an activated phenotype, which is characterized by the expression of CD69 and class II MHC molecule (Cao *et al*., 2003; Mottonen *et al*., 2005). However, conventional T cells in joint fluid of RA patients showed reduced susceptibility to Treg mediated suppression, compared to T cells in peripheral blood (van Amelsfort *et al*., 2004).

Controversy exists with regard to the frequency of CD4+CD25+ Treg in the peripheral circulation of patients with RA in comparison with healthy individuals. This discrepancy could be due to the fact that some researchers defined Treg as CD4+CD25+ cells (van Amelsfort *et al*., 2004) whereas others focused on CD4+CD25bright Treg cells (Cao *et al*., 2004; Ehrenstein *et al*., 2004). Ehrenstein *et al.* isolated CD4+CD25+ Treg from the peripheral blood of patients with active RA using MACS beads and showed that these Treg were anergic upon stimulation with anti CD3 and anti CD28 antibodies and suppressed the proliferation of effector T cells *in vitro* (Ehrenstein *et al*., 2004). However, they were unable to suppress the secretion of pro-inflammatory cytokines by activated T cells and monocytes or to convey suppressive activity to conventional effector T cells (Ehrenstein *et al*., 2004).

Treatment with anti tumour necrosis factor alpha (anti-TNF-α) restored the capacity of RA Treg to inhibit cytokine production and to convey suppressive activity to

conventional effector T cells. Furthermore, the frequency of Treg cells were higher in anti TNF-α (infliximab) responding patients compared with the same patients before treatment (Ehrenstein *et al*., 2004). The expanded population of Treg cells from infliximab treated patients were FoxP3+ but lacked CD62L expression, thereby distinguishing this Treg subset from natural Treg cells present in healthy individuals and patients with active RA (Nadkarni *et al*., 2007). These FoxP3+CD62L- Treg cells from infliximab treated RA patients mediated their suppressive function through TGF-β and to a lesser extent IL-10 (Nadkarni *et al*., 2007). *In vitro* infliximab stimulation of CD4+CD25- T cells from RA patients induced a CD62L- Treg population (Nadkarni *et al*., 2007). However, the natural CD62L+ Treg from infliximab treated RA patients remained defective (Nadkarni *et al*., 2007). It was later reported that peripheral blood Treg from RA patients expressed reduced CTLA-4 and regulation of TCR signalling by CTLA-4 was impaired in these patients indicating that the defective Treg function in RA patients could be associated with CTLA-4 defects (Flores-Borja *et al*., 2008).

1.7.2 Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a multi-organ autoimmune disease characterized by the presence of high titre of IgG auto-antibodies directed towards nuclear localised auto-antigens. Initial studies in human SLE reported decreased frequency of CD4+CD25+ Treg cells from peripheral blood of SLE patients (Crispin *et al*., 2003). Studies using CD4+CD25high regulatory cells confirmed this result, showing that Treg frequency was reduced in the peripheral blood of patients with active disease compared to healthy controls (Habibagahi *et al*., 2010; Crispin *et al*., 2003; Bonelli *et al*., 2008a). Treg from SLE patients displayed an activated phenotype

as determined by the expression of CD69, CD71 and HLA-DR (Bonelli *et al*., 2008a). Lee *et al.* showed that SLE Treg also expressed reduced CCR4 and that they had decreased CCR4 ligand mediated migration (Lee *et al*., 2008). However Miyara *et al* reported that Treg from active SLE were reduced in number during disease flares and that SLE Treg were more susceptible to Fas mediated apoptosis (Miyara *et al*., 2005). Treg from active SLE patients also expressed significantly less FoxP3 whereas Treg from inactive SLE expressed increased FoxP3, which was not significantly different than normal (Valencia *et al*., 2007).

CD4+CD25high Treg isolated from active SLE patients (by FACS sorting), were defective in suppressing the proliferation and cytokine production by conventional T cells while Treg from inactive SLE exhibited normal suppressive function (Valencia *et al*., 2007). However all these results were based on Treg isolated based on the expression of CD4 and CD25 only. By sorting Treg cells using CD127low expression to eliminate activated T cells, Venigalla *et al* reported that Treg derived from SLE patients were quantitatively and qualitatively normal and that the defect in T-cell suppression observed in active SLE was due to the resistance of effector cells to Treg mediated suppression and not to abnormal regulatory T cells (Venigalla *et al*., 2008).

1.7.3 Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a chronic inflammatory disease characterized by the infiltration of the lymphocytes and inflammation of the central nervous system (CNS) white matter. The frequency of Treg in the cerebrospinal fluid (CSF) from MS patients is significantly increased (Feger *et al*., 2007; Venken *et al*., 2008b; Venken *et al*., 2008b). However the frequency of Treg in the peripheral blood do not differ

between patients with MS and healthy controls (Venken *et al*., 2008b; Feger *et al*., 2007).

It has been also been shown that Treg from relapsing remitting MS (RR-MS) patients are functionally impaired, which restored their suppressive function during remission, while Treg from secondary progressive MS (SP-MS) patients remained normal (Venken *et al*., 2008b; Viglietta *et al*., 2004). Patients with MS have reduced levels of FoxP3 at the mRNA and protein levels suggesting an involvement of diminished FoxP3 expression in impaired Treg cell immunoregulation in MS (Venken *et al*., 2008b; Huan *et al*., 2005). Studies by Venken *et al.* and Haas *et al.* revealed that the frequency of recent thymic emigrating Treg defined by the expression of CD31 and CD45RA was reduced in the peripheral blood of RR-MS patients (Venken *et al*., 2008a; Haas *et al*., 2007).

However all the above studies were based on Treg cells isolated on the basis of CD4 and high CD25 expression. Michel *et al.*, later applied stringent CD25 gates and introduced CD127low expression to define Treg cells and observed that RR-MS patients have normal Treg function when cells expressing CD127 (IL7R-α) were excluded from the analysis (Michel *et al*., 2008). Fletcher *et al.* later observed that although both CD39+ and CD39- Treg suppressed the proliferation and IFN-γ production by effector T cells, only the CD4+CD25high CD39+ Treg suppressed IL-17 production, whereas CD4+CD25highCD39- Treg produced IL-17 (Fletcher *et al*., 2009). They also reported that there was reduction in the frequency and suppressive function of CD39+ Treg cells from MS patients which could play an important role in pathology of MS (Fletcher *et al*., 2009).

1.7.4 Type 1 Diabetes (T1D)

Type 1 diabetes is a T-cell mediated disease in which insulin producing pancreatic islet beta cells are destroyed selectively, resulting in a loss of insulin production and a subsequent inability to control glucose metabolism, leading to a life long dependency on insulin (Lawson *et al*., 2008). Many studies investigated the role of Treg in T1D, and found no difference in the absolute number or frequency of Treg between T1D patients and healthy controls (Lindley *et al*., 2005; Brusko *et al*., 2007) and there was no difference in the frequency of Treg between recent onset T1D patients and those with established disease (Brusko *et al*., 2005). Some groups suggested functional defects of Treg (MACS purified) in patients with T1D and this defect was shown to be associated with reduced production of IL-2 and TGF-β (Brusko *et al*., 2005; Lindley *et al*., 2005). But contrasting results were obtained when Putnam *et al.* used sorted Treg cells from T1D patients and found no defect in the functional capacity of Treg cells from these patients (Putnam *et al*., 2005).

However later studies involving crossover co-culture assays demonstrated a relative resistance of CD4+CD25- effector T cells to CD4+CD25high Treg mediated suppression while there appeared to be heterogeneity in the functional ability of Treg from these patients (Lawson *et al*., 2008; Schneider *et al*., 2008). Increased levels of apoptosis have also been reported in the Treg cells in recent onset T1D subjects and in subjects at high risk of this disease which could also contribute to defective regulation observed in T1D (Glisic-Milosavljevic *et al*., 2007). Kavvoura *et al.* performed a meta analysis of 33 studies examining polymorphisms associated with T1D and found that CTLA-4 polymorphism is associated with T1D (Kavvoura and Ioannidis, 2005).

Green *et al.* reported that highly potent Treg accumulate in the pancreatic lymph nodes during T1D (Green *et al*., 2002).

1.7.5 Inflammatory bowel disease (IBD)

IBD is a chronic relapsing and remitting inflammatory condition of the gastrointestinal tract that manifests as 2 distinct, but overlapping clinical entities- Crohns disease (CD) and ulcerative colitis (UC) (Maul *et al*., 2005). Earlier studies reported decreased frequency of Treg in the peripheral blood of patients with CD and UC during active disease suggesting an inverse correlation between disease severity and peripheral Treg frequency (Saruta *et al*., 2007; Maul *et al*., 2005; Takahashi *et al*., 2006). However lamina propria, mesenteric lymph nodes, and intestinal inflamed mucosa of patients with CD or UC have increased frequency of Treg which increase with disease activity (Maul *et al*., 2005; Saruta *et al*., 2007; Makita *et al*., 2004; Yu *et al*., 2007; Holmen *et al*., 2006). Treg from the peripheral blood as well as the inflamed mucosa maintain normal cell-contact dependant cytokine independent suppressive capacity (Holmen *et al*., 2006; Saruta *et al*., 2007; Yu *et al*., 2007; Makita *et al*., 2004; Maul *et al*., 2005). Recently Eastaff-Leung *et al.* observed a decrease in the Treg population and increase in Th17 population and a reduced ratio of Treg to Th17 cells in the peripheral blood of IBD patients (Eastaff-Leung *et al*., 2010). Infliximab treatment enhanced the number and function of FoxP3+ Treg in IBD (Boschetti *et al*., 2010).

Our understanding of the phenotypic and functional characteristics of Treg from the peripheral blood and the site of inflammation in various diseases are summarised in Table: 1.5.

Disease	Peripheral blood Treg	Treg at site of inflammation
RA	Decreased/ increased frequency, Decreased CTLA-4, Defective suppressive function (on cytokine production by Tconv)	Increased frequency, Increased CTLA-4 and GITR, Normal functional activity *in vitro*, decreased susceptibility of synovial Tconv cells to Treg suppression.
SLE	Decreased/increased frequency, Decreased expression of FoxP3 and CCR4, Tconv cells resistant to Treg mediated suppression	
MS	No difference in frequency, normal function, Decreased frequency and impaired function of Cd39+ Treg.	Increased frequency of Treg,
T1D	No difference in frequency, Tconv cells resistant to Treg suppression, increased level of apoptosis in Treg from recent onset diabetes	Increased frequency of Treg
IBD	Decreased frequency of Treg, normal suppressive capacity, Reduced Treg:Th17 ratio	Increased frequency of Treg

Table: 1.5 Phenotypic and functional characteristics of Treg in human diseases

Functional and phenotypic characteristics of Treg from peripheral blood as well as target site in human rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), type 1 diabetes (T1D) and inflammatory bowel disease (IBD). Conflicting reports exist about the frequency of Treg in the peripheral blood of RA patients as some papers report a decreased frequency of Treg (Cao,2004) whereas some studies report an increased frequency of Treg in peripheral blood of RA patients (Amelsfort, 2004). Similarly in SLE, some studies report a decreased frequency of Treg in the peripheral blood ((Habibagahi *et al*., 2010; Crispin *et al*., 2003; Bonelli *et al*., 2008a) and some reports show an increased proportions of Treg in patients with SLE (Suarez, 2006).

1.8 Hypothesis

As explained in the previous section, defects in Treg are associated with various systemic and autoimmune diseases including ocular inflammatory diseases. Either a decreased number and/or defective function of Treg have been shown in active uveitis patients. In this context, I hypothesised that there may be a deficiency in the number, phenotype and/or function of Treg from uveitis patients. Accumulation of Treg cells at other inflammatory sites prompted me to hypothesise that there may also be accumulation of Treg in the eye of uveitis patients.

1.9 Objectives

In this thesis I set out to identify and analyse Treg from different groups of uveitis patients such as chronic anterior (AU), chronic pan (pan), acute anterior (AAU) and acute pan (APU) uveitis and compare them with age and sex matched healthy controls (HC). The specific aims included:

1. Identify Treg cells as defined as CD4+CD25highCD127low T cells using flow cytometry from human subjects.
2. Characterise the phenotype of Treg from peripheral blood based on their expression of Treg markers such as FoxP3, CTLA-4 and CD39.
3. Isolate pure and functional Treg from peripheral blood and analyse their functional capacity
4. Analyse if there were any difference in the frequency, phenotype and/or function of Treg from uveitis patients compared to healthy controls.

5. Further analyse whether there were any difference in the frequency, phenotype and/or function of Treg between different uveitis entities (anterior and pan) as well as between acute and chronic patients.
6. Identify ocular Treg cells. As uvea samples from human patients are inaccessible, aqueous humor (AqH) samples from patients have to be analysed to identify ocular Treg.
7. Analyse whether there were any difference in the phenotype and/or function of ocular Treg from acute anterior uveitis patients compared to peripheral blood Treg

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), benzyl penicillin (40U/ml), streptomycin (0.4mg/ml) and HEPES buffer (10mM)
RPMI/ITS+3/NEAA/Na pyruvate (Serum Free Medium)	RPMI medium with 1% ITS+3 liquid media supplement, 1% Nonessential amino acids, 1% Sodium pyruvate , 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. Sodium pyruvate stock solution contains 100mM sodium pyruvate solution.
RPMI/0.5% BSA	RPMI medium with 0.5% bovine serum albumin (BSA)
RPMI/10% HIFCS	RPMI medium with 10% heat inactivated 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)
Dynal isolation buffer	Phosphate buffered saline (PBS) with 0.1%BSA, 2mM ethylenediamine tetra-acetic acid (EDTA)

2.1.2 Cytokines

Recombinant Human IL-2 (Immunotools)

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

Recombinant human IL-6 (Immunotools)

Recombinant human TNF-α (Peprotech)

Recombinant human TGFβ2 (Peprotech)

2.1.3 Antibodies

Target	Conjugate	Species	Isotype	Clone	Company	Cat No	Dilution
CD4	Pe-Cy7	Mouse	IgG1	SK3	BD Pharmingen	557852	1 in 10
CD127	FITC	Mouse	IgG1	eBioRDR5	Ebioscience	11-1278	1 in 5
FoxP3	PE	Rat	IgG2a	PCH101	Ebioscience	12-4776	1 in 5
CD127	None	Mouse	IgG1	eBioRDR5	Ebioscience	16-1278	1 in 5
CD39	FITC	Mouse	IgG1	eBioA1(A1)	Ebioscience	11-0399	1 in 10
CTLA-4	PE	Mouse	IgG_{2a}	BNI3	BD Pharmingen	555853	1 in 5
CD127	PE	Mouse	IgG1	R34.34	Beckman Coulter	IM1980U	1 in 5
CD4	PE	Mouse	IgG1	MEM-241	Immunotools	21270044	1 in 20
CD25	PE-Cy5	Mouse	IgG2a	B1.49.9	Beckman Coulter	IM2646	1 in 5
CD8	Pacific Blue	Mouse	IgG2a	3B5	Invitrogen	MHCD0828	1 in 50
CD25	None	Mouse	IgG1	M-A251	BD Pharmingen	555429	1 in 5
CD45RO	PETR	Mouse	IgG2a	UCHL1	Beckman Coulter	IM2712U	1 in 20
CD69	APC/Cy7	Mouse	IgG1	FN50	Biolegend	310913	1 in 10

Table 2.1 List of primary antibodies used for flow cytometry.

Abbreviation used Allophycocyanin (APC), Cyanine 5 (Cy5), Cyanine 7 (Cy7) Fluorescein isothiocyanate (FITC), Pacific blue (PB), Phycoerythrin (PE), Phycoerythrin Texas Red (PETR)

2.1.4 Other reagents

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

Heparin (CP pharmaceuticals, 25000 IU/ml)

MACS $CD4^+CD25^+$Regulatory T cell isolation kit (Miltenyi Biotec)

MACS Treg Suppression Inspector beads (Miltenyi Biotec)

Dynabeads® Human T-Activator anti CD3/CD28 beads (Invitrogen)

Carboxyfluorescein diacetate, sucinnimidyl ester (CFSE) (Invitrogen)

Dynabeads® Regulatory CD4+CD25+ T cell Kit (Invitrogen)

Click-iT™ EdU Flow Cytometry Assay Kit (Invitrogen)

Ethylenediaminetetraacetic acid (EDTA) disodium salt solution 0.5M (Sigma Aldrich)

Counting beads (CALTAG/Invitrogen, Paisley, UK)

Propidium iodide (Sigma-Aldrich)

PHA (Sigma Aldrich)

Anti-Mouse Igk/Negative Control (FBS) Compensation Particles Set (BD biosciences)

2.1.5 Other consumables

Neubauer haemocytometer (Weber Scientific, UK)

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

48-well adherent culture plates sterile flat bottom with lid

72-Well Mini Trays (VWR International Ltd)

2.2 Aqueous humour samples

Ethical approval for the collection of AqH and matched peripheral blood from patients with uveitis (acute and chronic patients) or cataract (controls) had previously been obtained in 1996 from Dudley Local Research Ethics Committee and, at the Trust level, from Sandwell and West Birmingham NHS Trust Research and Development Committee.

Patients attending 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. AqH sampling (40-100µl) from patients with active uveitis was performed at the slit lamp following a previously published protocol (Cheung *et al*., 2004).

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).

Detailed demographic data including classification of uveitis (Table: 1.3 & Table: 1.4), aetiology (where known), duration of current episode, duration of disease, whether unilateral or bilateral, current medical treatment (if any) and anterior chamber cellular activity (as per Standardization of Uveitis nomenclature 2005 classification (Jabs *et al*., 2005) were recorded at the time of sample collection.

Each sample was centrifuged (300g, 5mins, 20˚C), and the supernatant collected and frozen at -80ºC in aliquots. The cells were resuspended in 100µl of RPMI/10% heat inactivated fetal calf serum (HIFCS) for phenotypical or functional assays.

Peripheral blood samples (30ml) were also taken from patients attending regular uveitis clinic as well as healthy volunteers recruited from among colleagues. The peripheral blood mononuclear cells (PBMC) were then isolated as explained below which are then either frozen or used for phenotypic and functional assays.

2.3 Isolation of cells

2.3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Human PBMC were isolated using well established protocols. Heparinised blood samples were taken from donors (uveitis patients, cataract patients and healthy volunteers recruited from amongst colleagues). Informed consent was taken in accordance with the Human tissue Act 2004. Peripheral blood was diluted 1:1 with RPMI medium comprising of RPMI 1640 supplemented with L-glutamine (1.64mM), benzyl penicillin (40 U/ml), streptomycin (0.4 mg/ml) and HEPES buffer (10mM)(all sigma Aldrige). Diluted blood was layered on top of 8ml of ficol- Paque plus in 25ml universal tubes and centrifuged at 1200 rpm for 30 minutes at 20^{o}C without brake.

The buffy coat containing the PBMC was transferred to fresh universals containing using a serological pipette and washed three times in RPMI medium with centrifugation of 300g at 20°C for 8 mins each time.

The yield of PBMC were then calculated using a Neubauer haemocytometer (Weber Scientific, UK), as described by the manufacturer. The range of yield was about $5x10^5$ – $1.5x10^6$ per ml of peripheral blood.

2.3.2 Isolation of regulatory T cells (Treg)

Different techniques have been used for the isolation of regulatory T cells from PBMCs.

2.3.2.1 Isolation using Magnetic activated cell sorting (MACS)

MACS microbeads are super-paramagnetic particles, pre-coated with a specific antibody to enable positive or negative selection of cell populations when passed over a MACS column placed within a strong magnetic field. Regulatory T cells were isolated using MACS $CD4^+CD25^+$ Treg cells isolation kit (Miltenyi Biotec) as described by the manufacturer. The kit contains a cocktail of biotinylated antibodies and anti-biotin microbeads for depletion of non-$CD4^+$ T cells, and CD25 microbeads for subsequent positive selection of the $CD4^+CD25^+$ regulatory T cells.

PBMC were incubated with 90µl MACS buffer and 10µl of biotin-antibody cocktail per 10^7 cells for 10 minutes followed by 15 minutes incubation with anti biotin micro beads at 4°C. 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 biotin- antibody cocktails contained a cocktail of biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a (glycophorin A)). The cells were then washed once with MACS buffer (centrifugation of 300g, 4°C, and 8 mins), the supernatant pipetted off to a dry pellet, and cells resuspended in 500µl MACS buffer/10^8 cells. An LD MACS separation column was prepared by a single rinse with 2ml MACS buffer and placed on a QuadroMACS separator magnet. The resuspended cells were passed over the column, and the negative fraction (unbound $CD4^+$ cells) eluted with a series of two x 1ml MACS buffer washes.

The eluted cells were then centrifuged and resuspended in 90µl of MACS buffer and 10µl of $CD25^+$ micro beads per 10^7 cells and incubated in dark at 4°C for 15 minutes. The cells were then washed once with MACS buffer (centrifugation of 300g, 4°C, 8 mins), and resuspended in 500µl MACS buffer/10^8 cells. The cells were then passed through an MS column (prepared by single rinse with 500µl MACS buffer) and the negative fraction ($CD4^+CD25^-$ conventional T cells/ Tconv) eluted with a series of three x 500µl MACS buffer washes. The column was then removed from the magnet and the CD25-microbead positive fraction ($CD4^+CD25^+$ Treg cells) eluted with 1ml MACS buffer and firm column pressure from the plunger. To increase the purity of the $CD4^+CD25^+$ Treg cells, the eluted fraction was enriched over a second MS column.

The yield of $CD25^+$ Treg was calculated by use of the haemocytometer. The range of yield was 0.1- 0.5 x 10^5 Treg per ml of peripheral blood. The purities of Treg (Fig:

2.1a) and Tconv (Fig: 2.2a) populations were analysed by flow cytometry following staining with CD4, CD25 and CD127.

2.3.2.2 Isolation of Treg using Dynabeads

Dynabeads are superparamagnetic, monosized polymer particles, providing a solid-phase with liquid-phase kinetics. This is a gentle and tube-based magnetic separation where the cells are not exposed to the stress of going through a dense column, and hence yields more viable and functional cells.

PBMC were isolated and separated into two portions- one for $CD4^+CD25^+CD127^-$ Treg isolation and the other for $CD4^+CD25^-CD127^+$ conventional T cell isolation. The cells were then incubated for 20mins at 2-8°C with 500µl dynal isolation buffer, 200µl HIFCS (heat inactivated foetal calf serum – Sigma Aldrich) and 200µl of antibody mix human CD4 per 10^8 cells. 5ul of purified anti human CD127 antibody was added to the Treg fraction and 5ul anti CD25 antibody was added to the conventional T cell fraction. The antibody mix human CD4 contained mouse IgG antibodies to CD14, CD16 (specific for CD16a and CD16b), CD56, CD123, CD36, CD8, CD19 and Glycophorin A- supplied in PBS and 0.02% sodium azide. Dynal isolation buffer was filter-sterilized and consisted of phosphate buffered saline (PBS; Oxoid limited) supplemented with 0.1% bovine serum albumin of 98% purity (BSA; Sigma-Aldrich) and 2mM ethylenediamine tetra-acetic acid, (EDTA; Sigma-Aldrich). After the incubation, cells were centrifuged and resuspended in 1ml cold isolation buffer and 2ml depletion My One Dynabeads (uniform, super paramagnetic polystyrene beads of 1µm diameter) per 10^8 cells at room temperature under rolling and tilting for 15 minutes. The bead-bound cells were vigorously resuspended by vortexing and placed

on a DynaMag™ magnet. The supernatant containing the bead-free $CD4^+CD25^-$ conventional T cell (from conventional T cell fraction) or $CD4^+CD127^-$ T cells (from Treg fraction) were transferred to new tubes and any residual depletion beads were separated again on the magnet. The Tconv cells were counted and resuspended in RPMI with 10% HIFCS.

The $CD4^+CD127^-$ T cells for the Treg isolation were then centrifuged (300g, 4°C, 8 mins) and incubated for 25 minutes at 2-8^o C with rolling and tilting with 1ml isolation buffer and 200µl Dynabeads CD25 (uniform, super paramagnetic polystyrene beads of 4.5µm diameter) per 1.5 x 10^7 cells. The tube was then placed on the magnet and the supernatant was removed carefully and discarded. The bead bound $CD25^+$ cells were then washed twice on the magnet and incubated for 45 minutes at room temperature with tilting and rotation with 500µl RPMI with 1% HIFCS and 80µl DETACHaBEAD per 1.5 x 10^7 cells. DETACHaBEAD are polyclonal sheep anti-Mouse anti-Fab antibodies that bind to the antigen-binding region of the target antibodies (here anti CD25 antibody on the dynabeads) with high affinity, thereby out competing their binding to the cell. The tube was then placed on the magnet and the supernatant containing $CD4^+CD25^+$ cells were removed into a new tube. The Dynabeads CD25 were washed twice in 1 ml RPMI with 1% HIFCS to obtain the residual cells and the supernatant collected after separation on a magnet. The Treg cells were then centrifuged and resuspended in RPMI with 10% HIFCS.

The yield of $CD4^+CD25^+CD127^-$ Treg was calculated using a haemocytometer. The range of yield was usually 0.1- 0.4 x 10^5 Treg per ml of peripheral blood. The purities

of the isolated populations of Treg (Fig: 2.1b) and Tconv cells (Fig: 2.2b) were determined using flow cytometry.

2.3.2.3 Isolation of Treg by High speed cell sorting

The isolated PBMC were labelled with the antibodies to CD127 (FITC), CD4 (PE) and CD25 (PE-Cy5). The cells were sorted on the Mo-Flow cell sorter into two populations: $CD4^{+}CD127^{low}CD25^{high}$ (regulatory T cells) and $CD4^{+}$ $CD127^{high}$ $CD25^{low}$ (conventional T cells).

The yield of $CD25^{high}$ Treg was calculated using a haemocytometer. The yield was usually lower and the range of yield was usually $0.4 \times 10^{4} – 0.2 \times 10^{5}$ Treg per ml of peripheral blood. The purities of the isolated populations of Treg (Fig: 2.1c) and Tconv (Fig: 2.2c) were determined using flow cytometry.

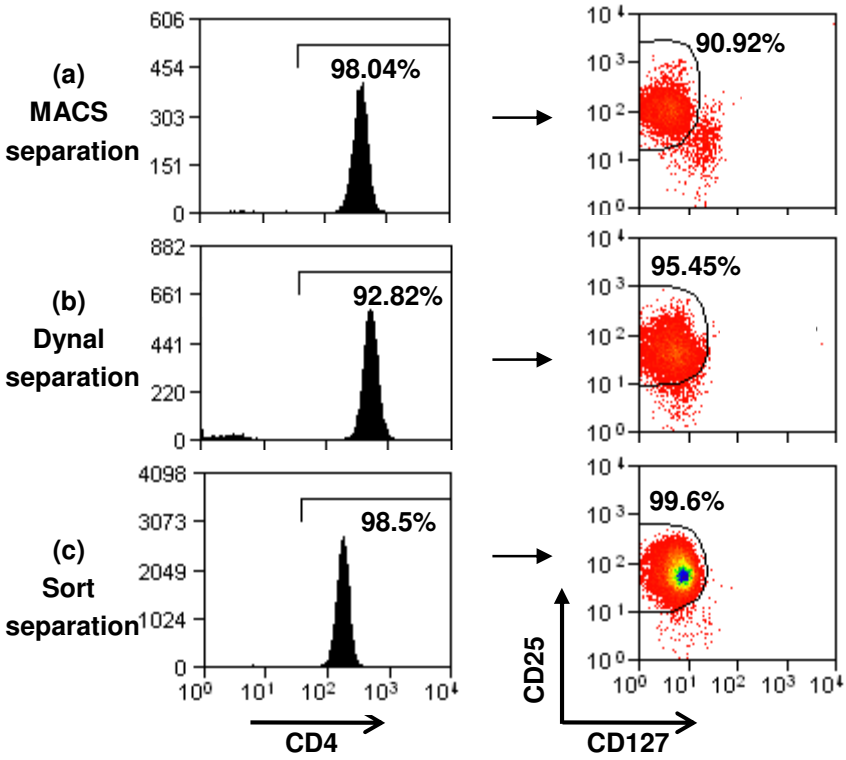


Fig: 2.1 Purity of Treg isolated using different techniques

Treg cells isolated using Macs beads, dynabeads and high speed cell sorting. Purified cells were labelled and gated on CD4+ cells to identify CD4+CD25highCD127low Treg. Purity of the cells depicted as percentage of CD4+ cells.

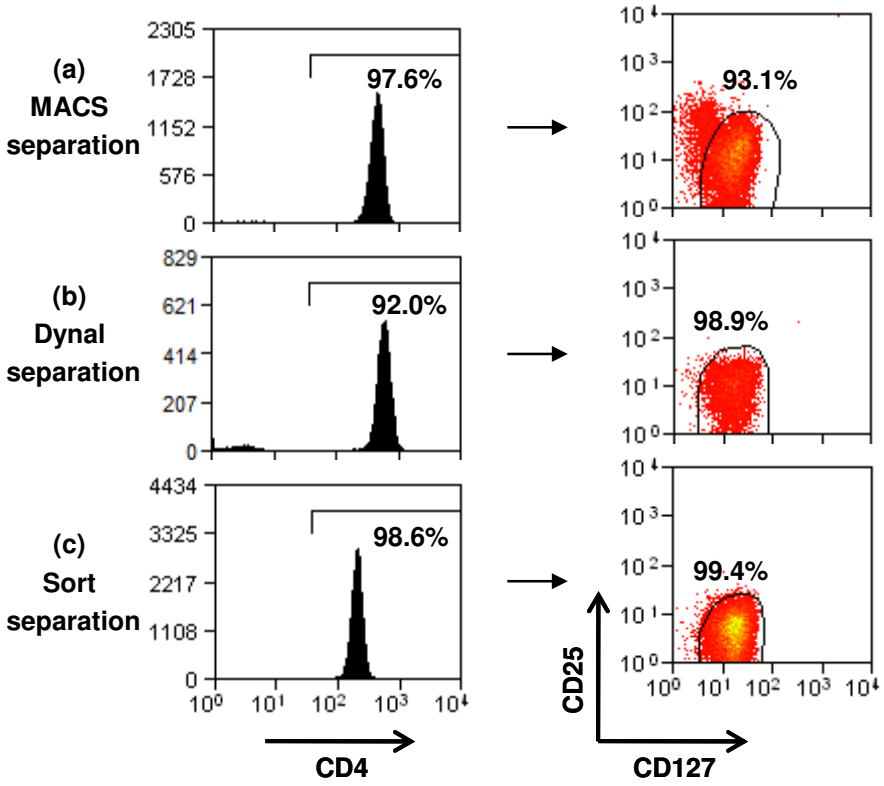


Fig: 2.2 Purity of conventional T cells isolated using different techniques

Tconv cells isolated using Macs beads, dynabeads and high speed cell sorting. Purified cells were labelled and gated on CD4+ cells to identify CD4+CD25lowCD127high Tconv cells. Purity of the cells depicted as percentage of CD4+ cells.

2.4 Cryopreservation of cells

A small portion of PBMCs were frozen as soon as they were isolated for the purpose of surface and intra cellular staining later on. (Functional analyses on the other hand were carried out always using freshly isolated cells). The cells were centrifuged at 1200 rpm for 8 minutes and the supernatant aspirated off. The pellet was then resuspended in freezing solution (10% DMSO in RPMI/10% HIFCS) at room temperature at a concentration of $1x10^6$ cells/ml. The cells were then aliquoted into cryovials. The cryovials were then placed into a room temperature Nalgene freezing container filled with isopropanol. The freezing container was then transferred to a -80ºC freezer for a minimum of 12 hours. After a minimum of 12 hours and maximum of 48 hours, the cryovials were transferred into liquid nitrogen tanks for indefinite storage.

To thaw the cells, the temperature in the cryovial was raised rapidly to between 25°C and 37°C using a water bath, while taking care to avoid contamination of the cells. The cells were then transferred slowly from the cryovials into a universal containing RPMI/10%HIFCS. The cells were then washed twice with RPMI/10% HIFCS and resuspended in appropriate buffer for staining.

2.5 Analysis of regulatory T cell surface phenotype by flow cytometry

The PBMC or the aqueous humour cells were aliquoted into marked wells at 2×10^5 – 1×10^6 cells/well and centrifuged. The cells were incubated with antibodies specific for CD4 (PE-Cy7), CD25 (PE-Cy5), CD127 (FITC), CD45RO (PE-Texas Red), CD8 (Pacific blue), CD69 (APC Cy7) and the appropriate isotype controls. Following 20

minutes incubation on ice, the cells were washed with PBS/ 0.5% BSA and the expression of the various molecules measured by flow cytometry. Treg were identified as CD4+CD25highCD127low T cells.

As shown in Fig: 2.3A, the overlap of each single surface marker used to define Treg (CD4, CD25) does not allow for accurate gating due to the inclusion of significant numbers of FoxP3 negative cells. However, when combining both CD25 and CD127 along with CD4, a clear visual distinction of a population of cells can be made containing >90% FoxP3 positive cells (Fig: 2.3B)

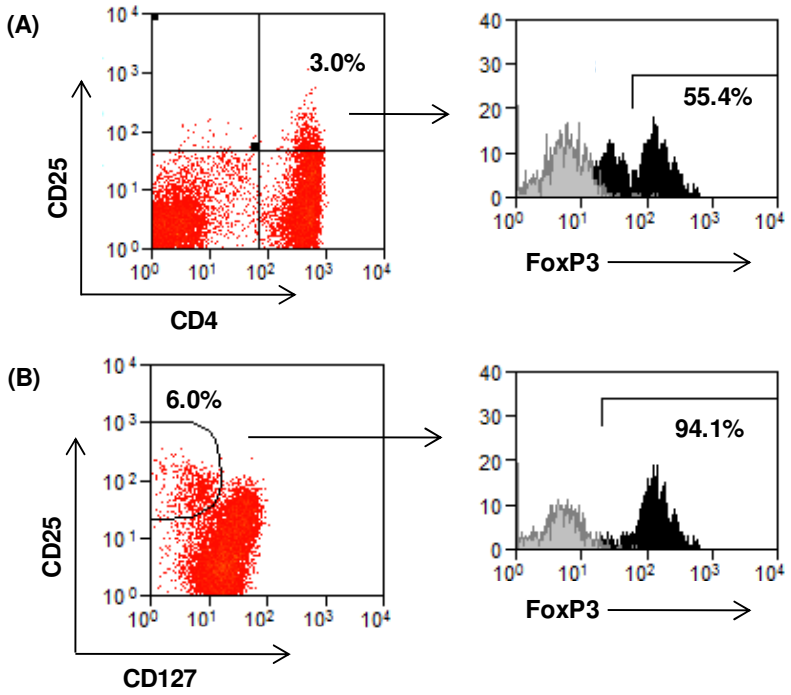


Fig: 2.3 Foxp3 expression of CD4+CD25high and CD4+CD25highCD127low Treg cells gated by the use of eye

(A) Lymphocytes were gated on CD4 and CD25 positive cells and the FoxP3 expression of CD4+CD25high Treg (3% of total lymphocytes) were analysed by flow cytometry. (B) CD4+ lymphocytes were gated on CD25highCD127low Treg cells (6% of CD4+lymphocytes and about 1% of total lymphocytes) and the FoxP3 expression was analysed by flow cytometry. Use of CD25 and CD127 to identify Treg cells (gated by the use of eye) helped to identify Treg population that contained the majority of FoxP3+ cells. Grey peaks represent isotype control and black peaks represent FoxP3 staining.

2.6 Analysis of FoxP3 and CTLA-4 expression of regulatory T cells

The PBMC or the aqueous humour cells were aliquoted into marked wells at 2 x 10^5 – 1 x 10^6 cells/well and centrifuged. The cells were incubated with antibodies to CD4 (PE-Cy7), CD127 (FITC), CD25 (PE-Cy5), CD45RO (PE-Texas Red), CD69 (Apc-Cy7) and CD8 (Pacific Blue) along with the appropriate isotype controls. Following 20 minutes incubation on ice the cells were washed with PBS/0.5% BSA. The fixation and permeabilisation buffers were made up according to manufacturer's instruction (e-bioscience FoxP3 staining kit). The cells were then incubated with fixation buffer for 30 minutes on ice followed by washing with PBS/0.5% BSA. The cells were then washed twice with permeabilisation buffer. 1µl of rat serum was added to the wells receiving FoxP3 antibody or its isotype control and incubated on ice for 15 minutes along with 30µl permeabilisation buffer in all the wells. After the incubation, the FoxP3 (PE) or the CTLA-4 (PE) antibodies and their isotype controls were added to the respective wells and incubated for 30 minutes followed by washing with permeabilisation buffer. The cells were resuspended in PBS/0.5% BSA and the expression of FoxP3 and CTLA-4 measured using flow cytometry.

2.7 Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) labelling of PBMC or Tconv cells.

The population of interest (either PBMC or $CD4^+CD25^-$ conventional 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. 1µl of 10M CFSE was added to 5ml of sterile PBS to give a 2µM solution. This was added at a 1:1 ratio to the cell suspension to give a final CFSE concentration of 1µM, and incubated for 10min at room temperature with periodic shaking. After 10mins, an equal volume of RPMI/10%HIFCS was added and left for further one minute. The cells were then washed once with PBS and twice with RPMI/10%HIFCS. The cells were then counted and resuspended in RPMI/10%HIFCS at the required concentration.

2.8 Functional assay for Treg

Regulatory T cells are capable of suppressing the proliferation and cytokine production by other immune cell types. The functional capacities of regulatory T cells were assayed by their ability to suppress the proliferation of Tconv cells. The isolated Treg were cultured with Tconv cells either in the presence or absence of antigen presenting cells (APC). For the assay in the presence of APCs, cells were stimulated with phytoheamaglutinin (PHA) which stimulates T cells in the culture. For functional assay in the absence of APCs, the cells were stimulated either with anti CD3 and anti CD28 antibodies or with anti CD3/CD2/CD28 coated Treg suppression inspector beads.

2.8.1 Suppression Assay in the presence of APC

The PBMC were labelled with CFSE as described earlier and cultured in 96 well round bottom plates at 2.5 x 10^4 cells per well along with either unlabelled $CD4^+CD25^-$ Tconv cells or $CD4^+CD25^+$ regulatory T cells at 1:1 CFSE labelled PBMC: unlabelled cells ratio (for sorted cells). The cells were stimulated with PHA

(4.5 ng/well) and cultured for 4 days. Different concentrations of PHA were tested to get the optimal proliferation of T cells and suppression by Treg (Fig: 2.4). The culture medium used was RPMI/10% HIFCS. After 4 days of culture the 96 well plate was centrifuged (300g, 4mins, 21°C) and 50-70µl 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 200µl PBS/0.5%BSA before being transferred to flow cytometry tubes containing 190µl PBS/0.5%BSA and 10µl (10000) counting beads. Flow cytometry was performed on the Dako Cyan ADP High Performance flow cytometer (Dako Colorado). When only a part of the sample is run, the absolute numbers of cells in the original sample can be calculated from the number of counting beads counted using the following equation:

Absolute number of total live cells = Number of proliferating cells counted x (total number of beads added/ total number of beads counted).

Undivided cells have the highest concentration of CFSE (and therefore highest signal) which 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 (i.e, of the highest signal).

2.8.2 Suppression Assay in the absence of APC

In this assay, instead of PBMC, the isolated $CD4^+CD25^-$ Tconv cells were labelled with CFSE and cultured in 96 well round bottom plates at 2.5 x 10^4 cells per well along with either unlabelled $CD4^+CD25^-$ Tconv cells or $CD4^+CD25^+$ regulatory T cells at 1:1 CFSE labelled Tconv: unlabelled cells ratio (for sorted cells) or a series of

doubling dilutions ranging from 1: 1 to 64:1 (for cells isolated by dynabeads). The cells were stimulated with equal number of anti CD3/CD2/CD28 coated Treg inspector beads (miltenyi biotech). After 4 days of culture the 96 well plate was centrifuged (300g, 4mins, 21°C) and 50-70µl 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 200µl PBS/0.5%BSA before being transferred to flow cytometry tubes containing 190µl PBS/0.5%BSA and 10µl counting beads. Flow cytometry was performed on the Dako Cyan ADP High Performance flow cytometer (Dako Colorado). The actual numbers of proliferated cells were calculated as described earlier

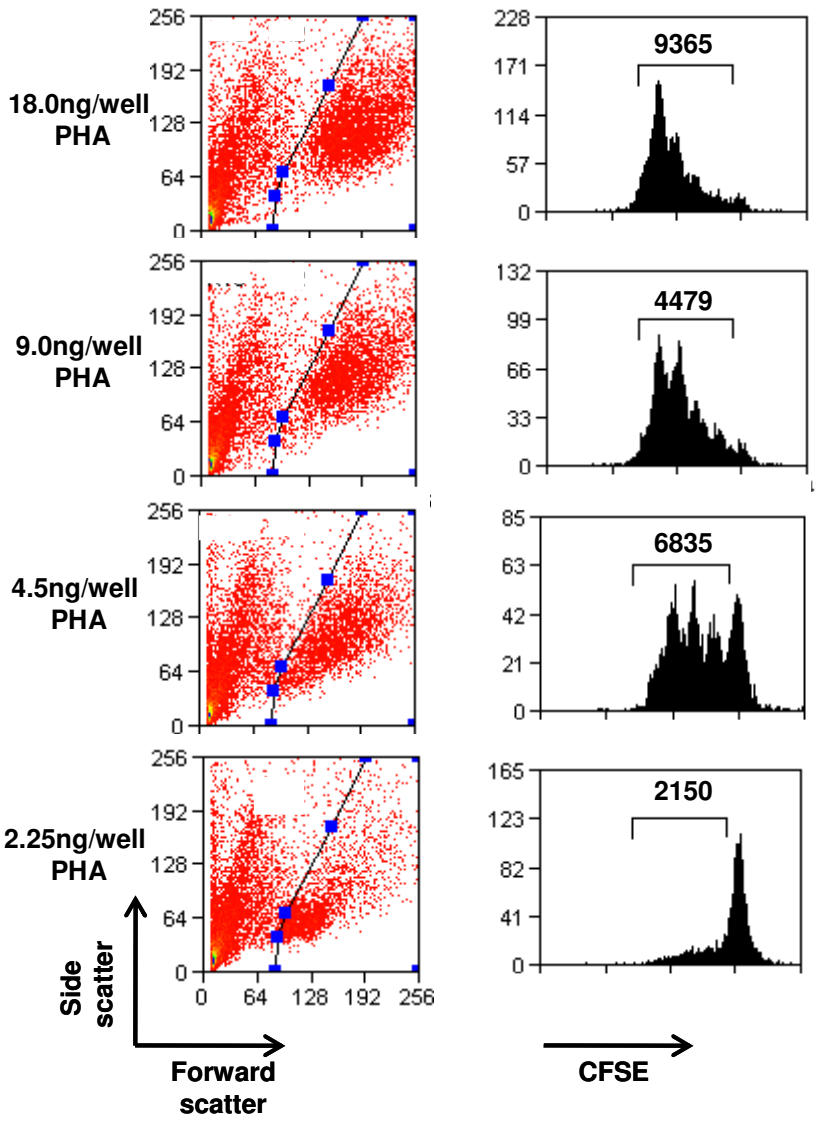


Fig: 2.4 Optimisation of PHA concentration for PBMC proliferation

PBMCs were labelled with CFSE and cultured with different concentrations of PHA such as (a) 18ng/well, (b) 9ng/well, (c) 4.5ng/well and (d) 2.25ng/well in a final volume of 200µl to find out the minimum concentration at which optimum proliferation of PBMC occur. Number of dividing CFSE labelled cells noted on the histogram. Optimum proliferation of cells with clear distinct CFSE peaks was observed at 4.5ng/well concentration of PHA.

2.8.3 Suppression assay in the presence of Aqueous humour (AqH)

Since the volume of individual samples of AqH is a limiting factor, these experiments were carried out in 72 well mini trays (from VWR International ltd) in a final volume of 20µl. Tconv cells were labelled with CFSE and cultured in 72 well mini trays at 1.0 x 10^4 cells per well with or without 10µl of individual uveitis or control AqH samples. The cells were stimulated with equal number of anti CD3/CD2/CD28 Treg suppression inspector beads (miltenyi biotech). The CFSE labelled Tconv cells were cultured with either unlabelled CD25- Tconv cells or $CD25^+$ regulatory T cells at 1:1 concentration. The cells were cultured in RPMI/ 10% HIFCS. At day 4, the cells were harvested and stained for 5 minutes with propidium iodide (PI - 4µg/ml final concentration) which acts as dead cell exclusion dye and washed twice with PBS/0.5% BSA. The cells were then resuspended in 200µl PBS/0.5%BSA before being transferred to flow cytometry tubes containing 199µl PBS/0.5%BSA and 1µl counting beads. 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 x (total number of beads added/ total number of beads counted).*

2.9 Studies on the effect of ocular microenvironment on FoxP3 expression

2.9.1 Effect of dexamethasone and inflammatory cytokines

$CD25^+$ Treg were isolated using dynabeads from a healthy control and cultured with recombinant serum cytokines (present in the uveitis AqH) at different concentrations. Cells were cultured with TNF-α (10ng/ml and 100ng/ml), IL-6 (20 ng/ml and 200 ng/ml), TGF-β2 (10ng/ml and 100ng/ml) and IL-1β (10ng/ml and 100ng/ml) and also with dexamethasone (topical glucocorticoid used to treat uveitis). The cells were cultured for 24 hours in serum free medium and the FoxP3 expression was analysed using flow cytometry.

2.9.2 Effect of uveitis AqH

CD25+ Treg isolated using dynabeads were cultured in 72 well micro well plates in 20µl volume with or without 10µl uveitis AqH or varying activity. The cells were cultured for 24 hours in serum free medium and the FoxP3 expression was analysed using flow cytometry.

2.10 Preparation of regulatory T cell line

CD25high Treg and CD25low Tconv cells were isolated from patient and control peripheral blood by sorting. The suppressive functions of Treg were determined on the day in the absence of APC as described in subsection 2.7.2. Treg cells were individually cultured in 48 well flat bottom plates with the addition of anti CD3/CD28 coated beads (Dynal T cell expander beads) at 3 beads: 1 cell ratio in culture medium containing RPMI/10%HIFCS/100 U/ml IL-2. The cells were cultured for 14 days. The medium was replaced every two days with fresh medium and the cells split between wells whenever necessary. On day 15, the suppressive capacity of these Treg cell lines were tested on the T cells isolated from a healthy control on the day in an APC free setting.

2.11 Treg depletion from very small number of cells

Regulatory T cells from the AqH of uveitis patients could not be isolated by traditional methods due to the very small sample volume and cell number. One possible way to analyse AqH Treg function is to deplete Treg from AqH using anti CD25 antibody coated beads and analyse the proliferative capacity of the residual Tconv cells.

Dynal sheep anti mouse IgG beads-M450 were first coated with mouse anti human CD25 antibody or its isotype control. For this, the beads were transferred to an eppendorf and mixed with equal volume of dynal isolation buffer and placed on DynaMag™ magnet for 3 minutes and supernatant discarded. 1µg of purified mouse anti human CD25 or isotype control was added to 25µl of the washed dynabeads and

incubated for ≥ 30 minutes at 2-8°C with gentle tilting and rotation after which the beads were washed on DynaMag™ magnet. After 2 similar washes, the dynabeads were resuspended in the same volume of buffer as the initial volume of dynabeads.

For optimal depletion of Treg, $5x10^3$ cells were incubated with different amounts of anti CD25 antibody coated beads for 30 minutes at 2-5 degrees under rolling and tilting. The beads bound cells were removed on a magnet and the remaining cells were cultured in 72 well mini trays for a different periods of time (24hr, 36 hr, and 48hr) along with click-iT EdU reagent and analysed using click-iT EdU assay (explained later).

To get maximum proliferation in the conical bottom mini tray, the plate was inverted and cells cultured as a hanging drop suspension in a moist chamber.

2.12 Click-iT™ EdU assay

Click-it EdU assay is an alternative to BrdU (Bromodeoxyuridine) for measuring cell proliferation or S-phase cells as it eliminates DNA denaturation and harsh permeabilisation steps required by BrdU assays, steps that damage sample morphology and integrity. EdU (5-ethynyl-2'-deoxyuridine) is a nucleoside analog to thymidine and is incorporated in DNA during active DNA synthesis. Detection is based on a click reaction, a copper catalyzed covalent reaction between an azide and an alkyne. In this application, the EdU contains the alkyne, while the Pacific Blue™ dye contains the azide. Standard flow cytometry methods are used for determining the percentage of cells in the population that are in S-phase.

The AqH cells were harvested after 48 hours and transferred to 96 well flexi plates and washed once with PBS/1% BSA. The cells were then incubated with 50µl of Click-iT™ fixative (which contains 4% para-formaldehyde in PBS) for 15 minutes at room temperature, protected from light followed by washing once with PBS/ 1% BSA. This was followed by incubation with 50µl of the 1X saponin-based permeabilisation and wash buffer for 30 minutes at room temperature, protected from light. The cells were then washed with 100µl of the 1X saponin-based permeabilisation and wash buffer, centrifuged (300g, 20°C, 8 minutes) and incubated for 30 minutes with 43.5µl of 1X Click-iT™ Reaction Buffer, 1 µl of copper sulphate (CuSO4), 0.5µl of fluorescent dye azide (here pacific blue azide) and 5µl of reaction buffer addictive at room temperature protected from light. After washing once with 100µl of the 1X saponin-based permeabilisation and wash buffer, the cells were incubated with 48.5µl of 1X saponin-based permeabilisation buffer, 1µl of ribonuclease- A and 0.5µl of Cell Cycle 488-red (7-AAD) antibody for 30 minutes at room temperature protected from light for detection of the DNA content and cell cycle phase. The cells were washed twice in 1X saponin-based permeabilisation buffer and resuspended in 200µl PBS/ 1%BSA before being transferred to flow cytometry tubes containing 199µl PBS/ 1%BSA and 1µl counting beads. Flow cytometry was performed on the Dako Cyan ADP High Performance flow cytometer (Dako Colorado). The fluorescent signal generated by the Pacific Blue™ azide was best detected with logarithmic amplification and that generated by the Cell Cycle stain (7AAD) with linear amplification.

2.13 AqH depletion/ mock depletion

Dynal sheep anti mouse IgG beads-M450 were first coated with mouse anti human CD25 antibody or its isotype control as described earlier (2.8). AqH from uveitis patients were spun down and the supernatant frozen. The cells were resuspended in 100ul dynal isolation buffer (PBS/ 0.1% BSA/ 2mM EDTA) and divided into two. 3µl of dynabeads coated with either anti CD25 antibody (depletion) or isotype control antibody (mock depletion) were added to each tube containing 50µl AqH cells and incubated for 30 minutes under rolling and tilting at 2-8°C. The tube was then placed in a magnet and the supernatant containing the unbound cells collected. The depleted and the mock depleted cells were spun down and resuspended in RPMI/10% HIFCS and cultured with 5x 10^3 anti CD3/CD2/CD28 coated Treg inspector beads and 2µM EdU at a final volume of 20µl in 72 well mini-tray. The mini- tray was then inverted to form a hanging drop suspension of cells and cultured in a moist chamber for 48 hours. After 48 hours the cells were harvested and the percentage of cells that has gone into S-phase of cell cycle is determined by Click-iT EdU detection assay.

3 REGULATORY T CELLS IN THE PERIPHERAL BLOOD OF CHRONIC NON INFECTIOUS UVEITIS PATIENTS

3.1 Introduction

Chronic uveitis can be differentiated from acute uveitis by its rate of progression and can be defined as active uveitis that persists longer than three months (Jabs *et al*., 2005; McCluskey *et al*., 2000). Chronic uveitis can be associated with other systemic conditions like Behcet's disease, sarcoidosis, juvenile chronic arthritis etc (McCluskey *et al*., 2000). It is also associated with high incidence of sight threatening complications like cataract, macular oedema, and glaucoma which may cause irreversible visual loss (McCluskey *et al*., 2000).

Phenotypic and/or functional impairment of Treg has been indicated in acute and chronic autoimmune diseases. The role of Treg in the control of inflammatory disease has generated considerable interest recently in uveitis studies, as well as in reports describing their role in systemic autoimmune conditions. Association of Treg with uveitis has been studied mostly in animal models of uveitis. Takeuchi *et al.* reported that thymectomised B6A mice, treated with anti CD25 antibody to deplete CD25+ Treg cells, developed spontaneous autoimmune uveitis (Takeuchi *et al*., 2004). In addition, supplementation of regulatory T cells has been shown to suppress EAU (Keino *et al*., 2007). Adoptive transfer of Treg to IRBP immunized mice ameliorated EAU even in the efferent phase (Keino *et al*., 2007). Grajewski *et al.* showed later that

even Treg of a different specificity could inhibit development of IRBP specific uveitogenic conventional T cells in a bystander fashion (Grajewski *et al*., 2006). In particular, it is intriguing that in a biphasic rat experimental uveitis model, Treg control the recurrence of the disease (Ke *et al*., 2008).

To date there has been very few reports on the role of Treg in human uveitis and almost none on the role of Treg in the chronic phase of the disease. Vogt-Koyanagi-Harada (VKH) syndrome is a multisystem inflammatory disorder with associated bilateral pan uveitis and shows a chronic intraocular inflammation with recurrent episodes. Decreased frequency and function of Treg has been reported to be associated with active uveitis in VKH syndrome (Chen *et al*., 2008). Recent studies in human uveitis have reported a decreased frequency of Treg in the peripheral blood of active uveitis patients compared to inactive patients (Yeh *et al*., 2009). However, none of these studies have made a clear division of uveitis as acute and chronic based on SUN classification criteria (Jabs *et al*., 2005) and haven't excluded the effect of associated systemic diseases on the frequency, phenotype and function of Treg.

One of the hypotheses of this thesis was that there could be quantitative and/or qualitative defect in the Treg from chronic uveitis patients. The aims of the series of experiments described in this chapter were (1) to identify and characterise Treg cells from peripheral blood of chronic uveitis patients, (2) to isolate and analyse their functional activity *in vitro* and (3) to determine whether these Treg maintain their phenotypic and functional characteristics in long term culture.

3.2 Identification of regulatory T cells

Regulatory T cells in the peripheral blood were identified by isolating PBMC and staining them with Treg specific surface and intracellular markers as described in chapter 2. Treg were defined as CD4+CD25highCD127low T cells whereas the conventional T cells (Tconv) were identified as CD4+CD25lowCD127high T cells (Fig: 3.1A). The vast majority of Treg were FoxP3+ and CD45RO+ (memory), as previously reported (Liu *et al*., 2006), compared to the conventional T cell population that was largely FoxP3- and consisted of both CD45RO+ and CD45RO- cells.

Consistent with the published literature (Fontenot *et al*., 2003; Wing *et al*., 2008), Treg cells expressed increased levels of intracellular FoxP3 as well as CTLA-4 compared to Tconv cells (Fig: 3.1B). Unlike FoxP3 and CTLA-4, CD39 was expressed by only a subset of Treg (Fig: 3.1B). Other Treg markers such as surface CTLA-4, CD73 and GITR were not expressed exclusively by Treg and were not much different from conventional T cells (Fig: 3.1B) by flow cytometric analysis. Hence these markers were not analysed as Treg markers further in this thesis.

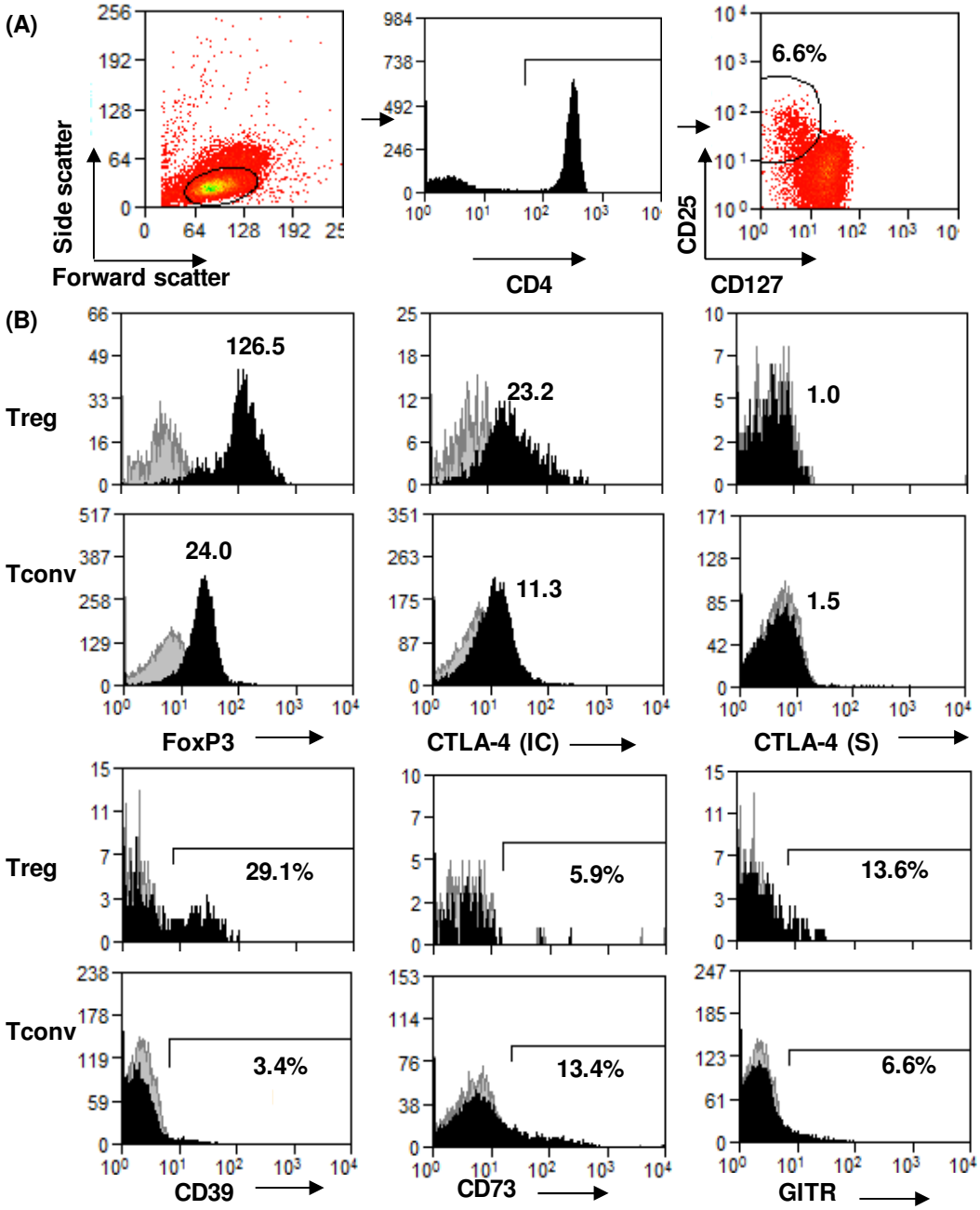


Fig: 3.1 Phenotypic analysis of Treg from normal peripheral blood

(A) Staining of PBMC with Treg markers. Lymphocytes gated on CD4+ cells to determine CD25highCD127low Treg and CD25lowCD127high Tconv. Frequency of Treg marked on the histogram. (B) Flow cytometric analysis of FoxP3, intracellular (IC) CTLA-4 and surface (S) CTLA-4 and surface CD39, CD73 and GITR from Treg and Tconv (black curves). Gray curves represent isotype control. MFI (median fluorescent intensity) or percentage of positive cells marked on histograms. Representative of n=2 assays.

3.3 Phenotypic analysis of Treg from chronic uveitis patients

Quantitative and qualitative defects in Treg have been attributed to the pathology of various autoimmune and inflammatory diseases. To analyze whether similar defects occur in uveitis patients, PBMC were isolated from the peripheral blood of chronic anterior and pan uveitis patients (see Table: 3.1 and Table: 3.2) as well as healthy controls (Table 3.3) and stained for Treg specific markers. Several patients in our initial cohort demonstrated evidence of systemic autoimmune diseases including sarcoidosis, multiple sclerosis, ankylosing spondylosis, psoriasis and Vogt-Koyanagi-Harada syndrome. Each of these conditions has been associated with abnormal populations of T-regulatory cells (Chen *et al*., 2008; Huan *et al*., 2005; Feger *et al*., 2007; Sugiyama *et al*., 2005). Hence to avoid the effect of any associated systemic inflammation on Treg phenotype, only idiopathic chronic uveitis patients with no known associated systemic diseases were analysed in this study. As systemic glucocorticoid treatment has been shown to affect the phenotype and function of Treg (Karagiannidis *et al*., 2004), those patients taking any kind of systemic immunosuppressants including corticosteroids were also excluded from the study.

Of the 16 idiopathic pan uveitis patients, 10 patients were undergoing topical glucocorticoid treatment whereas the other 6 were not on any kind of steroid treatments. In the idiopathic anterior uveitis group, 14 patients were on topical steroids and 4 not on treatment. The clinical details of these patients are summarized in Table: 3.1 and Table: 3.2. 15 age and sex matched healthy controls were recruited for this study, the details of whom are summarized in Table: 3.3.

Patient no: / Sex	Laterality	AqH cells	First/ recurrent episode	Age	Treatment
1/M	U	0	Recurrent	40.7	Dex 0.1%, Cyclo
2/F	B	2	first episode	41.8	Dex 0.1%
3/M	U	2	first episode	45.9	None
4/F	B	0	first episode	76.4	None
5/F	B	0	Recurrent	86.8	Dex 0.1%
6/M	B	1	Recurrent	22.8	Predforte
7/F	B	0	Recurrent	39.3	Predforte
8/F	B	0	Recurrent	55.6	Dipyramadole
9/M	B	0	Recurrent	26.1	Cosopt , Predforte
10/M	B	0	Recurrent	64.9	Prednisolone
11/M	B	0	Recurrent	67.8	Dex 0.1%
12/F	B	0	Recurrent	46.3	None
13/F	B	0	Recurrent	46.9	Dex 0.1%
14/F	B	1	Recurrent	51.2	Maxidex, Cyclo
15/F	B	1	Recurrent	59.9	Dex 0.1%
16/F	U	2	Recurrent	51.2	Vexol
17/F	B	1	Recurrent	59.9	None
18/M	B	0	Recurrent	25.7	Lotemax, Timolol

Table: 3.1 Baseline characteristics and Clinical features of chronic idiopathic anterior uveitis samples analyzed by flow cytometry for phenotypic analysis of Treg

Clinical details of the patients were recorded at the time of sampling. Anterior chamber cellular activity was graded as per Standardization of Uveitis Nomenclature 2005 classification criteria (Jabs *et al*., 2005) (B- bilateral, U- unilateral, Dex- dexamethasone, Cyclo- cyclopentolate)

Patient no: / Sex	Laterality	AqH cells	First/ recurrent episode	Age	Treatment
1/F	B	0	Recurrent	79.5	Pred 1%
2/F	B	0	Recurrent	60.8	maxidex, cosopt, cyclo
3/M	B	0	Recurrent	60.7	Azopt
4/F	B	0	Recurrent	68.2	Dex 0.1%
5/F	B	1	Recurrent	52.7	Dex 0.1%, cyclo 1%
6/F	B	2	Recurrent	42.7	None
7/F	B	0	Recurrent	67.7	Maxidex
8/F	B	1	Recurrent	59.7	Dex 0.1%
9/M	B	0	Recurrent	30.1	None
10/F	B	0	Recurrent	72.3	None
11/M	B	0.5	Recurrent	39.3	Vexol
12/F	B	1	Recurrent	53.2	Maxidex
13/M	B	0	Recurrent	32.0	None
14/F	B	0	Recurrent	29.8	None
15/F	B	3	Recurrent	37.9	Predforte
16/F	B	1	Recurrent	60.0	Cosopt

Table: 3.2 Baseline characteristics and Clinical features of chronic idiopathic pan uveitis samples analyzed by flow cytometry for phenotypic analysis of Treg

Clinical details of the patients were recorded at the time of sampling. Anterior chamber cellular activity was graded as per Standardization of Uveitis Nomenclature 2005 classification criteria (Jabs *et al*., 2005) (B- bilateral, U- unilateral, Pred- prednisolone, Dex- dexamethasone, Cyclo- cyclopentolate)

Control no:	Sex	Age
1	F	22.0
2	M	24.0
3	F	24.0
4	M	25.0
5	M	25.0
6	M	26.4
7	M	26.9
8	F	45.0
9	M	51.0
10	F	53.0
11	M	55.0
12	F	64.9
13	F	75.2
14	M	87.8
15	F	90.2

Table: 3.3 Baseline characteristics of healthy control (HC) samples analyzed by flow cytometry for phenotypic analysis of Treg

3.3.1 No difference in the frequency of Treg in chronic non infectious uveitis patients

CD4+ Treg from chronic idiopathic uveitis patients were identified and their frequencies determined to analyze whether there were any quantitative defects in the Treg cells from these subjects (Fig: 3.2A). Using a strict gating procedure to identify Treg, no difference was found in the frequency of CD4+CD25highCD127low cells between healthy control individuals (median±SD: 6.3±1.0) and patients with chronic anterior (median±SD: 6.7±1.7) or Pan uveitis (median±SD: 7.3±2.6) (Fig: 3.2B). An increase in the frequency of Treg with ageing has been reported (Gregg *et al*., 2005), but in this study there was no correlation between Treg frequency and age (Fig: 3.2C). An increased number of CD4+ Treg has been reported in patients with SLE and asthma following systemic treatment with corticosteroid (Karagiannidis *et al*., 2004; Suarez *et al*., 2006). The frequencies of Treg were also not different between patients undergoing topical glucocorticoid therapy and those who were not on treatment (Fig: 3.2D). The frequency of Treg was not affected by the differences in the sex or disease activity of the patients.

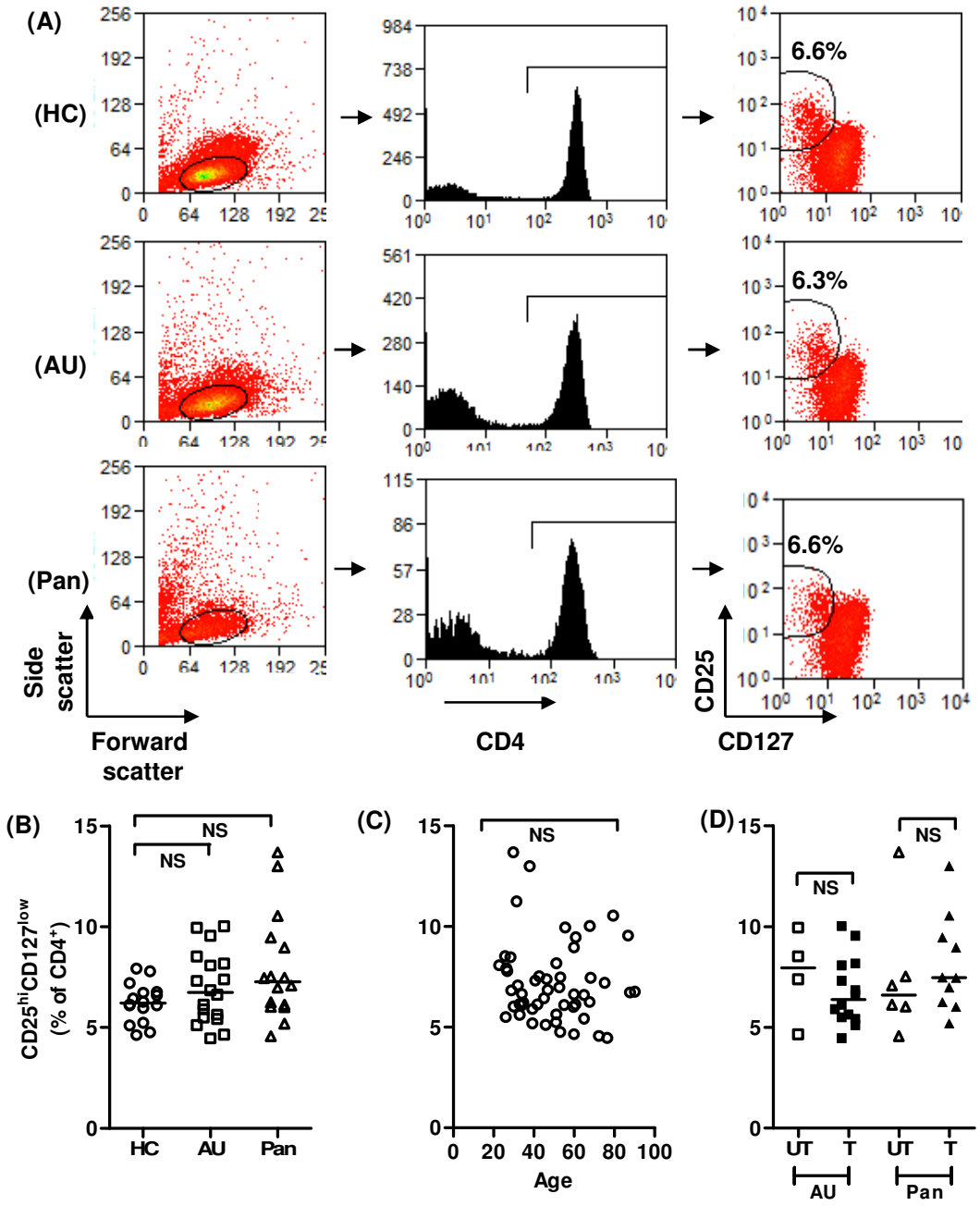


Fig: 3.2 Similar frequencies of Treg from chronic uveitis patients

(A) Staining of PBMC from healthy controls (HC), anterior uveitis (AU) and pan uveitis patients. PBMC gated on lymphocytes and then on CD4+ cells to determine CD25highCD127low Treg (B) Similar frequency of Treg from AU and pan patients compared to HC. (C) Frequency of Treg did not correlate with age. (D) Topical glucocorticoid treatment had no effect on the frequency of Treg in chronic uveitis patients. Horizontal bars represent median value. (Statistical tests used- Kruskal Wallis test and correlation, NS-not significant, UT-untreated, T-treated)

3.3.2 No difference in the FoxP3 expression of Treg from chronic uveitis patients

FoxP3 has been shown to be required for the differentiation, maintenance and function of CD4+ Treg. Alterations in the expression of FoxP3 have been associated with functional alterations in Treg cells. FoxP3 expressions of Treg (Fig: 3.3A) as well as Tconv cells (Fig: 3.3B) from the peripheral blood of chronic uveitis patients and healthy controls were analyzed. The population of naïve (CD45RO-) Treg cells was too small to allow precise analysis. No difference in the FoxP3 expression of memory (CD45RO+) Treg (Fig: 3.3C) from chronic idiopathic anterior uveitis (median±SD: 117.6±39.8) and pan uveitis patients (median±SD: 126.5±58.8) was observed compared to healthy controls (median±SD: 113.5±28.5). Interestingly some pan uveitis patients expressed increased levels of FoxP3. However this did not relate to their age, sex or disease activity.

In asthma patients, systemic as well as inhaled glucocorticoids have been shown to increase the FoxP3 expression of Treg (Karagiannidis *et al*., 2004). Interestingly in our patient group, chronic anterior uveitis patients who were receiving topical glucocorticoid treatment expressed significantly increased levels of FoxP3 as compared to untreated patients (Fig: 3.3E). The FoxP3 expression did not differ with the age, sex, or disease activity of the patients.

As expected the Tconv cell population showed no significant expression of FoxP3 and was not different between the patient groups (Fig: 3.3E). No significant differences were found in the FoxP3 expression of naive conventional T cell population.

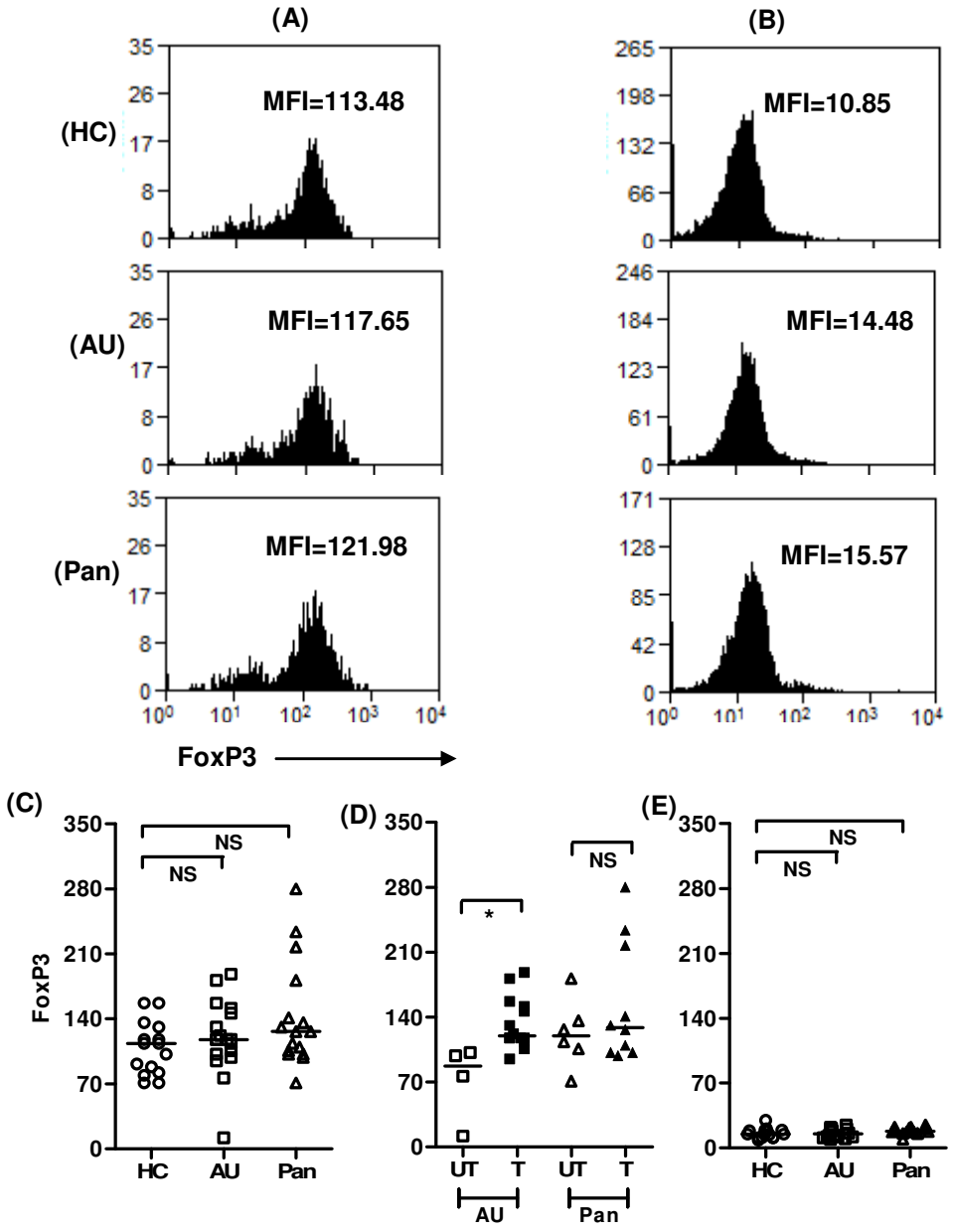


Fig: 3.3 No difference in the FoxP3 expression of Treg from chronic uveitis

FoxP3 expression of CD45RO+ (A) Treg and (B) Tconv from healthy controls (HC), anterior uveitis (AU) and pan uveitis patients. MFI (median fluorescence intensity) of FoxP3 for representative samples noted on histograms. (C) No significant difference in the FoxP3 expression of Treg between AU, pan and HC. (D) Higher levels of FoxP3 expression in Treg from AU patients undergoing topical glucocorticoid therapy (T) compared to untreated patients (UT). (E) Tconv from chronic uveitis patients showed no difference in FoxP3 expression as compared to healthy controls. Horizontal bars represent median value. (Statistical tests used- Kruskal Wallis test, NS- not significant, *-$p<0.05$).

3.3.3 No difference in the expression of CTLA-4 of Treg

Expression of intracellular CTLA-4 by Treg (Fig: 3.4A) as well as Tconv cells (Fig: 3.4B) were analyzed in chronic uveitis patients and healthy controls. Neither pan (median±SD: 39.8±21.3) nor anterior (median±SD: 42.0±10.3) uveitis patients showed significant differences in CTLA-4 expression of Treg population compared to healthy controls (median±SD: 39.8±11.5) (Fig: 3.4C). Similar to FoxP3, some pan uveitis patients expressed increased levels of CTLA-4. However this was not related to any difference in their age, sex, disease activity or treatment status. Topical glucocorticoid therapy did not have any effect on the CTLA-4 expression of Treg from either anterior or pan uveitis patients (Fig: 3.4D).

Interestingly the conventional T cells from pan uveitis patients (median±SD: 24.0±5.7), not anterior uveitis (median±SD: 18.9±5.9) expressed significantly higher CTLA-4 compared to healthy controls (median±SD: 16.7±5.0) (Fig: 3.4E).

3.3.4 No difference in the CD39 expression of Treg cells

Unlike CTLA-4 and FoxP3 which are expressed by all Treg, CD39 shows a biphasic expression within the Treg population and varies considerably between individuals (Fig: 3.5A). Conventional T cells showed no significant expression of CD39 (Fig: 3.5B). Although changes in CD39 expression have been indicated in other inflammatory diseases (Fletcher *et al*., 2009), there was no difference in the frequency of CD39+ cells in the Treg (Fig:3.5C) and was not affected by glucocorticoid treatment (Fig: 3.5D). There was no difference in the frequency of CD39+ Tconv cells (Fig: 3.5E) from chronic anterior or pan uveitis patients.

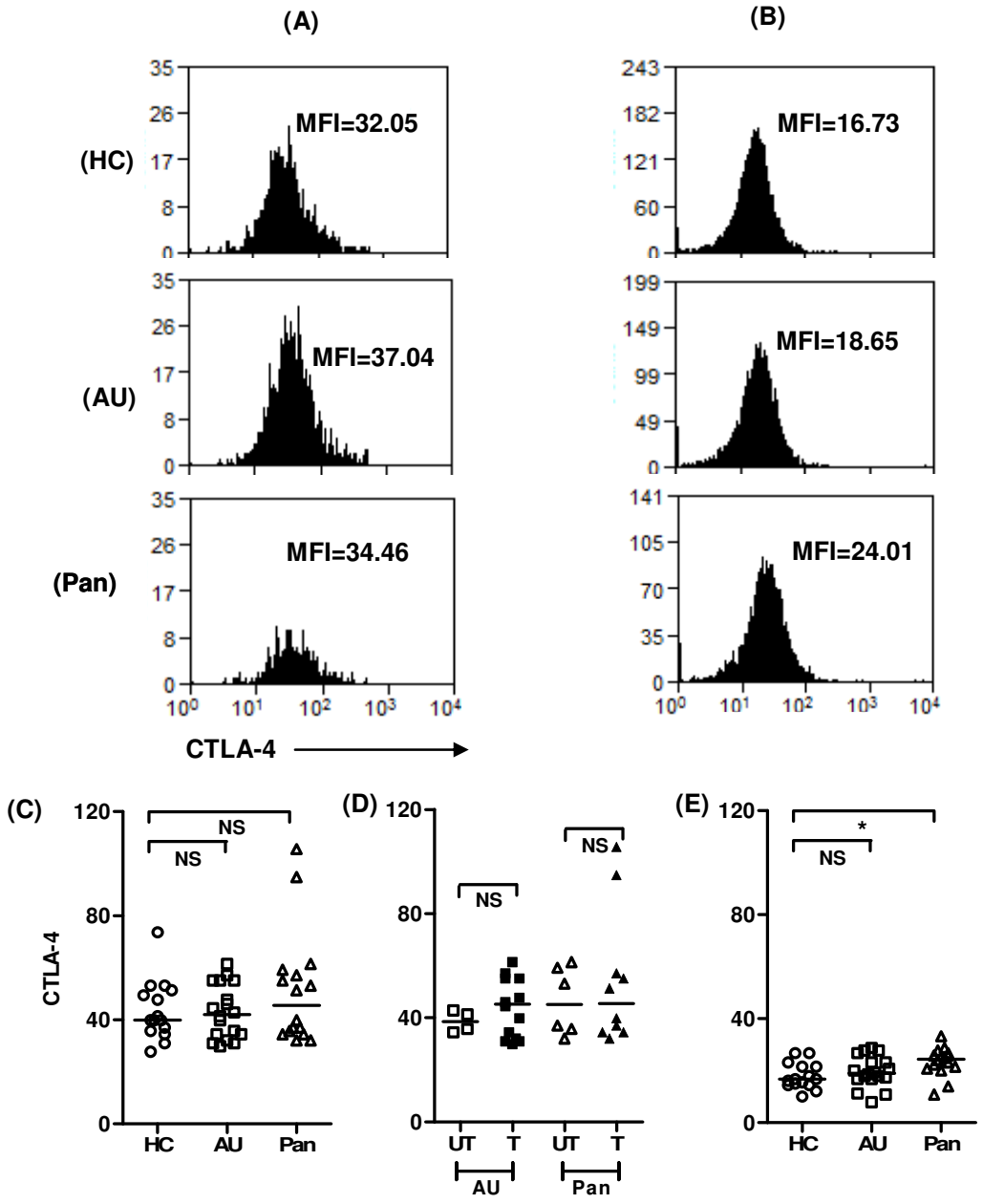


Fig: 3.4 No difference in CTLA-4 expression of Treg from chronic uveitis

CTLA-4 expression of CD45RO+ (A) Treg and (B) Tconv cells from healthy control (HC), anterior uveitis (AU) and pan uveitis patients. MFI for representative samples marked on histograms (C) No difference in CTLA-4 expression of Treg between AU, pan and HC. (D) Topical GC therapy did not affect the CTLA-4 expression of Treg in any patient groups. (E) Tconv cells from chronic pan uveitis patients showed increased CTLA-4 expression as compared to healthy controls. Horizontal bars represent median value. (Statistical tests used- Kruskal Wallis test, NS- not significant, **-p<0.01)

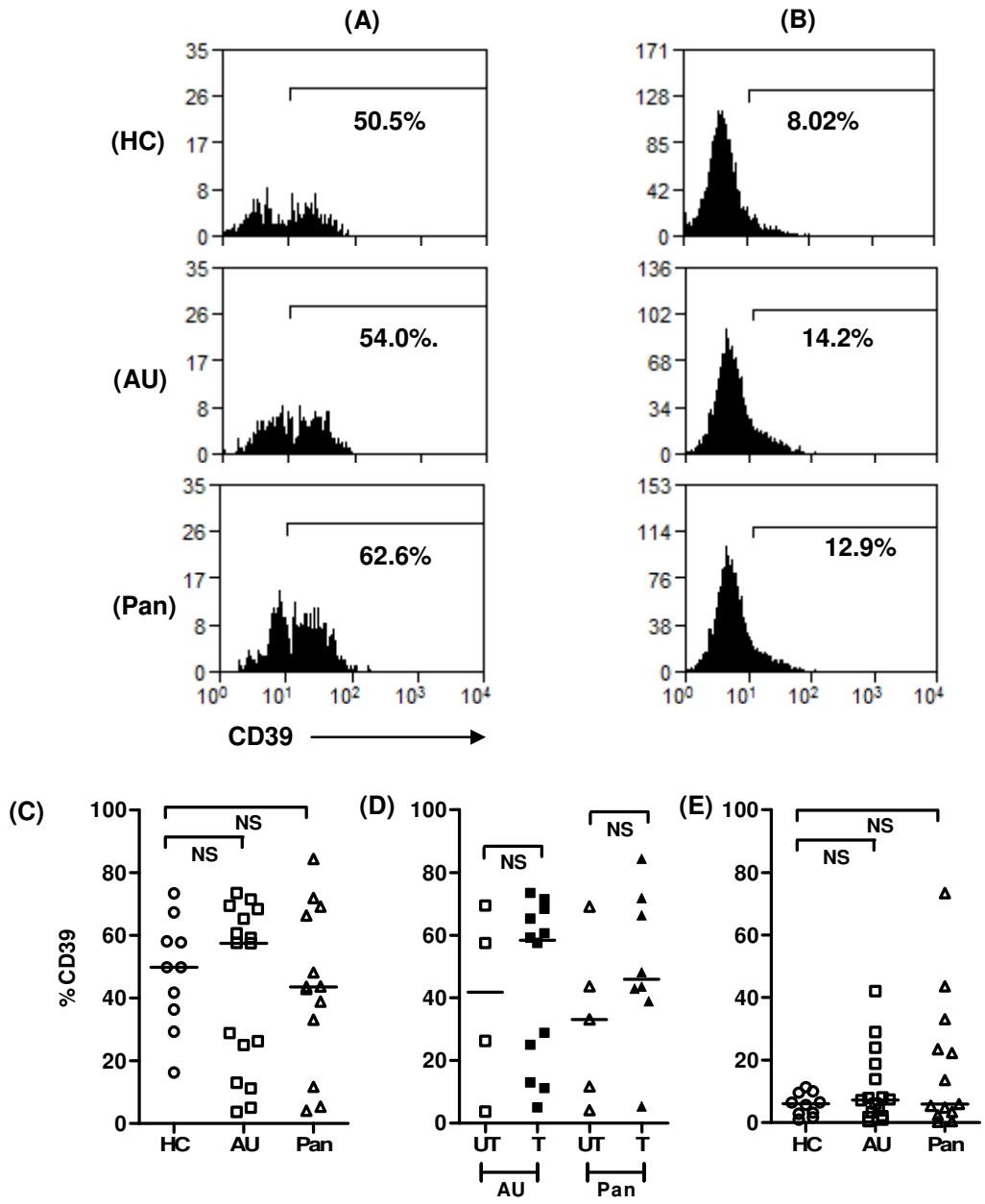


Fig: 3.5 No difference in CD39 expression of Treg from chronic uveitis patients

CD39 expression of CD45RO+ (A) Treg and (B) Tconv cells from healthy control (HC), anterior uveitis (AU) and pan uveitis patients. Percentage of CD39+ Treg for the representative samples marked on the histograms. (C) No difference in the frequency of CD39+Treg between AU, pan and HC (D) No difference in the frequency of CD39+Treg between patients undergoing topical GC therapy (T) and untreated patients (UT). (E) No difference CD39 expression of Tconv cells between AU, pan and HC. Horizontal bars represent median value. (Statistical tests used- Kruskal Wallis test, NS- not significant)

3.4 Functional analysis of Treg from chronic patients

Defects in the function of regulatory T cells have been implicated in various autoimmune and inflammatory diseases. The functional capacity of Treg from chronic uveitis patients were determined based on their ability to suppress the proliferation of CFSE labeled Tconv cells either in the presence or in the absence of antigen presenting cells (APC). The proliferation of stimulated CFSE-labelled Tconv cells (responding cells) was determined in the presence of highly purified unlabelled CD4+CD25highCD127low Treg or CD4+CD25lowCD127high Tconv cells. The addition of Treg, but not Tconv cells, suppressed the proliferation of responding Tconv cells.

3.4.1 No Defect in the suppressive activity of Treg from uveitis patients in the presence of APC

In this assay, the proliferation of PHA-stimulated CFSE-labelled PBMC was determined in the presence of CD4+ Treg or Tconv cells, isolated from the peripheral blood of healthy controls, and anterior or pan uveitis patients, by high-speed cell sorting. The purity of the sorted Treg populations was always >95% as shown in Fig: 3.6. The proliferation of CFSE labeled cells were analyzed by flow cytometry following a 4-day culture. (Fig: 3.7A). The use of counting beads during the flow cytometric analysis allowed us to determine the exact number of proliferated cells (Fig: 3.7B). The anergic state of the Treg appeared to be maintained in patients with uveitis, as shown by the relatively small unlabelled (CFSE-negative) peak when CD25high Treg were added, as compared to that of the large peak of CD25low Tconv cells (Fig:3.7A). This is also evident from the low percentage of Treg survived

following 4 days in culture from anterior uveitis patients (median % survival ±SD: 25.7±15.4), pan uveitis patients (median % survival ±SD: 13.9±18.9) and healthy controls (median % survival ±SD: 47.0±39.6).

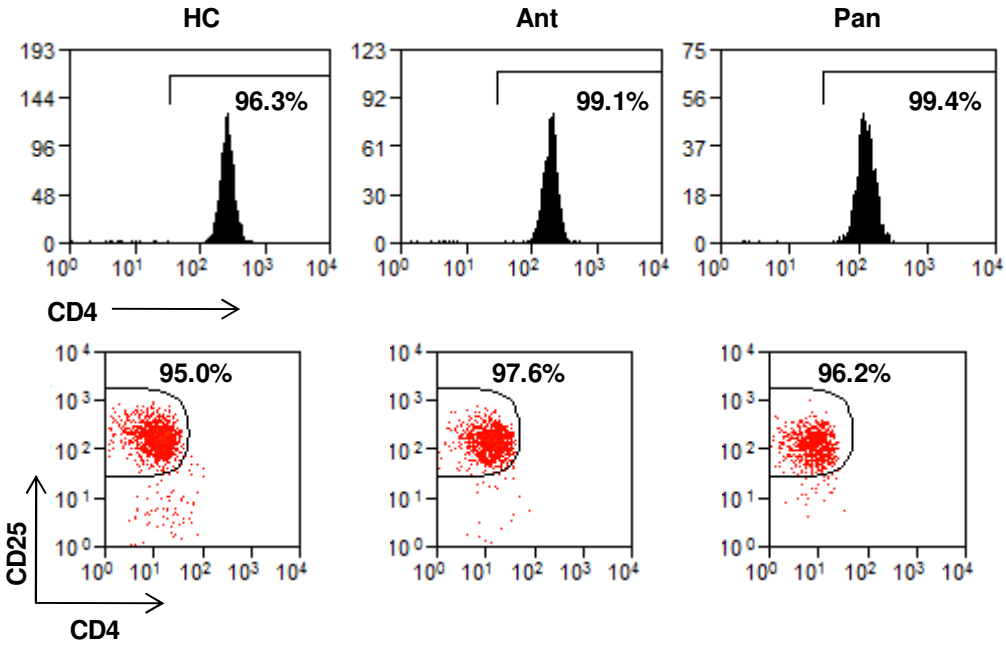


Fig: 3.6 Purities of Treg isolated using cell sorting for suppression assay in the presence of APC

Sorted Treg cells from healthy controls (HC), anterior uveitis (Ant) and pan uveitis (pan) patients, were stained with anti CD4, CD25 and CD127 antibodies to analyse the purity of CD4+CD25highCD127low Treg cells. Purity of isolated cells represented as percentage of CD4+ T cells.

Of the 7 chronic anterior uveitis patients, 5 were on topical glucocorticoid treatment and 2 patients were not on any kind of treatment. Of the 6 pan uveitis patients, 3 were on topical treatment and 3 were not on treatment. Patients taking any kind of systemic immunosuppressants including corticosteroids were excluded from this study as systemic glucocorticoid therapy has been shown to affect Treg functions and restored the impaired suppressive capacity of Treg in MS patients (Xu *et al.*, 2009). The

clinical features of patients used in this functional assay are summarized in Table: 3.4 and the healthy controls used in this assay are summarised in Table: 3.5.

Patient no: / Sex	Uveitis	Laterality	AqH cells	First/ recurrent episode	Age	Treatment
1/F	A	U	0	Recurrent	32.3	maxidex
2/M	A	U	0	Recurrent	40.7	Dex 0.1%, cyclo 1%
3/F	A	B	2	First episode	41.8	Dex 0.1%
4/M	A	B	0	Recurrent	45.9	None
5/M	A	B	0	Recurrent	22.5	None
6/M	A	B	1	Recurrent	22.8	Predforte
7/F	A	B	0	Recurrent	39.3	Predforte
8/F	P	B	0	Recurrent	53.6	Dex 0.1%
9/F	P	B	0	Recurrent	60.8	maxidex, cosopt, cyclo
10/M	P	B	0	Recurrent	60.7	Azopt
11/F	P	B	0	Recurrent	68.2	Dex 0.1%
12/F	P	B	1	Recurrent	52.7	Dex 0.1%, carteolol
13/F	P	B	2	Recurrent	42.7	None

Table: 3.4 Clinical features of patients samples used in Treg functional assay in the presence of APC (using sorted cells)

Clinical details of the patients were recorded at the time of sampling. (A-anterior uveitis, P-pan uveitis, B- bilateral, U- unilateral, Pred- prednisolone, Dex- dexamethasone, Cyclo- cyclopentolate).

Control No:	Sex	Age
1	M	34.0
2	F	28.9
3	M	32.9

4	M	34.8
5	F	53.0
6	M	51.0
7	F	45.0

Table: 3.5 Features of healthy control samples used in Treg functional assay in the presence of APC (using sorted cells)

As shown in Fig: 3.7, the suppressive capacities of Treg isolated from anterior (median±SD: 39.5±15.9) and pan (median±SD: 12.8±27.0) uveitis patient were not significantly different from that of healthy controls (median±SD: 50.0±26.3). As majority of the patients were on topical steroid treatment, it was not possible to analyze the effect of topical glucocorticoid treatment on the suppressive function of peripheral Treg. Interestingly some of the pan uveitis patients showed a clearly diminished suppressive capacity, but failed to reach statistical significance. As Treg from pan uveitis patients expressed the least survival rate in this assay (median % survival ±SD: 13.9±18.9), it is not clear whether the reduced suppressive function seen in cultures of pan uveitis Treg were a reflection of their reduced survival. Even the percentage proliferation rate of CFSE labeled Tconv cells from pan uveitis patients (median % proliferation± SD: 22.6±25.6) were lower compared to healthy controls (median % proliferation± SD: 83.9±45.2) and anterior uveitis patients (median % proliferation± SD: 32.8±29.6).

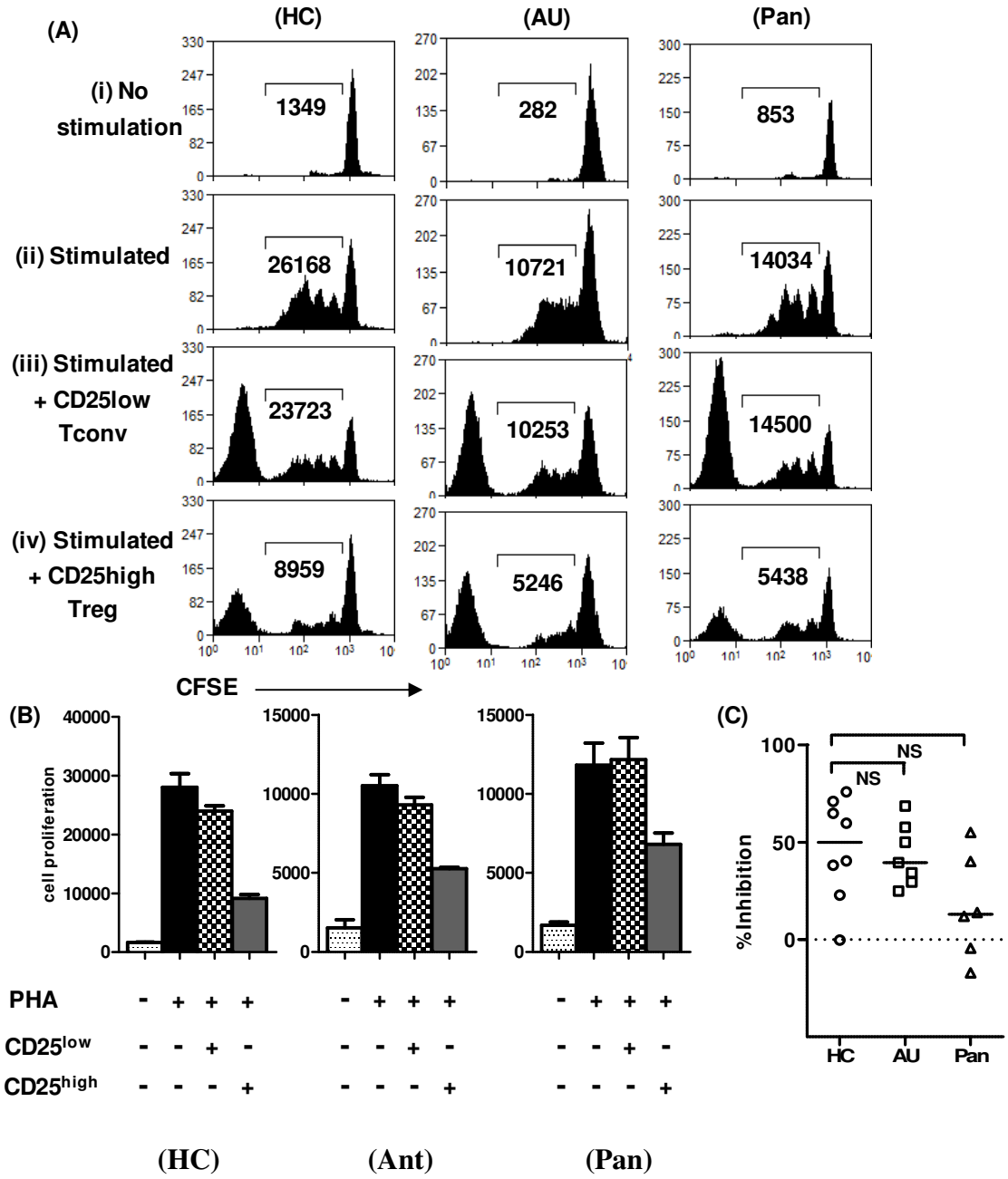


Fig: 3.7 Functional assay for Treg in the presence of APC

(A) FACS plot showing the proliferation of CFSE labeled PBMC with/without PHA in the presence of Tconv cells or Treg from healthy controls (HC), anterior uveitis (AU) and pan uveitis (pan) patients. Number of proliferating cells for the representative individuals marked on the histogram. (B) Graphical representation of proliferation of autologous Tconv cells from HC, AU and pan uveitis patients in the presence or absence of Treg/ Tconv cells. Results are expressed as mean ± SD. (C) No significant difference in the suppressive capacity of Treg from chronic anterior or pan uveitis patients compared to healthy controls. Horizontal bars represent median values. (Statistical test used- Kruskal Wallis test, NS- not significant)

T cell proliferation has traditionally been measured by their uptake of [^{3}H]-thymidine following antigenic or polyclonal stimulation *in vitro*. The CFSE based assay used here is also similar to this traditional method but also enables accurate determination of percentage and actual number of proliferating cells with the added advantage of not having to use radioactive materials. In thymidine incorporation assays, cells are stimulated over a period of 3 days with [^{3}H]-thymidine pulse being added for the final few hours. In CFSE assays however, the cells are labelled initially and the cell division is represented by the progressive halving of CFSE intensity which can be measured directly by flow cytometry after 4 days in culture.

Just before running the cells through the cytometer, 10,000 counting beads were added to each tube. Since the concentration of beads is known, the absolute count of positive cells could be obtained by relating the number of cells counted to the total number of fluorescent bead events. This allowed us to calculate the absolute number of cells in the sample only after running a part of the samples (approximately 25,000 cells). The absolute number of proliferating CFSE labelled cells (CFSE positive cells excluding the highest CFSE peak which represents the initially added CFSE labelled cells) is marked in Fig: 3.7A. The percentage inhibition was calculated based in the absolute number of cells using the following equation:

% inhibition= [(No: of proliferating cells in *CFSE PBMC+PHA* – No: of proliferating cells in *CFSE PBMC+Treg*) / No: of proliferating cells in *CFSE PBMC+PHA*] X 100

In a classic suppression assay however, the percentage inhibition is calculated based on the percentage of dividing cells rather than the absolute number of cells. Similar

analysis carried out with CFSE based assay also gave the same final results and validated the above results. Here the percentage of CFSE labelled cells that are proliferating were analysed for each sample and the percentage inhibition was calculated using the following equation.

% inhibition= [(% of proliferating cells in *CFSE PBMC+PHA* – % of proliferating cells in *CFSE PBMC+Treg*) / % of proliferating cells in *CFSE PBMC+PHA*] X 100

No significant difference was observed in the percentage inhibition between Treg from anterior and pan uveitis patients compared to healthy controls (Fig: 3.8). Thus calculating percentage inhibition using classic method also gave similar results validating my results based on absolute number fo cells.

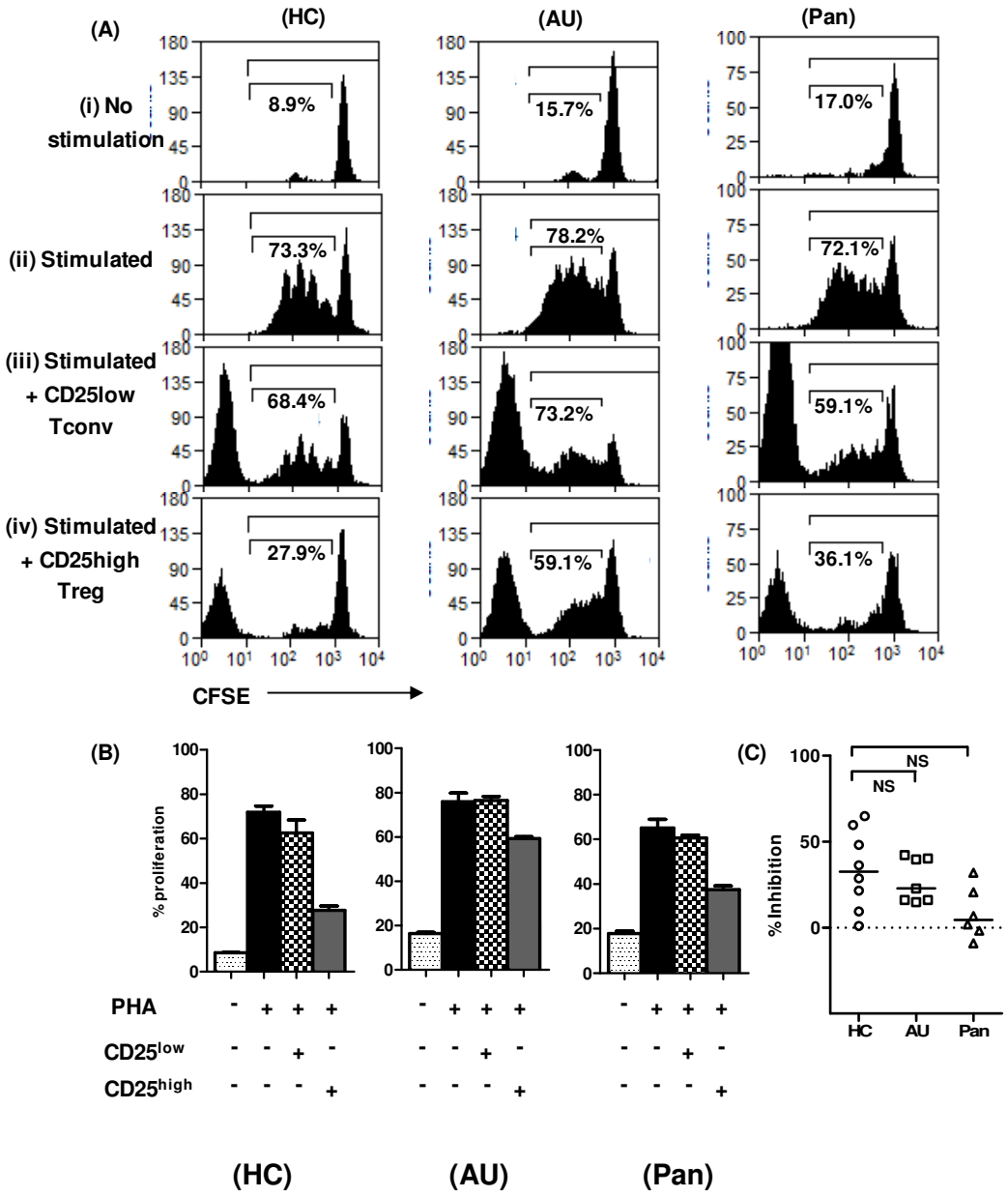


Fig: 3.8 Classic method of CFSE analysis using percentage of dividing cells

(A) FACS plot showing the proliferation of CFSE labeled PBMC with/without PHA in the presence of Tconv cells or Treg from healthy controls (HC), anterior uveitis (AU) and pan uveitis (pan) patients. Percentages of proliferating cells for the representative individuals are marked on the histogram. (B) Graphical representation of % proliferation of autologous Tconv cells from HC, AU and pan uveitis patients in the presence or absence of Treg/ Tconv cells. Results are expressed as mean ± SD. (C) No significant difference in the suppressive capacity of Treg from chronic anterior or pan uveitis patients compared to healthy controls. Horizontal bars represent median values. (Statistical test used- Kruskal Wallis test, NS- not significant)

3.4.2 No resistance of conventional T cells to Treg mediated suppression

Although the above data suggests that Treg from some of the chronic pan uveitis patients tend to have diminished suppressive capacities, it is also possible that the responding Tconv cells from these patients are more resistant to regulation, as has been recently shown for Type 1 diabetes (Lawson *et al*., 2008) and SLE (Vargas-Rojas *et al*., 2008). To assess these distinct possibilities, Treg functional assays were performed where the Treg and Tconv cells could be isolated from different donors.

Unlike the suppression assay described above, where antigen presenting cells were present in the culture system, this assay used only purified T cells stimulated with anti CD2/CD3/CD28 coated suppression inspector beads, to avoid any effect of other cells in the system as well as any adverse effect by the use of PHA for stimulation (Duarte *et al*., 2002; Li *et al*., 1994).

Treg and Tconv cells were isolated by cell sorting. The purities of isolated cells were always >95% as shown in Fig: 3.9. Tconv cells were then CFSE labeled and cultured with or without unlabelled Treg or Tconv cells in the presence of polyclonal stimulation using Treg suppression inspector beads for 4 days (Fig:3.10A) and the number of proliferated CFSE labeled cells were determined by flow cytometry (Fig: 3.10B).

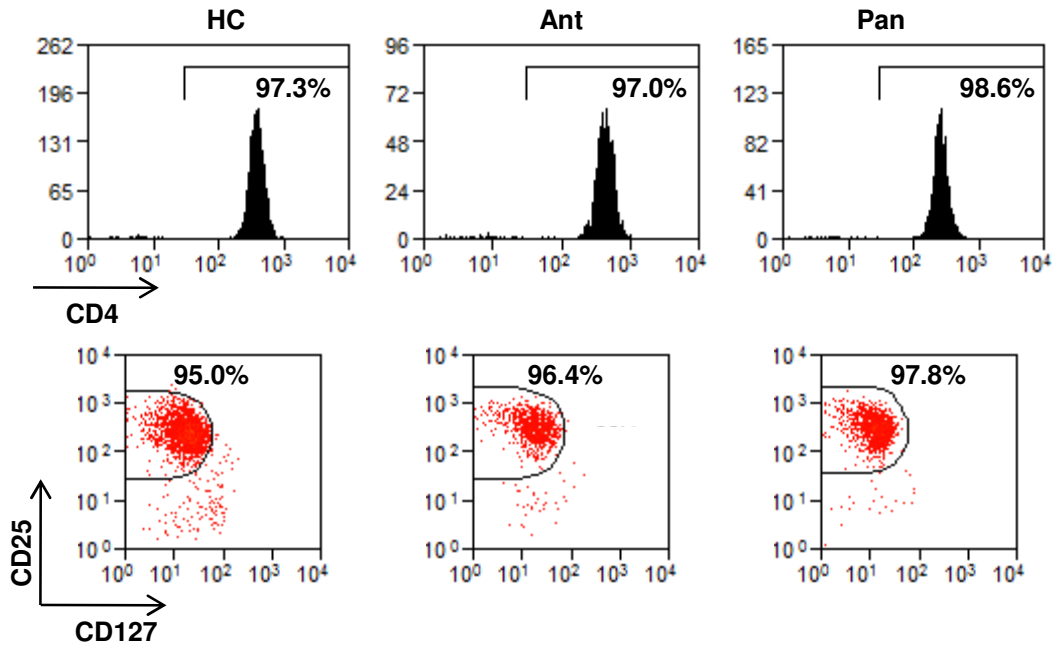


Fig: 3.9 Purities of Treg isolated using cell sorting for polyclonal suppression assay in the absence of APC

Sorted Treg cells from healthy controls (HC), anterior uveitis (Ant) and pan uveitis (pan) uveitis patients, were stained with anti CD4, CD25 and CD127 antibodies to analyse the purity of CD4+CD25highCD127low Treg cells. Purity of isolated cells represented as percentage of CD4+ T cells.

4/6 anterior uveitis patients were on topical glucocorticoid treatment and 2 were not on any treatment whereas 5/6 pan uveitis patients were on topical treatment and 1 patient not on treatment. The clinical features of healthy controls and patients used in this functional assay are summarized in Table: 3.6 and Table: 3.7 respectively.

Control No:	Sex	Age
1	F	30.1
2	M	24.5
3	F	45.0
4	M	26.9
5	M	34.0
6	M	34.0

7	M	26.4

Table: 3.6 Features of healthy control samples used in cross over co-culture assay (using sorted cells)

Patient no: / Sex	**Uveitis**	**Laterality**	**AqH cells**	**First/ recurrent episode**	**Age**	**Treatment**
1/M	A	B	0	Recurrent	22.5	None
2/M	A	B	1	Recurrent	22.8	Predforte
3/F	A	B	0	Recurrent	39.3	Predforte
4/F	A	B	0	Recurrent	55.6	aspirin, dipyramadole
5/M	A	B	0	Recurrent	64.9	G Pred 0.5%
6/M	A	B	0	Recurrent	26.1	G Predforte, Cosopt
7/F	P	B	0	Recurrent	67.7	maxidex
8/F	P	B	1	Recurrent	59.7	Dex 0.1%
9/F	P	B	0	Recurrent	68.2	Dex 0.1%
10/F	P	B	1	Recurrent	52.7	Dex 0.1%, cyclo 1%
11/F	P	B	2	Recurrent	42.7	None
12/M	P	U	0	Recurrent	45.2	G. Cyclo 1%, G Predforte

Table: 3.7 Clinical features of patients samples used in cross over co-culture assay (using sorted cells)

Clinical details of the patients were recorded at the time of sampling. (A-anterior uveitis, P-pan uveitis, B- bilateral, U- unilateral, Pred- prednisolone, Dex- dexamethasone, Cyclo- cyclopentolate).

As was seen in the suppression assay in the presence of APC, there was no significant defect in the suppressive function of Treg from pan or anterior uveitis patients (Fig: 3.10C) in this assay. Although some of the pan and anterior uveitis patients showed a

diminished suppressive capacity, this did not reach significant levels. The suppressive capacity was not affected by the age, sex, treatment or disease activity of the subjects. Here also the percentage proliferation of CFSE labeled Tconv cells from cultures of pan uveitis T cells were also low ((median % proliferation±SD: 42.0±241.1) as compared to healthy controls (median % proliferation±SD: 135.8±54.4) and anterior uveitis patients (median % proliferation±SD: 231.6±97.0). The percentage proliferation was calculated by the following equation:

% proliferation= No: of proliferating CFSE positive cells/ No: of cells added x 100

The percentage survival of Treg (median % survival±SD: 28.5±31.0) from pan uveitis patients was very low compared to healthy controls (median % survival±SD: 69.4±35.7) as well as anterior uveitis patients (median % survival±SD: 52.3±29.6). The percentage survival was calculated by the following equation:

% survival= (absolute no: of CFSE negative (Treg) cells/ Total no: of Treg added) x 100

To address any potential resistance of the uveitis T cells to be suppressed, a cross over co-culture assay was performed at the same time where Tconv from chronic pan uveitis patients were cultured with either autologous Treg cells or those from healthy controls (Fig: 3.11A) and vice versa (Fig: 3.11B). There was no significant difference in the suppressive capacity of Treg on autologous conventional T cells from pan uveitis patients compared to healthy controls (Fig: 3.11C). However conventional T cells from pan uveitis patients could be suppressed more effectively by Treg from

healthy controls (Fig: 3.11D).There was no difference in the suppressive capacity of Treg from these patients on conventional T cells from healthy controls (Fig: 3.11E). Similar assays were conducted on Treg from anterior uveitis patients, where patients T cells were co-cultured with autologous Treg or Treg from healthy controls (Fig: 3.12A) and vice versa (Fig: 3.12B). No significant difference could be found in the suppressive function of Treg from anterior uveitis patients on autologous T cells compared to healthy controls (Fig: 3.12C). There was no difference in the suppressive capacity of Treg from healthy controls on T cells from anterior uveitis patients (Fig: 3.12D) as well as the suppressive capacity of Treg from anterior uveitis patients on conventional T cells from healthy controls (Fig: 3.12E).

It has to be noted that almost every time a patient sample was analyzed, a healthy control sample was also analyzed in parallel as an experimental control. Healthy controls in these assays were age and sex matched with anterior uveitis patients. Pan uveitis patients on the other hand were older than the healthy controls. (For example, the median age ±SD of healthy controls, anterior uveitis and pan uveitis patients in PBMC assays were 35.8±9.7, 34.4±10.9 and 57.2±8.7 respectively.) Suppressive function of Treg has been shown to diminish with age (Tsaknaridis et al., 2003). This may account for the lower suppressive function seen in some of the pan uveitis patients although no significant correlation could be found between age and suppressive function in these patients.

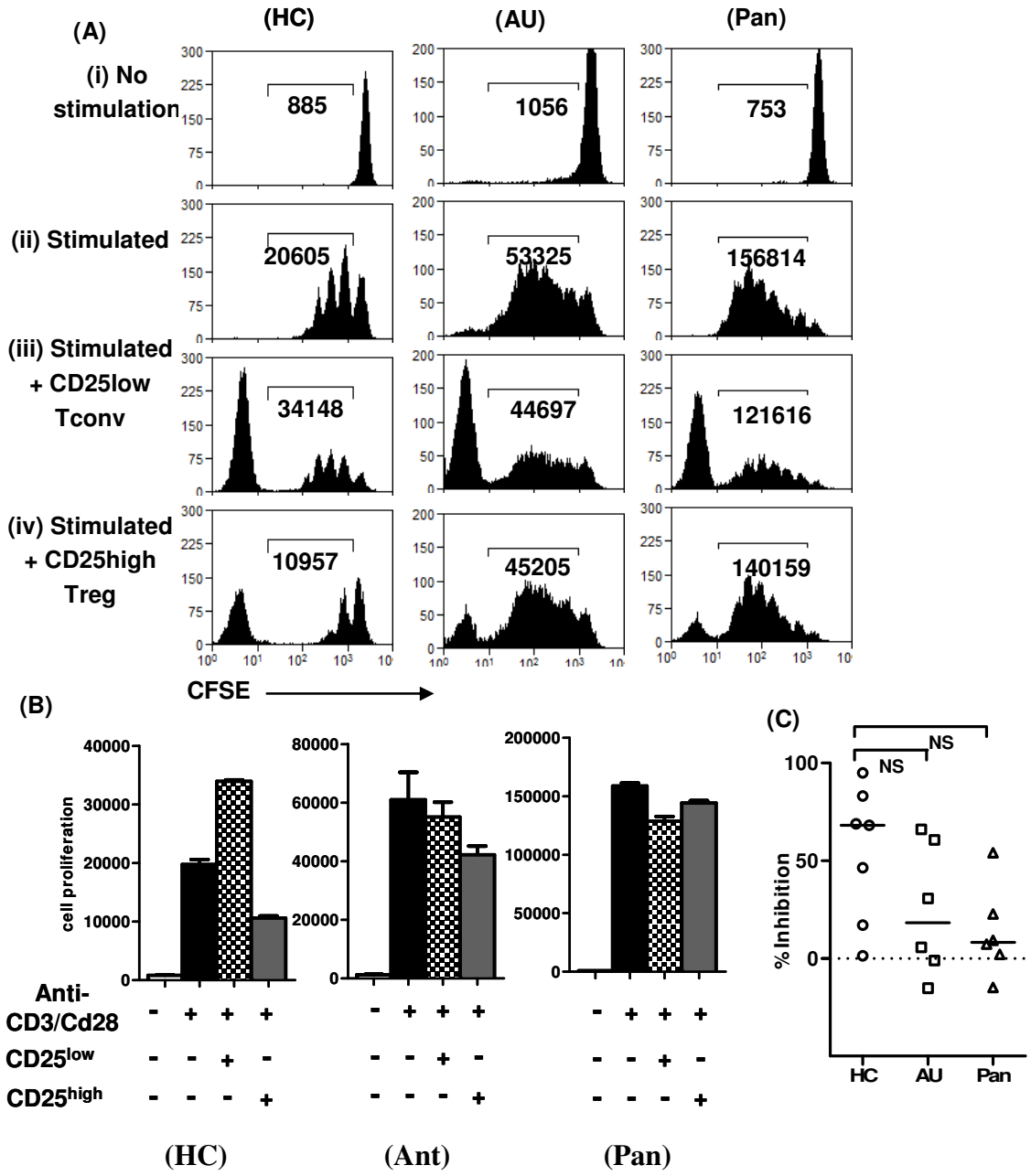


Fig: 3.10 Polyclonal suppression assay for Treg in the absence of APC

(A) Representative data of functional assay for Treg from healthy controls (HC), anterior uveitis (AU) and pan uveitis (pan) patients. CFSE labeled Tconv cells cultured with or without unlabeled Treg or Tconv cells. Number of proliferating cells for the representative data shown in the histogram. (B) Graphs representing proliferation of autologous Tconv cells from healthy controls, anterior and pan uveitis patients in the presence or absence of Treg/ Tconv cells. Results are expressed as mean ± SD. (C) No significant difference in the suppressive capacity of Treg from chronic anterior or pan uveitis patients compared to HC. Horizontal bars represent median values. (Statistical test used- Kruskal Wallis test, NS- not significant).

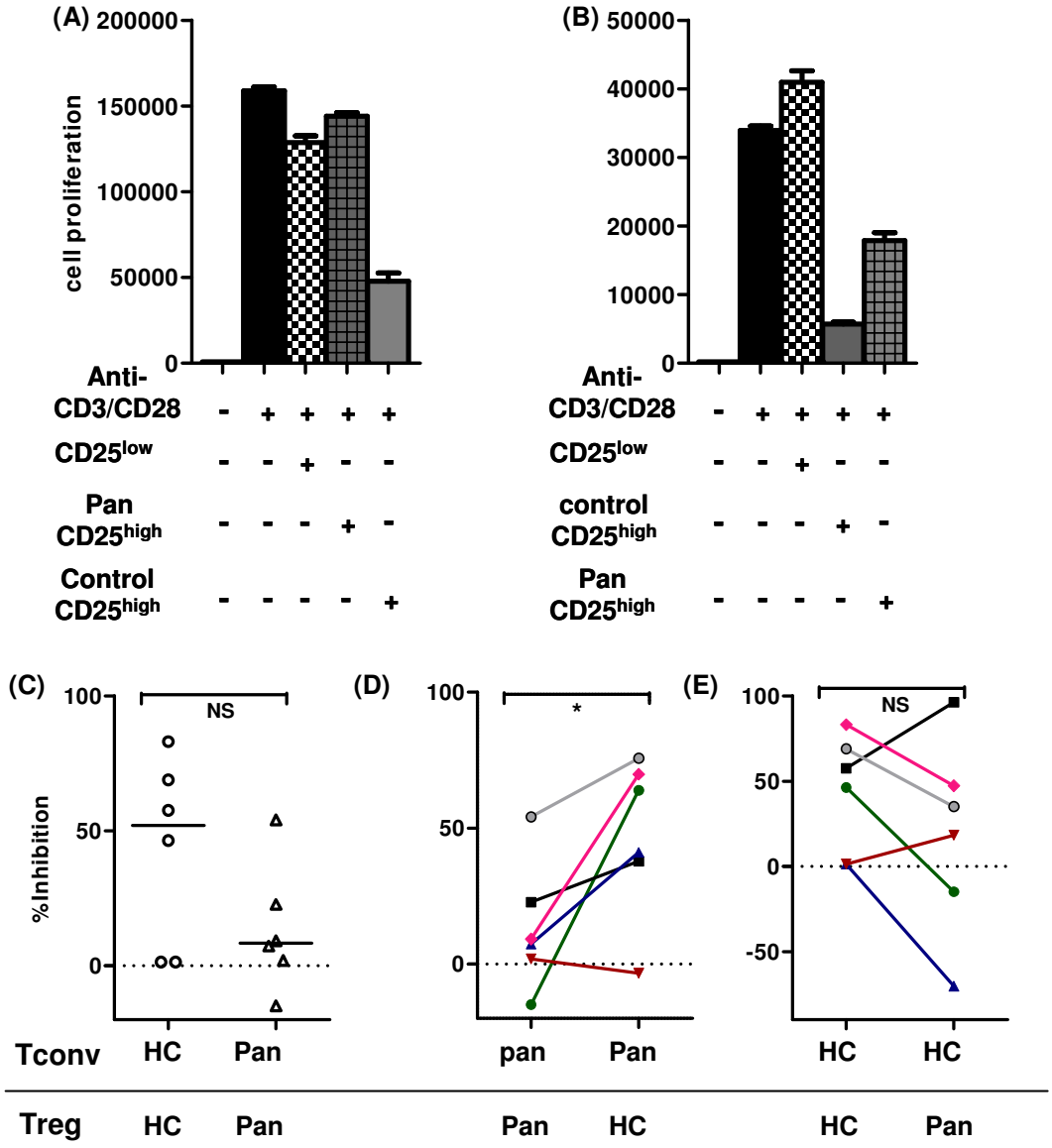


Fig: 3.11 Cross over co-culture assay with pan uveitis

(A) Proliferation of CFSE-Tconv cells from pan uveitis patient co-cultured with autologous Treg or Treg from a healthy control. (B) Proliferation of CFSE-Tconv cells from healthy control co-cultured with autologous Treg or pan uveitis Treg. (C) No difference in the suppressive activity of Treg from pan uveitis patients compared to healthy control (HC). (Horizontal bars represent median values). (D) Treg from HC suppressed Tconv cells from patients more effectively. (E). No significant difference in the suppressive activity of Treg from patients on Tconv cells from HC. (Statistical tests used- Mann Whitney test and two way ANOVA, NS- not significant, *-p≤0.05).

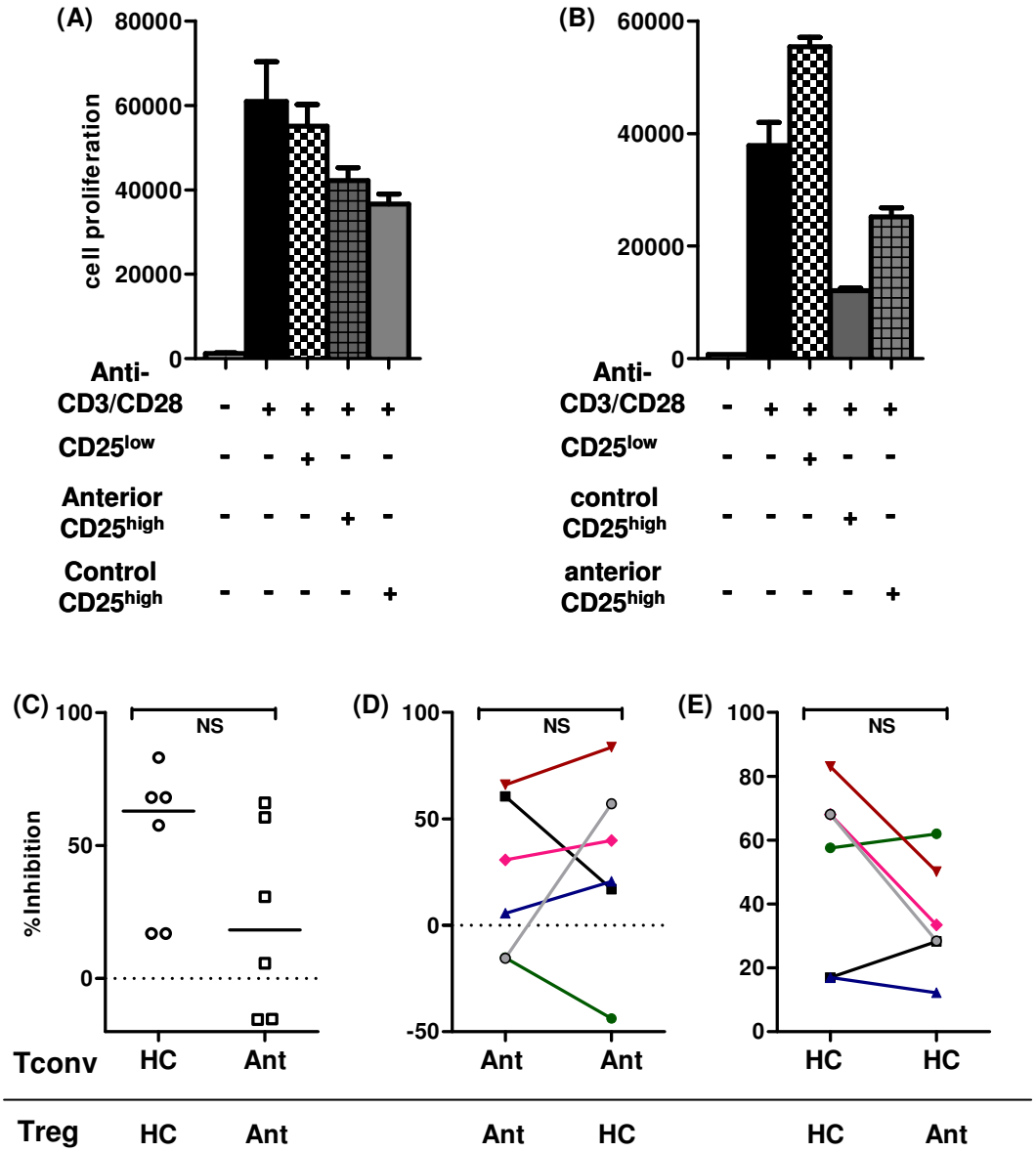


Fig: 3.12 Cross over co-culture assay with anterior uveitis

(A) Proliferation of CFSE-Tconv cells from AU patient co-cultured with autologous Treg or Treg from a healthy control. (B) Proliferation of CFSE-Tconv cells from healthy control co-cultured with autologous Treg or Treg from AU patient. (C) No difference in the suppressive activity of Treg from AU patients compared to healthy control (HC) (Horizontal bars represent median values). (D) Treg from HC did not suppress the Tconv cells more effectively. (E) No significant difference in the suppressive activity of Treg from AU patients on Tconv cells from HC (Statistical tests used- Mann Whitney test and two way ANOVA, NS- not significant)

3.5 Treg up regulated FoxP3 and maintained/restored their suppressive activity in long term culture

In order to determine if the suppressive capacity of regulatory T cells in patients with chronic uveitis was maintained following *in vitro* culture in the presence of IL-2, Treg cell lines were established from highly purified CD4+CD25highCD127low Treg cells of healthy controls and patients with chronic uveitis. Following *in vitro* culture, the cell lines up regulated their CD25 and FoxP3 expression (Fig: 3.13A). The suppressive capacity of the cell lines were tested on purified CFSE labelled Tconv cells from a different donor. For all individuals where there was a normal level of suppression using *ex vivo* freshly isolated Treg, the cell lines also maintained their suppressive capacity, and invariably showed increased regulatory activity. In patients where there was diminished Treg activity from freshly isolated cells, the suppressive activity was completely restored following long term culture (Fig: 3.13B).

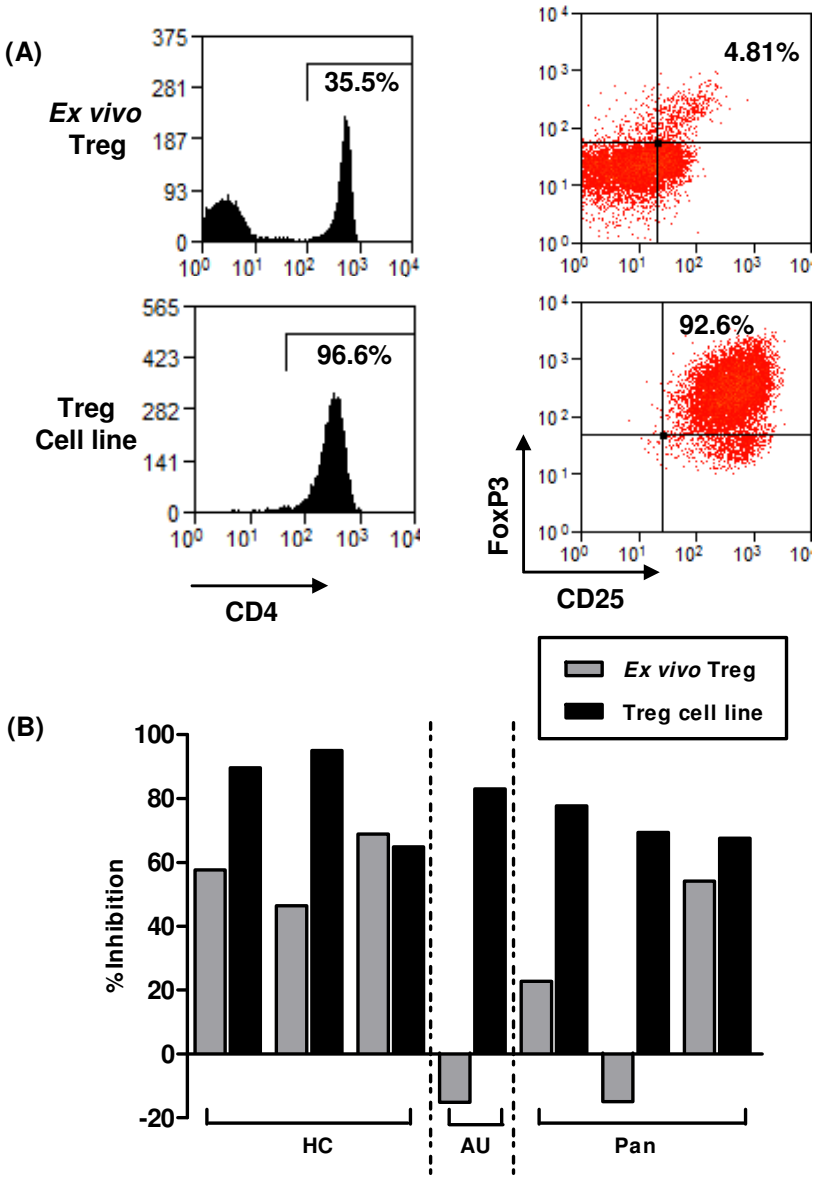


Fig: 3.13 Treg up regulated FoxP3 expression and suppressive capacity in long term culture

(A) Flow cytometric analysis of CD4, CD25 and FoxP3 expression of (A) *ex vivo* Treg and Treg cell line. Treg up regulated their FoxP3 and CD25 expression following long term culture (B) Graph representing suppressive function of *ex vivo* Treg (grey bars) and Treg cell line (black bars) from healthy controls (HC), anterior uveitis (AU) and pan uveitis (pan) patients. Treg with normal suppressive activity maintained their function whereas those with diminished activity restored normal suppressive activity in long term culture.

The yield of Treg following high speed cell sorting was very low which makes it difficult to study the suppressive effect of Treg at various Tconv: Treg ratios. Besides, high speed sorting can also affect viability of cells undergoing this ordeal. In many assays using sorted cells, the recovery of cells after 4 days in culture were very low. This could be because the conventional T cells and especially Treg isolated by high speed cell sorting were less healthy and more prone to cell death in culture. This may also account for the overall low suppressive function observed in these assays. Assays with very low survival rates were excluded from the final analysis. Hence dynabeads were used next to isolate pure CD4+CD25highCD127low Treg cells using a tube based isolation protocol and the capacity of these Treg to suppress the proliferation of conventional T cells in culture at different Tconv to Treg ratio were analyzed.

3.6 Bead separated Treg cells from chronic anterior uveitis patients are defective in function

Here cells with ≥95% purity were isolated using dynabeads (see Fig: 3.14). As before, Tconv cells from healthy controls, chronic anterior and chronic pan uveitis patients were CFSE labeled and cultured along with unlabelled Tconv cells or Treg in the presence of anti CD2/CD3/CD28 coated suppression inspector bead for 4 days. The major advantages of this method was the less harsh method of Treg isolation as well as the higher yield of Treg cells following isolation which allowed us to analyze the suppressive function of Treg at different concentrations. The recovery of cells after the 4 days culture was much better compared to sorted cells. This was supported by the higher percentage proliferation of CFSE labeled Tconv cells in this assay from anterior uveitis (median % proliferation± SD: 86.6±71.2), pan uveitis (median %

proliferation± SD: 86.1±61.9) and healthy controls (median % proliferation± SD: 88.2±72.0).

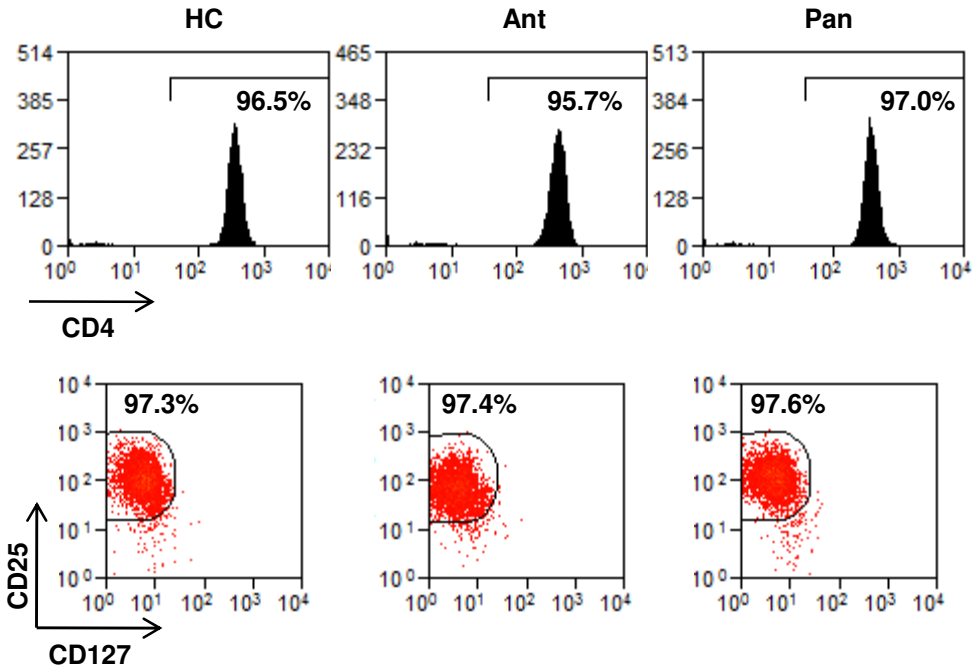


Fig: 3.14 Purities of Treg isolated using dynabeads for polyclonal suppression assay

Isolated Treg cells from healthy controls (HC), anterior uveitis (Ant) and pan (pan) uveitis patients were stained with anti CD4, CD25 and CD127 antibodies to analyse the purity of CD4+CD25highCD127low Treg cells.

The chronic anterior uveitis group included 5 patients on topical glucocorticoids and 2 patients not on any kind of treatment. All the 7 pan uveitis patients were on topical glucocorticoids. Clinical details of the patients in this assay are summarized in Table: 3.8 and healthy controls in Table: 3.9. Patients who were on systemic immunosuppressants including corticosteroid treatment were excluded to avoid any effect of the treatment on Treg function. Suppressive activity of Treg at different

Tconv: Treg ratios (1:1/2, 1:1/4, 1:1/8, 1:1/16, 1:1/32 and 1:1/64) were analyzed. Interestingly, Treg from pan uveitis patients in this assay showed no defect in their suppressive function at any of the ratios compared to age and sex matched healthy controls. In fact the Treg from these patients suppressed autologous T cells more effectively than those from anterior uveitis patients. Treg from anterior uveitis patients on the other hand showed a diminished suppressive function at various ratios compared to age and sex matched healthy controls especially at lower Tconv:Treg ratios. The suppressive function of Treg was not affected by the age, sex or disease activity of the patients.

Patient no: / Sex	**Uveitis**	**Laterality**	**AqH cells**	**First/ recurrent episode**	**Age**	**Treatment**
1/M	A	U	3	Recurrent	63.1	G Vexol
2/F	A	B	0	Recurrent	46.9	G Dexa0.1%
3/F	A	U	0	Recurrent	37.0	None
4/F	A	B	0.5	Recurrent	51.2	G.Maxidex, G Cyclo
5/F	A	B	1	Recurrent	59.9	G Dex 0.1%
6/F	A	B	0	Recurrent	46.3	None
7/M	A	B	0	Recurrent	25.7	G Lotemax, Timolol
8/M	P	U	0	First episode	31.5	G Predforte
9/F	P	B	1	Recurrent	53.2	G Maxidex
10/F	P	B	1	Recurrent	48.3	G Predforte
11/M	P	B	0	Recurrent	37.5	G Predforte
12/M	P	B	0	Recurrent	37.4	G.Dex 0.1%
13/F	P	B	3	Recurrent	38.0	G Predforte
14/M	P	B	0.5	Recurrent	39.4	G Vexol

Table: 3.8 Clinical features of patients samples used in functional assay using Treg isolated using dynabeads

Clinical details of the patients were recorded at the time of sampling. (A- anterior uveitis, P-pan uveitis patients, B- bilateral, U- unilateral, Pred- prednisolone, Dex- dexamethasone, Cyclo- cyclopentolate)

Control No:	**Sex**	**Age**
1	F	28.5
2	M	32.9
3	F	38.0
4	F	90.2
5	M	34.9
6	M	36.6
7	M	34.8

Table: 3.9 Clinical features of healthy control samples used in functional assay using Treg isolated using dynabeads

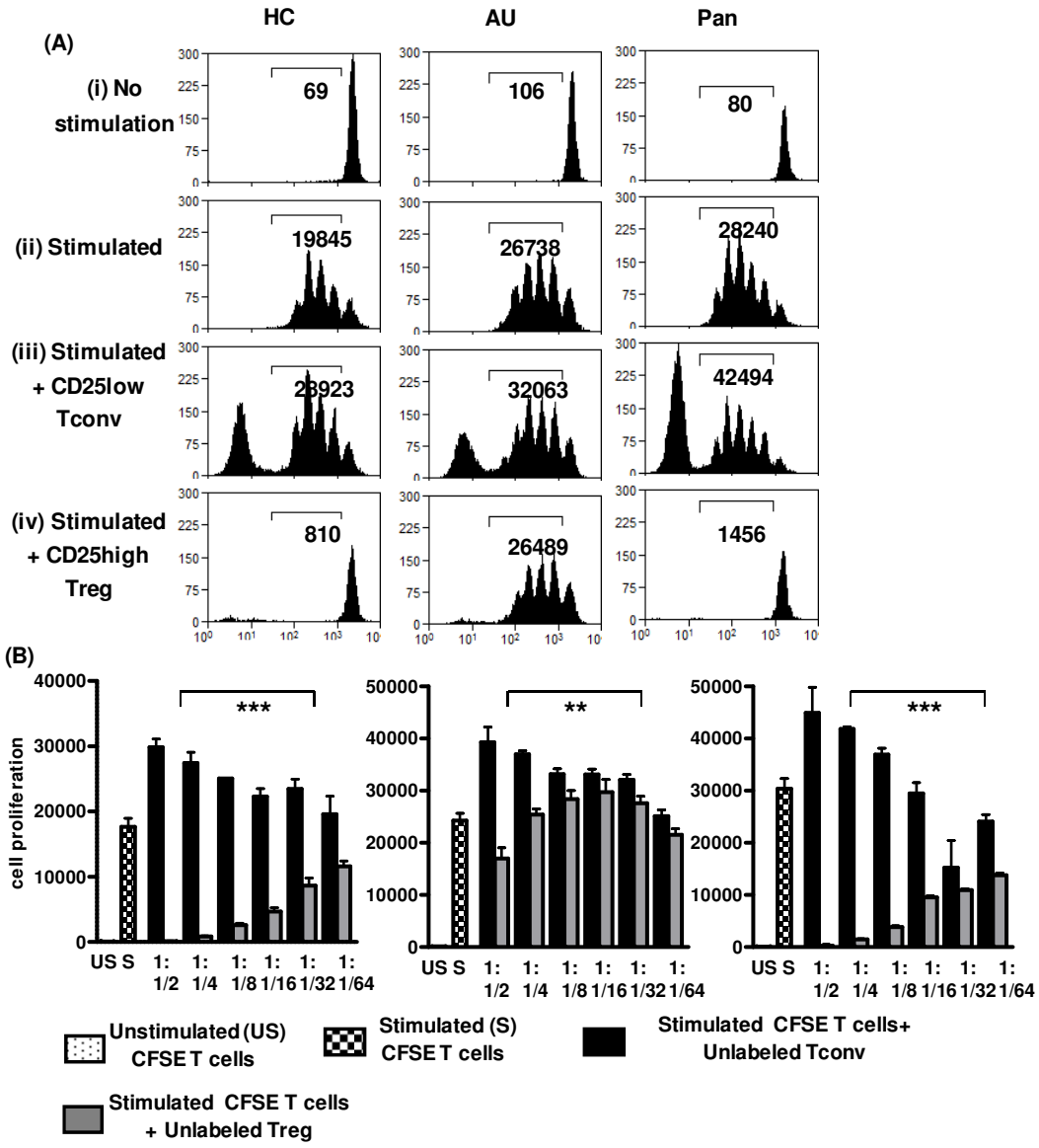


Fig: 3.15 Polyclonal suppression assay for Treg isolated using dynabeads

(A) Representative histograms showing proliferation of CFSE-Tconv cells from healthy controls (HC), anterior uveitis (AU) and pan uveitis (pan) patients at either unstimulated, stimulated or with Tconv: Treg ratio of 1:1/4. No: of proliferated CFSE Tconv cells for individual samples noted on histograms. (B) Graphs representing proliferation of autologous CFSE labelled Tconv cells from HC, AU and pan uveitis patients in the presence or absence of different ratios of Tconv cells: Treg. Results are expressed as mean ± SD. Each bar represents the mean of triplicate experiments. (Statistical test used –Kruskall wallis test, ***- $p \leq 0.001$, **- $p \leq 0.01$).

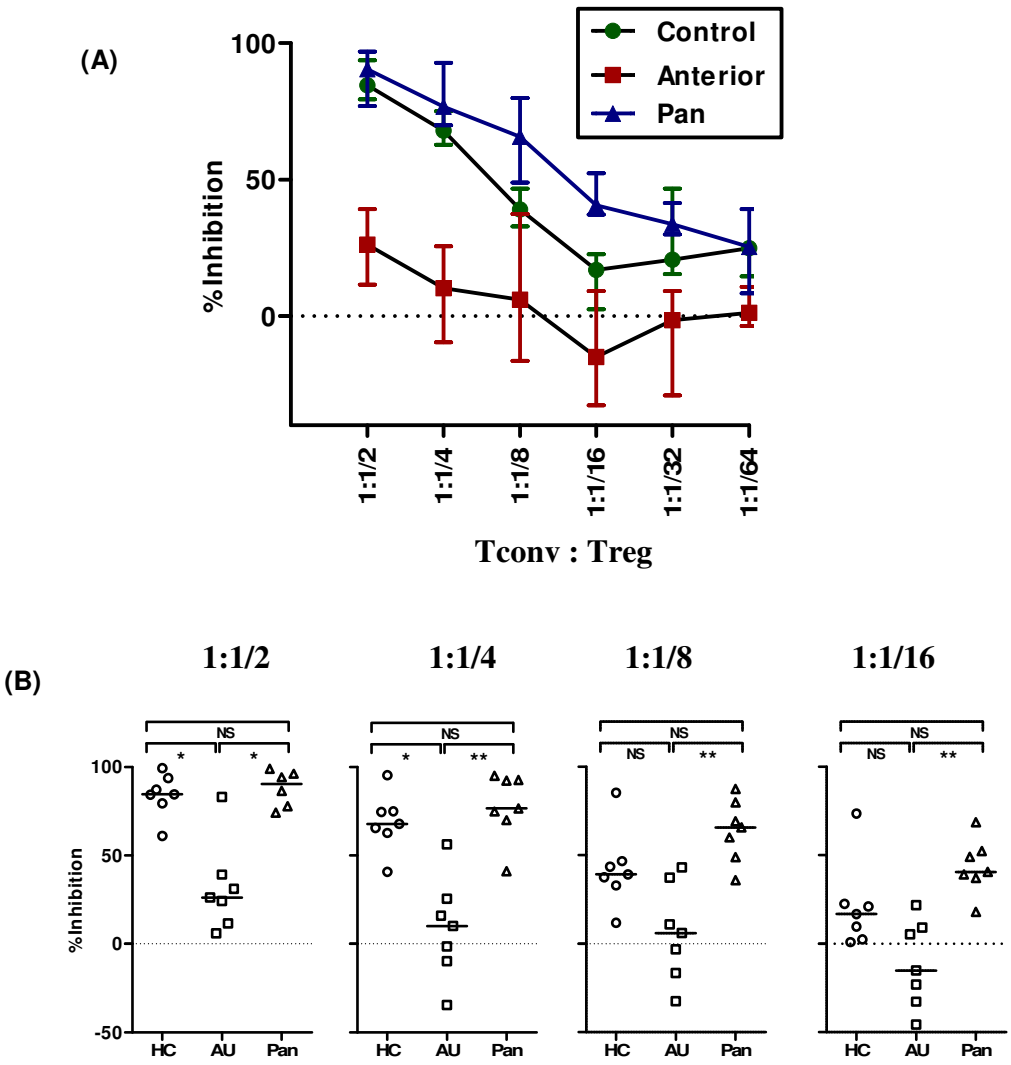


Fig: 3.16 Bead purified Treg from chronic anterior uveitis patients are defective in vitro

(A) Graph representing the percentage inhibition of proliferation of Tconv cells by Treg isolated using dynabeads at Tconv: Treg ratios of 1:1/2. 1:1/4, 1:8, 1:1/16, 1:1/32 and 1:1/64. The results are represented as interquartile range (B) Significantly defective Treg function in anterior uveitis (AU) patients at lower Tconv: Treg ratio compared to healthy controls. Treg from pan uveitis patients, showed no change in suppressive capacity compared to HC. Horizontal bars represent median values. (Statistical test used –Mann Whitney test, NS-not significant, *- $p \leq 0.05$, **- $p \leq 0.01$).

3.7 Discussion

The involvement of Treg in the pathology of human autoimmune diseases has been reported. Either a reduced frequency or impaired function of Treg has been reported in patients with a number of autoimmune diseases including multiple sclerosis, rheumatoid arthritis, SLE etc (Viglietta *et al*., 2004; Ehrenstein *et al*., 2004; Venigalla *et al*., 2008; Lyssuk *et al*., 2007).

In VKH disease a reduced frequency of regulatory T cells have been shown to be associated with active uveitis (Chen *et al*., 2008). Regulatory T cell frequency has been shown to be reduced during active uveitis in humans (Yeh *et al*., 2009). However, no studies have been conducted on the role of Treg in human chronic uveitis. In this study, no difference was found in the frequency of Treg from the chronic uveitis patient cohort compared to healthy controls. It has to be noted that the majority of the chronic uveitis patients in my chronic uveitis group had no or only relatively mild anterior chamber activity at the time of sampling, as compared to those that present with acute exacerbations of their disease or before initial treatment. I have also shown that Treg from anterior uveitis patients undergoing topical glucocorticoid therapy expressed increased levels of FoxP3 compared to the untreated patients (Fig: 3.3 E). Systemic glucocorticoid treatment has been shown to enhance the regulatory capacity and increase the FoxP3 expression in asthma and MS patients (Karagiannidis *et al*., 2004; Xu *et al*., 2009). A considerable amount of systemic absorption of topically applied glucocorticoids has been reported in a number of body areas (Burch and Migeon, 1968; NURSALL, 1965). Hence one could speculate that the increased FoxP3 expression of Treg from anterior uveitis patients undergoing topical glucocorticoid treatment may be due to the systemic effect of the treatment.

I also observed an increased CTLA-4 expression on the conventional T cells from pan uveitis patients. Along with being a Treg marker, CTLA-4 is also an activation marker for Tconv cells. Hence the increased CTLA-4 on the Tconv cells from pan uveitis patients might be an indicator of the increased activation status of T cells, as pan uveitis patients have more severe and extensive inflammation compared to anterior uveitis patients.

In this study, I have shown that the Treg isolated by high speed cell sorting from chronic uveitis patients did not show any significant defect in their suppressive function *in vitro* in the presence or absence of APC. One of the main disadvantages of the assay was that PHA, which was used to stimulate T cells in the system, may have adverse effect on the cells of the system which in effect could interfere with the final result. The expansion of T cells with PHA has been shown to skew the distribution of effector and memory subsets and impair their functions (Duarte *et al*., 2002). Apoptosis has been reported in human lymphocytes cultured in the presence of PHA (Li *et al*., 1994; O'Donovan *et al*., 1995). Along with this, human Treg have been shown to be more prone to apoptosis in culture (Ohara *et al*., 2002; Taams *et al*., 2001) which could account for the low survival rates seen in these cultures. High speed cell sorting as well as CFSE labelling could also affect the viability of cells in culture. This may also be the reason for the low rate of proliferation of CFSE labeled Tconv cells after 4 days in culture in most of these assays.

Cross over co culture assays with cells from healthy controls revealed that the diminished suppressive capacity seen in some of the pan uveitis patients was not due to a resistance of Tconv cells to Treg mediated suppression. Again, the main problem

with using sorted cells was the low rate of proliferation of cells in the culture. It is interesting that Tconv and Treg from pan uveitis patients showed the least rate of survival and proliferation respectively in both the above assays. It is not clear whether the decreased survival rates in pan uveitis is because the T cells from pan uveitis patients are more prone to apoptosis during high speed cell sorting. 50-70µl of supernatant were taken from some of these assays and frozen. Multiplex bead immunoassay for the detection of cytokines such as IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-13 and IL-17 were also carried out on these supernatants. However, very low levels of cytokines were detected in these supernatants (not shown). This may be because the stimuli used in these assays (PHA or suppression inspector beads) were insufficient to stimulate cytokine production by cells.

Treg cell lines developed from the chronic uveitis patients expressed normal functional capacity following long term culture *in vitro*. Hoffmann and co workers have shown that although the Treg cell compartment in adult peripheral blood comprises of naive as well as memory cells, it is solely the naive CD45RA subpopulation that gives rise to homogeneous Treg-cell lines (Hoffmann *et al*., 2006). In this study, the possibility that there might be an outgrowth of naïve Treg cells that give rise to functional Treg lines could not be ruled out.

Using a less harsh and more reliable method of isolation using Dynabeads, it was found that Treg from chronic anterior uveitis patients showed defective suppressive function *in vitro* especially at lower Tconv: Treg ratios. Even at higher Tconv: Treg ratios, Treg from most of the anterior uveitis patients expressed lower suppressive function compared to healthy controls, though not statistically significant. A

longitudinal analysis of those patients that express lower suppressive function at all ratios will be required to identify whether this reflects a true defect in their Treg population. Treg from pan uveitis patients on the other hand showed suppressive capacities similar to that of healthy controls.

What causes the difference between Treg function from pan and anterior uveitis patients is not known. Even though pan uveitis patents have a more clinically severe disease, their Treg appeared to be functional in vitro. It has to be noted that I have only tested the cell contact dependent suppressive function of Treg from these patients in vitro. It is also possible that other mechanisms of Treg mediated suppression (cytokine mediated and cytotoxicity) may be defective in pan uveitis patients. Involvement of Th17 in uveitis progression has been reported recently (Amadi-Obi *et al*., 2007). In autoimmune dry eye disease, Treg cells were unable to suppress pathogenic Th17 cells (Chauhan *et al*., 2009a). Whether such resistance of Th17 cells to Treg mediated suppression exist in pan uveitis patients has not been analysed in this study. The ability of FoxP3+ Treg cells to produce IFNγ has also been reported (Dominguez-Villar *et al*., 2011; McClymont *et al*., 2011). Given the clinical severity of pan uveitis, it would be interesting to analyse whether such plasticity of Treg cells are more prominent in these patients.

Cell isolation using dynabeads produced highly pure cells which tend to be more viable and healthy in the culture and hence could be used as a reliable method of Treg isolation for functional assays, allowing us to analyze the suppressive capacity of Treg at different concentrations. The contradictory results obtained in assays using cells isolated by cell sorting could be attributed to the decreased proliferation and survival

rate of cells in those assays. Also the heterogeneity of patients cohorts used in both sets of assays could not be ignored.

Systemic glucocorticoid treatment have been shown to enhance the regulatory capacity of Treg and restore the impaired suppressive function in MS patients (Xu *et al*., 2009). However, whether topical glucocorticoid treatment has any systemic effect on Treg is not yet known. I was unable to ascertain if topical glucocorticoid treatment has any effect on the peripheral blood regulatory T cell activity, as the vast majority of patients were on topical GC therapy at the time of sampling.

In many studies in uveitis where Treg cells have been shown to be defective including the one by Chen *et al.* where a defective Treg function was reported in VKH patients, Treg cells were isolated on the basis of their CD4 and CD25 expression only (Chen *et al*., 2008). Study by Yeh *et al* where frequency of Treg was shown to be decreased in active uveitis patients defined Treg by their CD4 and FoxP3 expression only (Yeh *et al*., 2009). Activated conventional T cells can also upregulate their CD25 and FoxP3 expression transiently, and activated memory cells expressing IL7 receptor α (CD127) which are present in the CD4+CD25+ cell population could potentially interfere with classical suppression assays for measuring Treg suppressive properties. Michel *et al* in 2008, reported that in patients with relapsing remitting multiple sclerosis, where a defective function of Treg cells was associated with the pathogenesis of the disease (Viglietta *et al*., 2004; NURSALL, 1965), Treg cells expressed normal suppressive function when IL-7 receptor α expressing cells were excluded from the system (Michel *et al*., 2008).

In this study I have used stringent gating strategies and expression of CD127 to identify and isolate Treg and found that chronic uveitis patients have similar frequency and phenotype of peripheral blood Treg as compared to healthy controls. Treg from chronic anterior uveitis but not pan uveitis patients showed a diminished capacity *in vitro* to suppress the proliferation of autologous Tconv cells as compared to healthy controls.

4 REGULATORY T CELLS IN THE PERIPHERAL BLOOD OF IDIOPATHIC ACUTE UVEITIS PATIENTS

4.1 Introduction

Acute anterior uveitis (AAU) is the most common form of uveitis and is characterised by the breakdown of blood ocular barrier and acute inflammation of the anterior structures of the eye. Majority of patients have no underlying disorder and are termed as idiopathic. In EAU, Treg have been shown to have an enhanced frequency and function at the peak of the acute disease (Sun *et al*., 2010a; Sun *et al*., 2010b). The study of Treg in human uveitis is very limited due to the diversity of the disease and heterogeneity of the patient cohort. Yeh *et al.* investigated the role played by Treg in human active non infectious uveitis patients and observed a decrease in the frequency of Treg in active uveitis patients compared to the inactive ones (Yeh *et al*., 2009). In VKH patients, decreased frequency and impaired function of Treg has been shown to be associated with active uveitis (Chen *et al*., 2008). However, these studies included all uveitis entities- infectious and non infectious with other associated systemic diseases. Also these studies did not use the SUN classification while classifying the disease type.

In the body of work described in the previous chapter, I presented evidence showing that Treg from chronic anterior uveitis patients have similar phenotype, but have

reduced suppressive function compared to healthy controls. However these were the patients with no or very low disease activity on the day of sampling.

The aim of the experiments described in this chapter is to analyse the Treg from idiopathic non infectious acute uveitis patients, classified according to the SUN classification (Jabs *et al*., 2005) presenting themselves to the A&E with an acute inflammation in the eye, characterised by redness, pain and photophobia. As glucocorticoid treatment as well as other systemic autoimmune diseases have been shown to affect the phenotype and function of Treg (Karagiannidis *et al*., 2004; Xu *et al*., 2009; Bonelli *et al*., 2008a), those patients undergoing systemic therapy as well as those with other autoimmune diseases were excluded from the analysis. As most of the pan/posterior uveitis patients were on systemic glucocorticoid therapy, our cohort included mainly acute anterior uveitis (AAU) patients and only 4 pan uveitis patients. Patients with infectious uveitis as well as those who were suspected to be clinically associated to HLA-B27 were also excluded. Treg from acute idiopathic non infectious uveitis patients were analysed for their phenotype as well as function *in vitro*.

4.2 Increased frequency of Treg from the peripheral blood of acute uveitis patients

PBMC was isolated from peripheral blood of acute anterior uveitis (AAU) and acute pan uveitis (APU) patients as well as healthy controls and stained with Treg specific markers to identify CD4+ CD25high CD127low Treg and CD4+CD25lowCD127high Tconv cells (Fig: 4.1A). The frequencies of CD4+ Treg in the peripheral blood of 24 idiopathic AAU patients, 4 pan uveitis patients and 23 healthy controls were analysed. The clinical features of patients recruited in this study are detailed in Table: 4.1 and the details of healthy controls in Table: 4.2. Increased frequency of Treg was observed in the peripheral blood of AAU patients (median±SD: 8.1 ± 2.0) and APU patients (median±SD: 11.1±2.3) compared to healthy controls (median±SD: 6.2 ± 0.9) (Fig: 4.1B). The frequency of naïve Treg cells were very low, and was not different in AAU or APU compared to age and sex matched healthy controls (Fig: 4.1C). The increased frequency was seen only in the memory Treg compartment from AAU patients. Among the AAU patients analysed, 6 patients were undergoing topical glucocorticoid treatment and the remaining 18 were not on treatment. The increased frequencies of Treg in AAU patients were more prominent in untreated patients than in those undergoing topical glucocorticoid treatment compared to healthy controls (Fig: 4.1D). Of the 4 pan uveitis patients analysed, 3 of them were on topical glucocorticoid therapy and hence it was not possible to study the effect of topical treatment on the Treg frequency in this patient group. The increase in frequency was also more prominent in AAU patients with recurrent disease than those presenting with the first episode (Fig: 4.1E).

Patient no: / Sex	Laterality	Ant/ Pan	AqH cells	First/ recurrent episode	Age	Treatment
1/M	U	A	2	first episode	80.8	None
2/M	U	A	3	Recurrent	51.8	None
3/F	U	A	2	Recurrent	41.2	None
4/M	U	A	3	Recurrent	52.0	None
5/M	U	A	3	first episode	67.7	None
6/F	U	A	3	first episode	24.7	None
7/F	U	A	2	first episode	57.3	None
8/M	U	A	2	first episode	40.1	Predforte, Cyclo
9/F	U	A	3	first episode	30.4	None
10/F	U	A	3	first episode	57.1	Maxidex, Cyclo
11/M	B	A	3	Recurrent	48.1	None
12/M	U	A	1	Recurrent	49.1	Vexol
13/M	U	A	2	Recurrent	29.0	Vexol
14/F	U	A	2	Recurrent	48.5	None
15/M	U	A	3	Recurrent	27.8	None
16/M	U	A	2	Recurrent	57.8	None
17/M	U	A	3	Recurrent	23.4	Dex1%,cyclo
18/M	U	A	1	Recurrent	49.4	None
19/M	U	A	2	Recurrent	45.1	None
20/M	U	A	3	first episode	66.2	Ludosemide
21/F	U	A	3	Recurrent	51.6	None
22/M	U	A	3	Recurrent	61.0	None
23/F	U	A	2	Recurrent	46.9	Predforte
24/M	B	A	2	Recurrent	62.5	None

25/F	U	P	3	Recurrent	36.0	Predforte
26/M	U	P	3	Recurrent	35.8	None
27/M	B	P	3	Recurrent	56.1	Rimexolone
28/F	U	P	3	Recurrent	36.3	Predforte, Cyclo

Table 4:1 Baseline characteristics and clinical features of idiopathic acute uveitis samples analyzed by flow cytometry.

Laterality of the disease (unilateral/U or bilateral/B), type of uveitis (anterior/A or pan/P), anterior chamber activity recorded at the time of sampling, first/recurrent episode, age of the patient and treatment shown above. (Cyclo-cyclopentolate, Dex-dexamethasone).

Control no:	**Sex**	**Age**
1	M	34.0
2	F	28.9
3	M	32.9
4	M	24.2
5	F	22.0
6	M	34.8
7	F	53.0
8	M	51.0
9	M	23.0
10	F	45.0
11	M	26.9
12	F	24.3

13	M	34.0
14	M	26.4
15	F	75.2
16	F	90.2
17	M	87.8
18	F	28.5
19	F	64.9
20	M	32.9
21	M	24.0
22	F	21.0
23	M	55.0

Table 4:2 Baseline characteristics of healthy controls analyzed by flow cytometry.

Features of healthy controls analysed by flow cytometry. The sex and age of the controls is shown above.

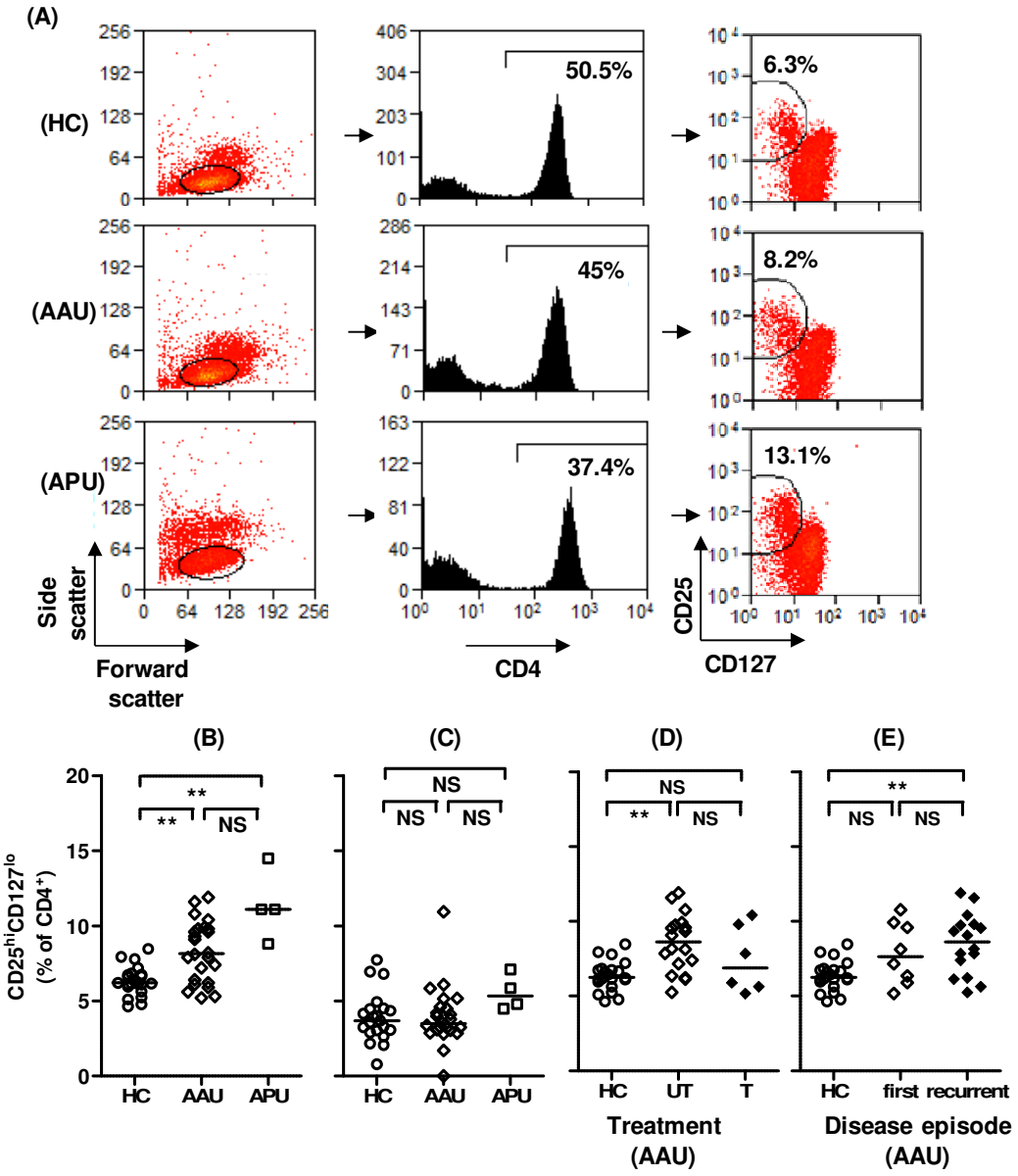


Fig: 4.1 Increased frequency of Treg from acute uveitis patients

(A) PBMCs were gated on lymphocytes and then on CD4+ cells to determine CD25highCD127low Treg from healthy controls (HC), acute anterior uveitis (AAU) and acute pan uveitis (APU) patients. (B) Increased frequency of primed Treg from AAU as well as APU patients compared to HC. (C) No difference in the frequency of naïve Treg from AAU and APU patients compared to HC. (C) Increased frequency of Treg evident in untreated (UT) AAU patients but not in topically treated (T) patients. (E) AAU patients with recurrent episode of disease had significantly higher Treg frequency compared to healthy controls and those with first episode of the disease. Horizontal bars represent median values. (Statistical tests used- Kruskal Wallis test, NS-not significant, **- $p \leq 0.01$)

4.3 No difference in the FoxP3 expression of Treg from acute uveitis patients

Chen *et al.* observed decreased FoxP3 expression of Treg from VKH patients with active uveitis (Chen *et al*., 2008). As the regulatory activity of Treg has been shown to reside in the CD45RO+ (memory) population (Jonuleit *et al*., 2001), only memory Treg and Tconv cells were analysed for phenotype in this study. Also the population of naive Treg cells was too small to allow precise analysis as most Treg had a memory phenotype. Hence the FoxP3 expression of only memory Treg and Tconv cells from the peripheral blood of AAU and APU patients were analysed. Almost all the CD127lowCD25high Treg were positive for FoxP3. Of the 19 AAU patients analysed for FoxP3 expression, 5 were on topical glucocorticoid treatments and the remaining 14 were not on treatment (Table: 4.1). 23 age and sex matched healthy controls were analysed for FoxP3 expression. There was no difference in the FoxP3 expression of memory Treg from the peripheral blood of AAU patients (median MFI±SD: 113.5 ± 35.5) compared to healthy controls (median MFI±SD: 113.5 ± 34.5) (Fig: 4.2A&C). Only 2 APU samples were analysed for FoxP3 expression where one was on topical treatment and the other one not on any treatment. FoxP3 expression of Treg from AAU did not differ between patients presenting with first or recurrent episodes of the disease (Fig: 4.2D).

As expected the Tconv cells showed no significant expression of FoxP3 and were not different between patients and healthy controls (Fig: 4.2B&E). No significant differences were found in the FoxP3 expression of naive Tconv cell population.

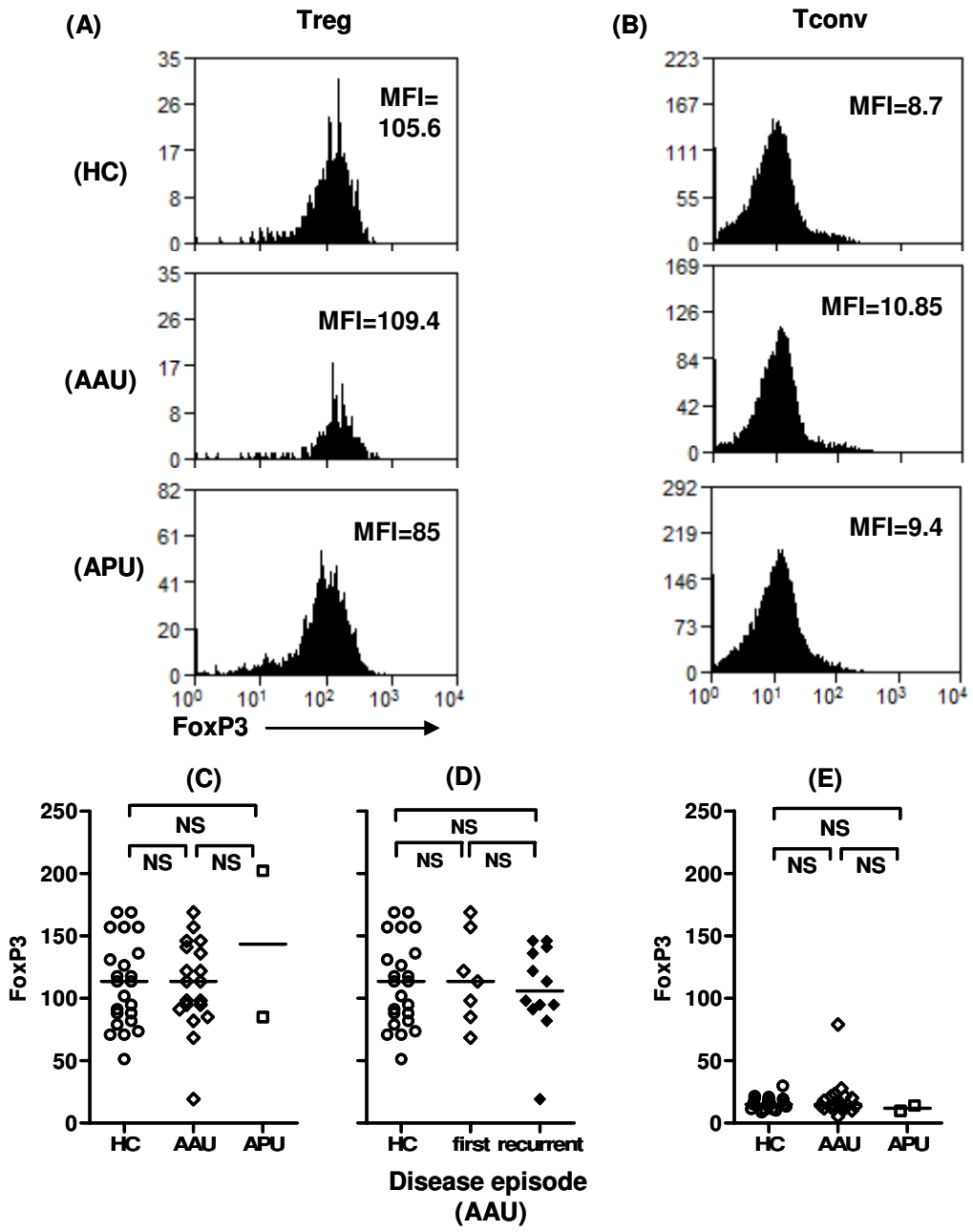


Fig: 4.2 No difference in the FoxP3 expression of Treg from acute uveitis

FoxP3 expression of CD45RO+ (A) Treg and (B) Tconv from healthy control (HC), acute anterior uveitis (AAU) and acute pan uveitis (APU) patients. FoxP3 MFI (median fluorescence intensity) for individual samples noted on histograms. (C) No difference in FoxP3 expression of Treg between AAU, APU and HC. (D) Treg from AAU presenting with first episode and recurrent episode of disease showed similar FoxP3 expression compared to HC. (E) No difference in FoxP3 expression of Tconv cells between AAU, APU and HC. Horizontal bars represent median values. (Statistical test used- Kruskal Wallis test, NS- not significant)

4.4 No difference in the CTLA-4 expression of Treg from acute uveitis patients

Intracellular CTLA-4 expression of memory Treg and Tconv cells from 16 AAU patients, and 22 healthy controls were also analysed. Similar to FoxP3, expression of intracellular CTLA-4 was also not different between Treg from AAU (median MFI±SD: 37.04 ± 9.7) and age and sex matched healthy controls (median MFI±SD: 42.03 ± 10.3) (Fig: 4.3A&C). CTLA-4 expression was also not different between patients with first and recurrent episodes of disease (Fig: 4.3D). Tconv cells showed no significant expression of CTLA-4 and was not different between patients and healthy controls (Fig: 4.3B&E). Only 1 pan uveitis patient was analysed for CTLA-4 and hence could not give any conclusive results (not shown).

4.5 No difference in the CD39 expression of Treg from acute uveitis patient

Expressions of surface CD39, which showed a biphasic expression on Treg were analysed from 17 AAU patients and 14 healthy controls. No difference in the frequency of Treg expressing CD39 was observed between AAU patients (median %±SD: 55.4 ± 19.4) and age and sex matched healthy controls (median %±SD: 49.8 ± 17.4) (Fig: 4.4A&C). CD39 expression did not differ between patients with first or recurrent episodes of the disease (Fig: 4.4D). There was also no difference in the CD39 expression of Tconv cells from AAU patients compared to healthy controls (Fig: 4.4B&E). Only 1 pan uveitis patient was analysed for CD39 and hence could not give any conclusive results (not shown).

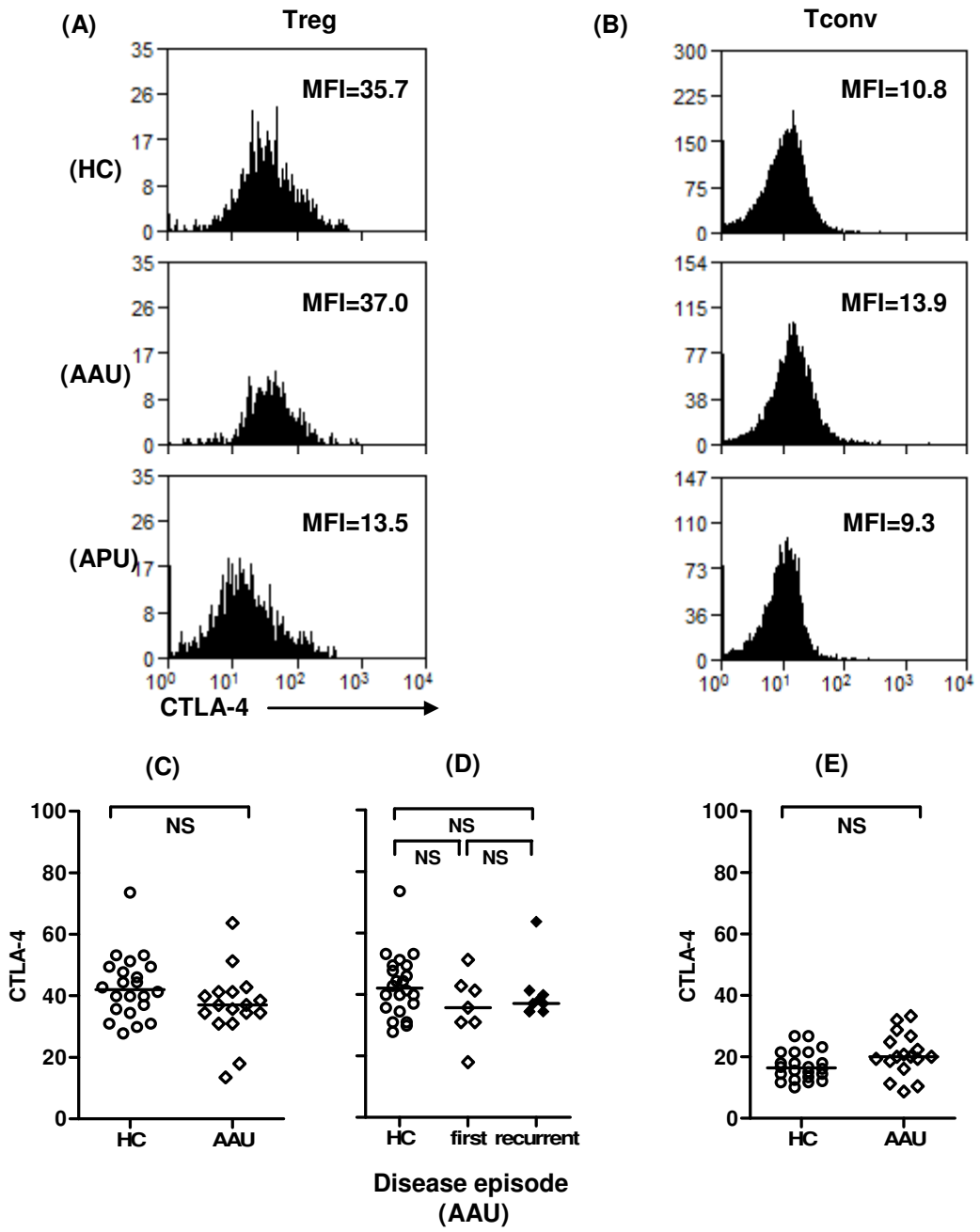


Fig: 4.3 No difference in the CTLA4 expression of Treg from AAU patients

CTLA-4 expression of CD45RO+ (A) Treg and (B) Tconv from healthy control (HC) and acute anterior uveitis (AAU) patients: CTLA-4 MFI (median fluorescence intensity) for individual samples noted on histograms. (C) No difference in CTLA-4 expression of Treg from AAU patients compared to HC (D) Treg from AAU patients with first episode and recurrent episodes of disease showed similar CTLA-4 expression compared to HC. (E) Tconv from AAU showed no difference in CTLA-4 expression compared to HC. Horizontal bars represent median values. (Kruskal Wallis test, Mann Whitney test, NS- not significant)

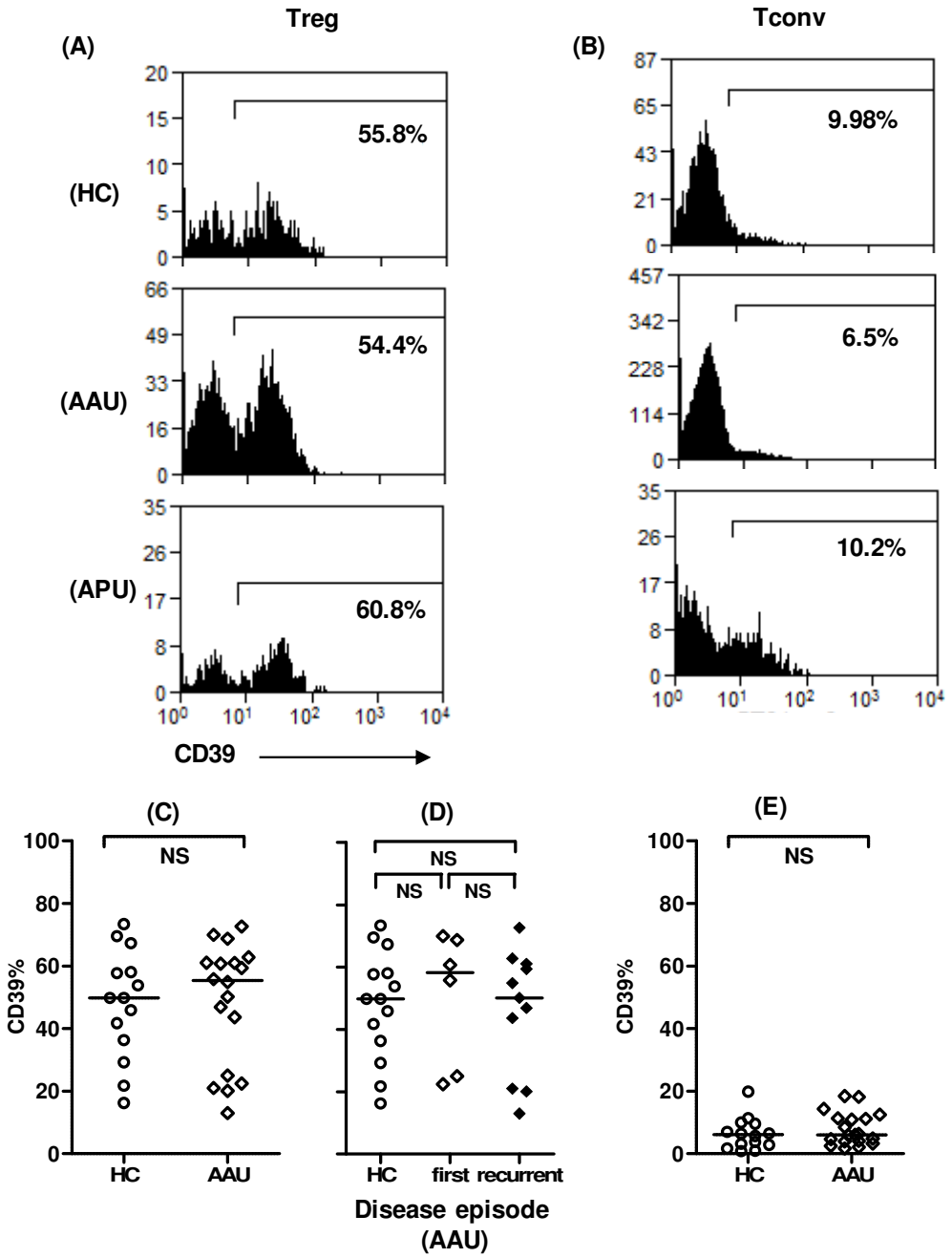


Fig: 4.4 No difference in the CD39 expression of Treg from AAU patients

CD39 expression of CD45RO+ (A) Treg and (B) Tconv from healthy control (HC) and acute anterior uveitis (AAU) patients: (C) No difference in the frequency of CD39+ Treg from AAU compared to HC. (D) Treg from AAU patients with first episode and recurrent episode of disease showed similar frequencies of CD39+ Treg compared to HC. (E) Tconv from AAU showed no difference in the CD39 expression as compared to HC. Horizontal bars represent median values. (Statistical test used- Kruskal Wallis test, Mann Whitney test, NS- not significant)

4.6 Comparison of phenotypes between acute and chronic uveitis patients

The phenotype and function of Treg from chronic uveitis patients were analysed in chapter 3. The clinical features and phenotype of chronic anterior uveitis (AU) patients are detailed in chapter 3, Table: 3.1. When comparing the phenotypes of Treg between patients with acute and chronic uveitis, it was observed that AAU patients expressed increased frequencies of Treg compared to those with chronic disease (Fig:4.5Ai) whereas acute pan uveitis (APU) patients showed no significant difference in the frequency of Treg compared to chronic pan uveitis (Pan) patients (Fig:4.5Aii). This may be due to the very small number in the APU cohort. However, no difference in the FoxP3, CTLA-4 or CD39 expression was observed in the Treg or Tconv cells between AAU and AU patients (Fig 4.5 B&C).

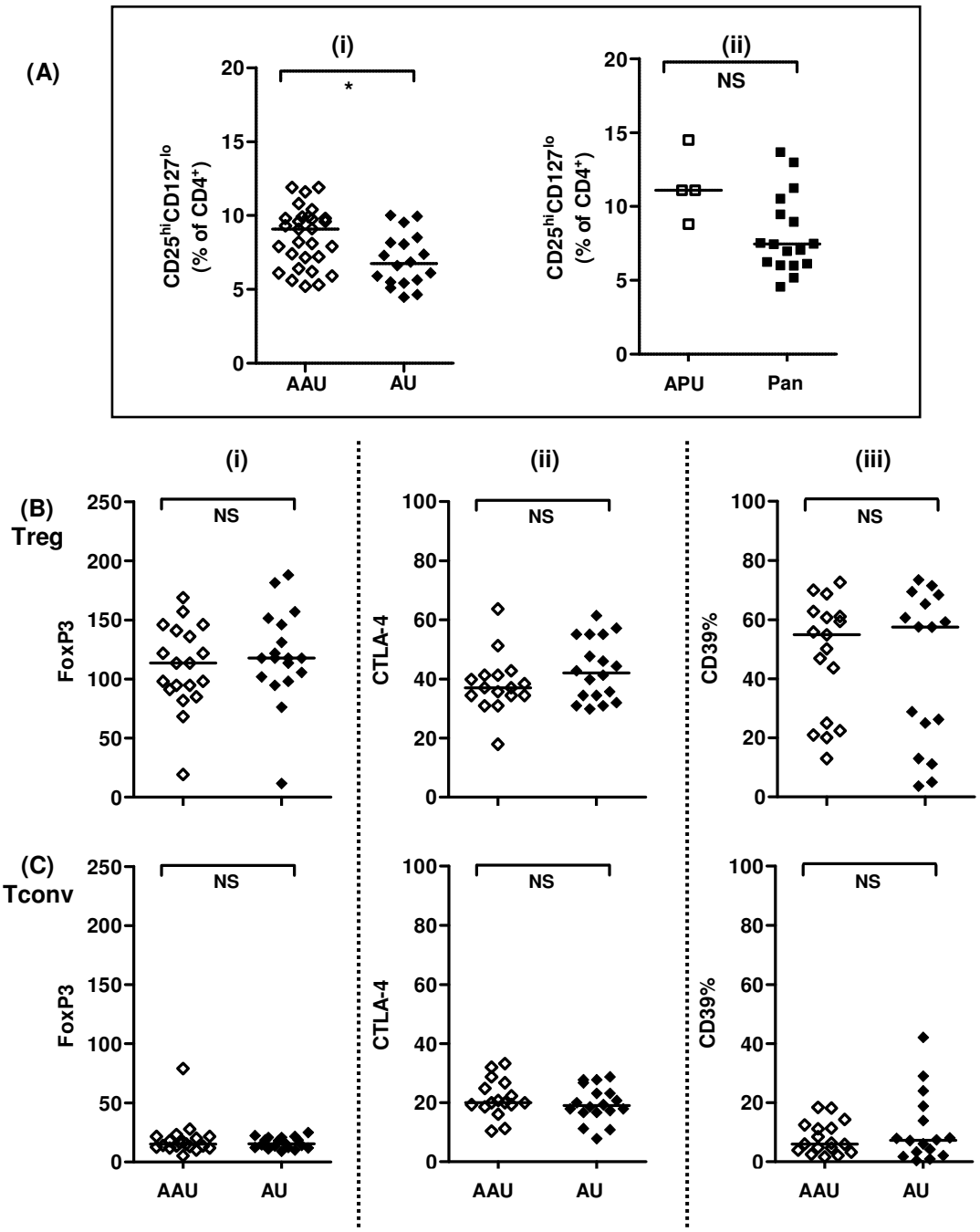


Fig: 4.5 Comparison of Treg from acute and chronic uveitis

(A) Increased frequency of Treg from (i) acute anterior uveitis (AAU) compared to chronic anterior uveitis (AU), but (ii) similar frequency of Treg from acute pan uveitis (APU) as compared to chronic pan uveitis (pan) patients. No difference in the expression of (i) FoxP3, (ii) CTLA-4 and (iii) CD39 of (B) Treg or (C) Tconv between AAU and AU. Horizontal bars represent median values. (Mann Whitney test, NS- not significant, *- $p \leq 0.05$)

4.7 Defective suppressive effect of Treg from AAU

Defective Treg mediated suppression of polyclonal proliferation has been shown to be associated with various autoimmune inflammatory diseases including active uveitis is VKH patients (Chen *et al*., 2008). Here, Treg from AAU patients as well as healthy controls were isolated using dynabeads and their suppressive effect on the proliferation of CFSE labelled conventional T cells were analysed. The dynabeads selected Treg were of ≥95% purity as shown in Fig: 4.6.

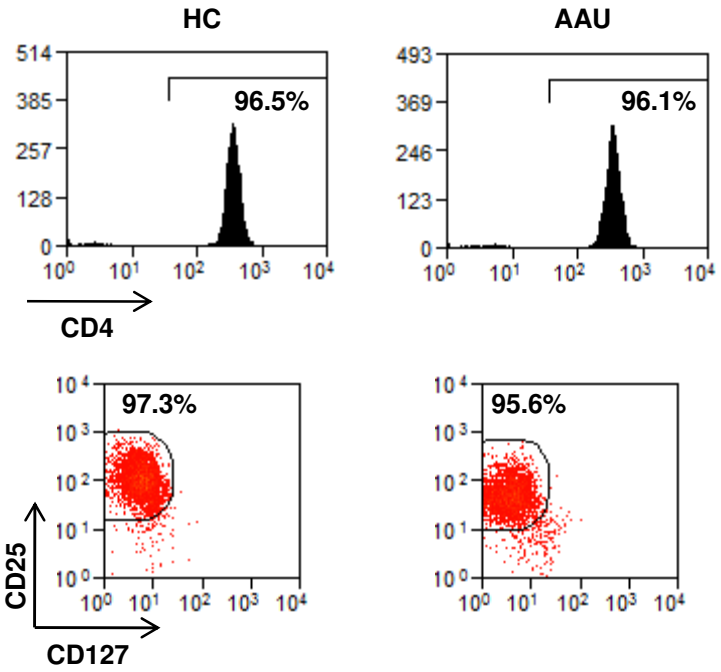


Fig: 4.6 Purities of Treg isolated using dynabeads for polyclonal suppression assay

Isolated Treg cells from healthy controls (HC) and acute anterior uveitis (AAU) patients were stained with anti CD4, CD25 and CD127 antibodies to analyse the purity of CD4+CD25highCD127low Treg cells. The purity of the isolated cells represented as percentage of CD4+ T cells.

As explained in chapter 3, Tconv cells were CFSE labeled and cultured along with unlabeled T cells or Treg with anti CD2/CD3/CD28 coated suppression inspector beads for 4 days. The proliferation of CFSE labeled cells were analyzed by flow cytometry (Fig: 4.7A).

Treg isolated from 9 AAU patients and 7 healthy controls were analyzed for their suppressive function at different Tconv: Treg ratios such as 1:1/2, 1:1/4, 1:1/8, 1:1/16, 1:1/32 and 1:1/64 (Fig:4.7B). All of the patients had idiopathic AAU and none were on systemic glucocorticoid therapy. It was observed that Treg from AAU showed diminished suppressive capacity compared to age and sex matched healthy controls (Fig: 4.8A) which became statistically significant at a ratio of 1 Tconv: 1/4 Treg (Fig: 4.8B). Of the 9 AAU patients, 5 were on topical glucocorticoid treatment and 6 were presented with a first episode of the disease. The suppressive capacity was not affected by the treatment status (Fig: 4.9A) or disease activity (Fig: 4.9B) of the patient. However the defective suppressive capacity was more prominent in patients with recurrent disease (Fig: 4.9C). The suppressive activity did not correlate with age of the patient or frequency, FoxP3, CTLA-4 and CD39 expression of Treg (Fig: 4.9D-F).

Patient no: / Sex	Laterality	AqH cells	First/ recurrent episode	Age	Treatment
1/M	U	2	first episode	40.1	Predforte, Cyclo
2/F	U	3	first episode	57.1	Maxidex, Cyclo
3/M	U	2	Recurrent	45.1	None
4/M	U	3	Recurrent	52.0	None
5/F	U	2	Recurrent	41.2	None
6/M	B	3	Recurrent	48.1	None
7/M	U	1	Recurrent	49.1	Vexol
8/M	U	2	Recurrent	29.0	Vexol
9/M	U	3	first episode	67.7	Chloramphenicol

Table 4:3 Baseline characteristics and clinical features of non infectious idiopathic AAU samples analyzed for the suppressive function of Treg.

Laterality of the disease (unilateral/U or bilateral/B), anterior chamber activity recorded at the time of sampling, first/recurrent episode, treatment and mean age shown above. (Cyclo-cyclopentolate)

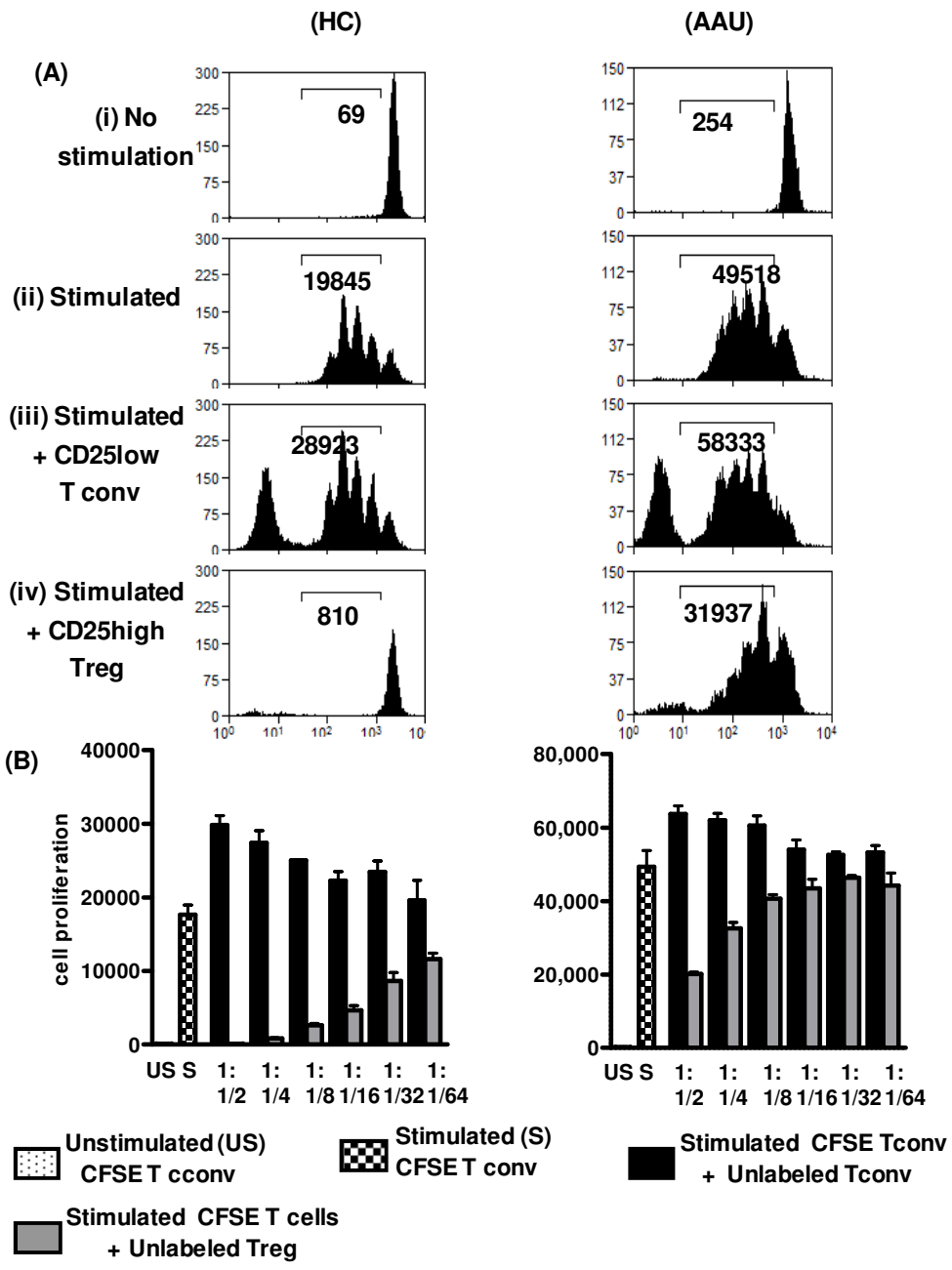


Fig: 4.7 Polyclonal suppression assay for Treg from AAU and HC

(A) Representative histograms showing proliferation of CFSE-Tconv cells from healthy controls (HC) and anterior uveitis (AU) patients either unstimulated, stimulated, or with Tconv cells or Treg at 1:1/4 ratio. No: of proliferated cells for individual samples noted on histograms. (B) Graphs representing proliferation of autologous Tconv cells from healthy controls and AAU patients in the presence or absence of polyclonal stimulation at different Tconv: Treg ratios. Results are expressed as mean ± SD.

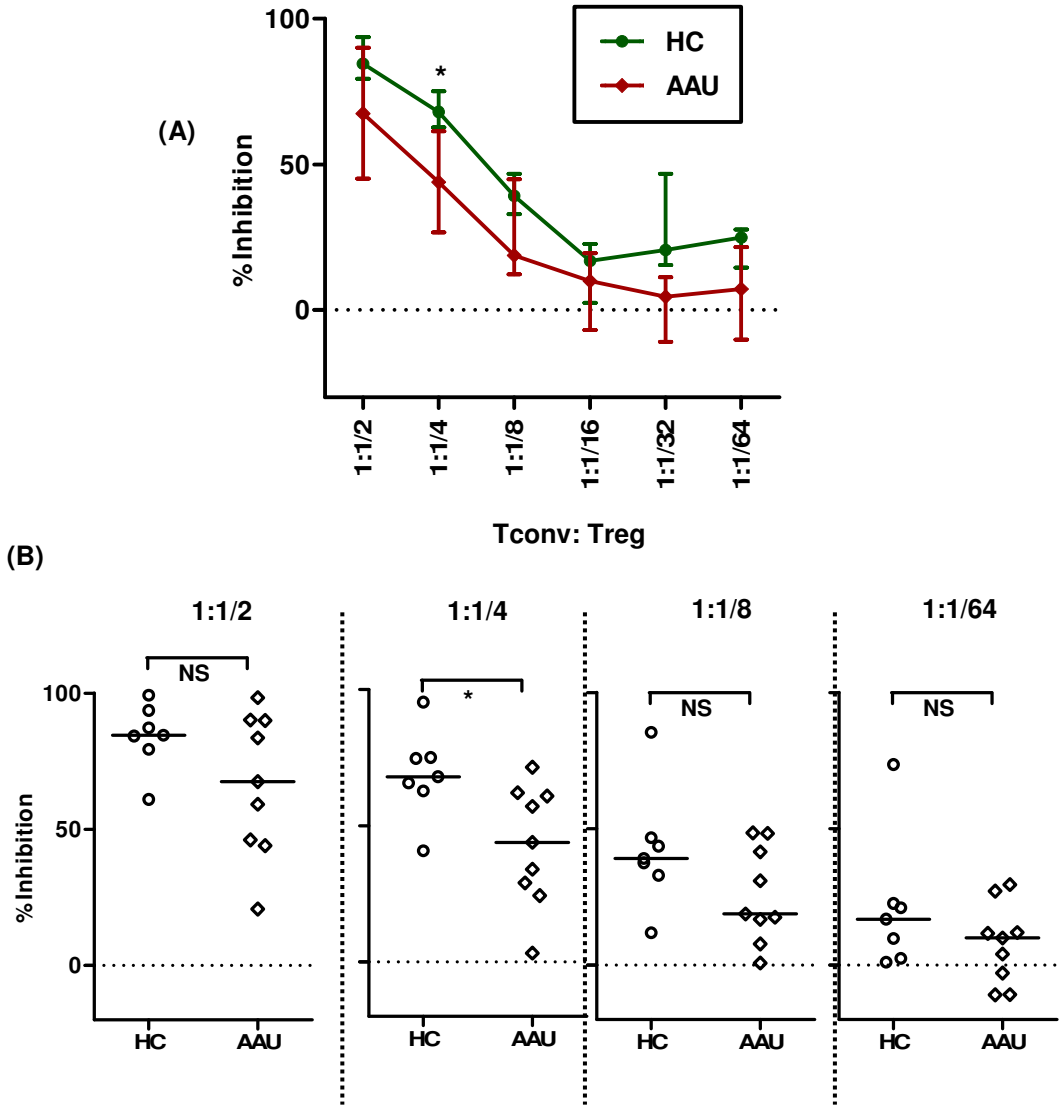


Fig: 4.8 Defective suppressive function of Treg from AAU

(A) Graph representing the percentage inhibition of proliferation of Tconv cells by Treg isolated using dynabeads at Tconv: Treg ratios of 1:1/2, 1:1/4, 1:1/8, 1:1/16, 1:1/32 and 1:1/64. The results are represented as median with interquartile range. (B) Significantly reduced suppressive capacity of Treg from AAU compared to HC at Tconv: Treg ratio of 4:1. Many AAU patients showed diminished suppressive capacity at other T cell: Treg ratios even though it did not reach statistical significance. Horizontal bars represent median values. (Statistical tests used –Two way ANOVA, Mann Whitney test, NS-not significant, *- $p \leq 0.05$).

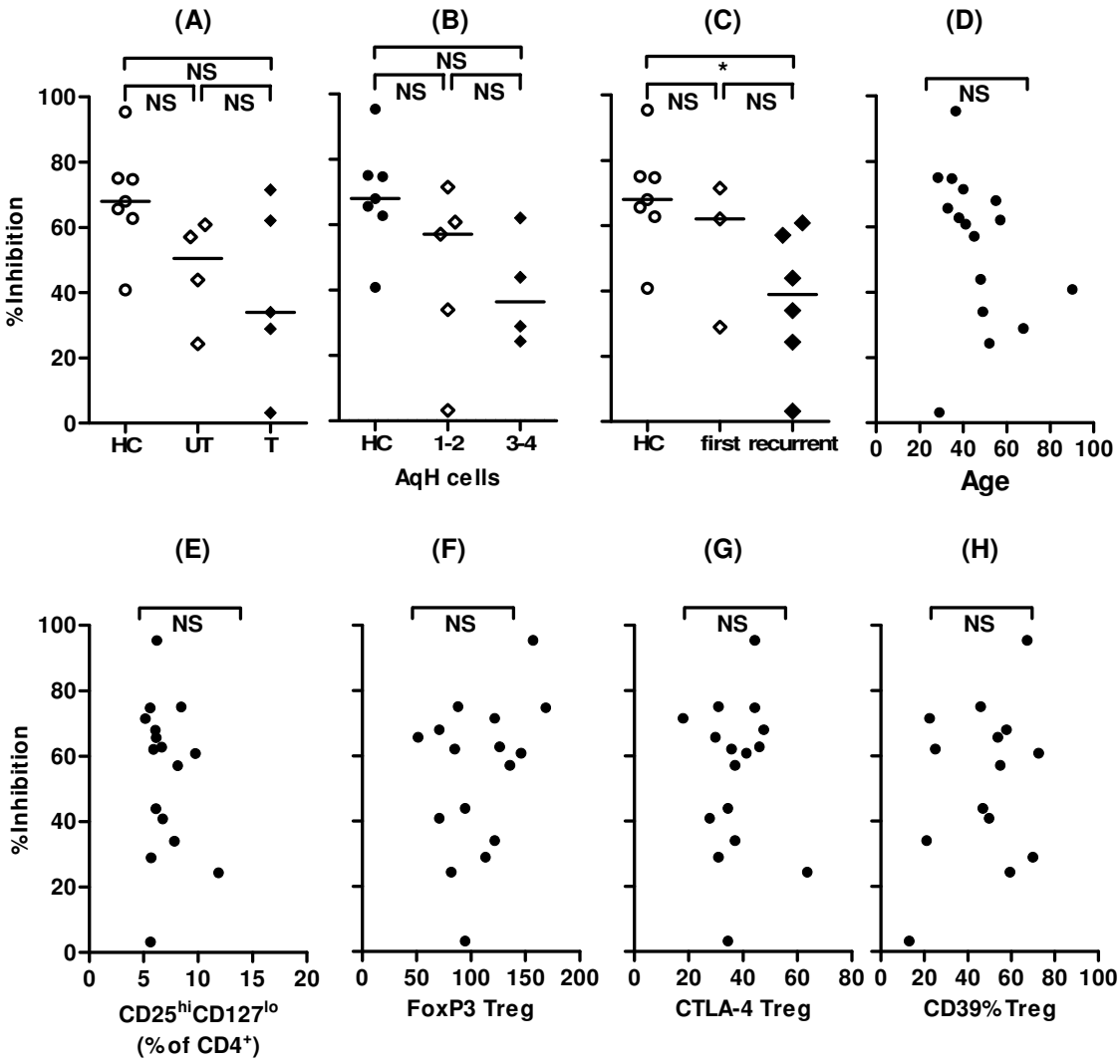


Fig: 4.9 Functional analysis of Treg from different groups of AAU patients and correlation with phenotype

The suppressive activity of Treg from AAU at T cells: Treg ratio of 4:1 was not affected by (A) treatment (untreated-UT and topically treated-T) or (B) disease activity (based on AqH cells). (C) The defective suppressive function of Treg was more prominent in patients presenting with recurrent episodes of disease. Horizontal bars represent median values. The suppressive activity did not correlate with (D) age of the sample, (E) frequency of peripheral blood Treg and (F) FoxP3, (G) CTLA-4 and (H) CD39 expression of Treg. (Statistical test used- Kruskal Wallis test, correlation, NS- not significant, *- $p \leq 0.05$)

4.8 Comparison of Treg function from chronic and acute anterior uveitis

Treg have been shown to control the transition from acute to chronic disease in a murine model of arthritis (Frey *et al*., 2010). AU patients were shown to have a defective suppressive function compared to healthy controls in the previous chapter (Fig 3.15). When comparing the suppressive activity of Treg from AAU and AU patients, it was observed that Treg from chronic patients showed significantly diminished suppressive function compared to those from AAU especially at lower Tconv: Treg ratios (2:1 and 4:1) (Fig:4.10 A&B).

Patient no: / Sex	**Laterality**	**AqH cells**	**First/ recurrent episode**	**Age**	**Treatment**
1/M	U	3	Recurrent	28.5	Predforte, Cyclo
2/F	B	0	Recurrent	32.9	Maxidex, Cyclo
3/F	U	0	Recurrent	54.1	None
4/F	B	1	Recurrent	75.2	None
5/F	B	1	Recurrent	55.0	None
6/F	B	1	Recurrent	34.9	None
7/M	B	0	Recurrent	36.6	Vexol

Table 4:4 Baseline characteristics and clinical features of chronic anterior uveitis samples analyzed for the suppressive function of Treg.

Laterality of the disease (unilateral/U or bilateral/B), anterior chamber activity recorded at the time of sampling, first/recurrent episode, treatment and mean age shown above. (Cyclo-cyclopentolate)

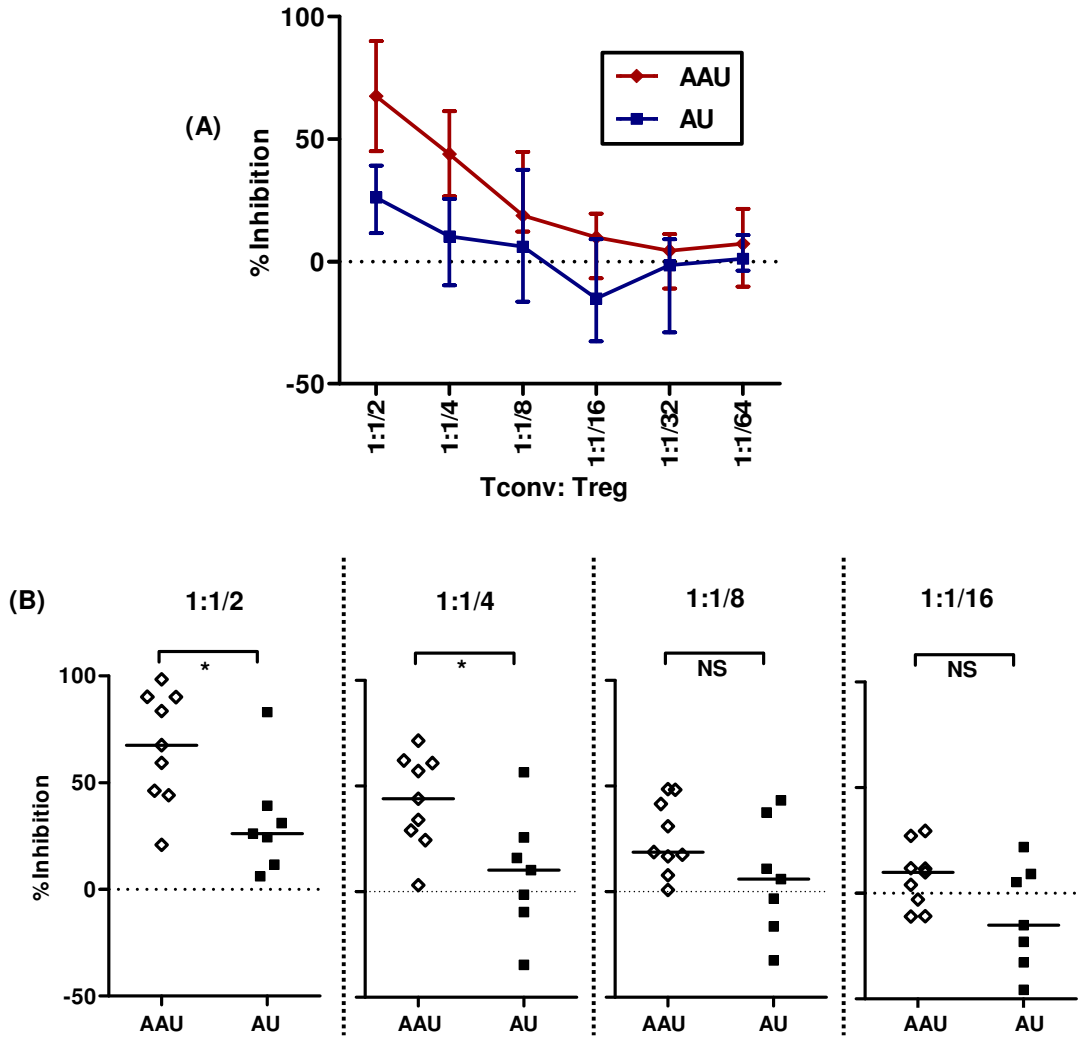


Fig: 4.10 Comparison of suppressive function between Treg from AAU and AU

(A) Graph representing the percentage inhibition of proliferation of Tconv by Treg isolated using dynabeads at Tconv: Treg ratios of 1:1/2, 1:1/4, 1:1/8, 1:1/16, 1:1/32 and 1:1/64 from AAU and AU. The results are represented as median with interquartile range (B) Treg from AU patients showed significantly reduced suppressive capacity compared to those from AAU especially at low Tconv: Treg (1:1/2 and 1:1/4) ratios Horizontal bars represent median values. (Statistical tests used –Two way ANOVA, Mann Whitney test, NS-not significant, *- $p \leq 0.05$).

4.9 Discussion

The body of work presented in this chapter showed an increased frequency of Treg in the peripheral blood of idiopathic non infectious AAU and APU patients (Fig: 4.1). This was similar to the findings in EAU where they showed an increased frequency of Treg during the peak of inflammation (Sun *et al*., 2010a; Sun *et al*., 2010b). Also in Behcet's disease an increased frequency of Treg was observed in patients with active disease compared to those in remission stage and healthy controls (Hamzaoui *et al*., 2006). Similar results were found in SLE where an increased frequency Treg cells in the peripheral blood of active patients were observed (Yan *et al*., 2008; Bonelli *et al*., 2008b).

Interestingly, the increased frequency of Treg observed in idiopathic acute uveitis is in contrast to what was already published in human uveitis by Yeh *et al.* where a decreased frequency of Treg in the active uveitis patients (Yeh *et al*., 2009) was shown. Several factors account for this difference. While Yeh *et al.* analysed mainly Treg from pan and intermediate uveitis patients, my study involved mainly non-infectious idiopathic AAU patients and some APU patients. Also the patients in the Yeh *et al.* study demonstrated evidences of associated autoimmune diseases such as sarcoidosis, multiple sclerosis, VKH, Behcet's disease and mixed connective tissue disease, each of which has been associated with abnormal Treg cell populations (Idali *et al*., 2008; Venken *et al*., 2008b; Chen *et al*., 2008; Hamzaoui *et al*., 2006; Barath *et al*., 2006). These factors may account for the different results in mine and Yeh et al's study. A decreased frequency of Treg has also been observed in VKH patients by Chen *et al.* (Chen *et al*., 2008). This study also included patients with other systemic autoimmune diseases and analysed Treg only on the basis of CD4 and CD25

expression. In the body of work described in this chapter, I have introduced more stringent gating strategy including CD4, CD25 and CD127 to define Treg and the patients included in the study described in this chapter were all idiopathic in nature to avoid the effect of the associated systemic autoimmune disease on Treg phenotype and function.

It was also shown in SLE patients as well as asthma patients that glucocorticoid therapy could increase the frequency of Treg in the peripheral blood (Karagiannidis *et al*., 2004; Suarez *et al*., 2006). In this study however, patients undergoing systemic immunosuppressive treatment including corticosteroids were excluded. It is not yet clear whether topically applied glucocorticoid could have any systemic effect on Treg frequency, phenotype and/or function. Glucocorticoid induced TNF receptor (GITR) is generally considered to be a Treg specific marker. However, my preliminary analysis revealed that GITR expression was not exclusive to the Treg population (Fig: 3.1) and hence this marker was not further analysed. In this study, the increased frequency of Treg was more prominent in patients who were not on any form of treatment, showing that it was not an artefact of topical glucocorticoid therapy. Interestingly, the increased frequency of Treg was more prominent in patients with recurrent diseases (Fig: 4.1E). In chronic myeloid leukaemia patients, increased frequency of Treg has been shown to correlate with disease relapse following allogenic stem cell transplant (Nadal *et al*., 2007). It would be interesting to see if the increased frequency of Treg in the peripheral blood could be used as a marker for recurrence in AAU. However this would require longitudinal analysis of a series of patients over a longer period of time.

The frequency of peripheral blood Treg in acute anterior uveitis patients were also higher compared to those with chronic disease (Fig: 4.5A). Similar to that was seen in chronic uveitis patients, there was no difference in the phenotype of Treg in AAU patients as compared to healthy controls. In Behcet's disease, the frequency of Treg has been shown to be decreased just before ocular attack and has been postulated as a possible marker for ocular attack (Nanke *et al*., 2008). In this study, only Treg from patients in the post-attack phase were analysed. It would be interesting to follow up some idiopathic AAU patients and analyse and compare the frequency and phenotype of their Treg cells in the pre and post ocular attack phase. In an EAU model, increased frequency and immuno-regulatory activity of Treg has been shown to be associated with the development and regression of the disease (Sun *et al*., 2010a). This is the first study in human uveitis to report an increased frequency of Treg in patients with acute, but not chronic idiopathic non infectious anterior uveitis. It has to be noted that there appeared to be a difference in the frequency of Treg between acute (APU) and chronic (pan) pan uveitis patients, but the small number of samples in the APU cohort might be the reason that it did not reach statistical significance.

The defective function of Treg cells have been associated with the active stages of various autoimmune inflammatory diseases (Valencia *et al*., 2007; Chen *et al*., 1994; Chen *et al*., 2008). In VKH disease, diminished suppressive function of Treg has been observed in patients with active uveitis (Chen *et al*., 2008). However, as stated earlier most of these studies isolated Treg solely on the basis of CD4 and CD25 expression only. In this study, I have employed stringent gating conditions including CD127 expression and investigated the functional capacity of CD4+CD25highCD127low Treg from idiopathic AAU patients as well as healthy controls to suppress the

proliferation of Tconv cells in response to polyclonal stimulation over a range of Tconv: Treg ratios. Treg from AAU patient showed a diminished suppressive capacity at a T cell: Treg ratio of 1:1/4. Even at other ratios, some of the patients showed a relatively lower suppressive capacity (Fig: 4.8). It is not clear whether these are the patients who have a higher chance of relapsing. Interestingly the defective Treg suppression evident at 4:1 ratio was more prominent in patients with recurrent disease.

This is the first study in human uveitis to show a diminished suppressive function of Treg in idiopathic acute anterior uveitis. In a recent report, Frey *et al.* showed that Treg controlled the transition from acute self—limiting to non- remitting destructive disease in a glucose-6-phosphate induced (G6PI) arthritis model (Frey *et al*., 2010). In this model they showed that transient depletion of Treg before immunisation prevented spontaneous resolution of disease which then developed into a chronic immune effector phase characterized by destructive joint disease which could no longer be controlled by Treg cells (Frey *et al*., 2010). This data suggested that Treg could not interfere in the acute phase but instead control the resolution of initial phase. It is interesting to note that in my study, the diminished capacity of patient Treg to suppress the proliferation of autologous T cells was more prominent in chronic uveitis patients as compared to acute uveitis patients. In this context, it would be interesting to follow up AAU patients and see if the Treg frequency and function could be an indicator of their tendency to develop into chronic disease.

5 REGULATORY T CELLS IN THE AQUEOUS HUMOR OF ACUTE UVEITIS PATIENTS

5.1 Introduction

Inflammatory activity in the anterior chamber is one of the main features of most forms of uveitis. Under resting non inflammatory conditions, the blood ocular barrier ensures that aqueous humor (AqH), the clear watery fluid that fills the space between cornea and iris (anterior chamber) is transparent and devoid of any cells. However under inflammatory conditions, the blood-ocular barrier breaks down (observed as cloudiness of AqH, known as flare) and leukocytes are recruited into the anterior chamber (Deschenes *et al*., 1988). Recruitment of lymphocytes with an activated phenotype into an otherwise immune privilege site is one of the fundamental paradoxes of ocular immunology. It has been shown that there was a predominance of T cells compared to B cells in the AqH of uveitis patients (Muhaya *et al*., 1998). An increased CD25 expression which was thought to be an activation marker was also observed in AqH T cells from uveitis patients (Deschenes *et al*., 1988; Dick *et al*., 1999). However CD25 is also a marker for Treg cells. Accumulation of Treg cells at the site of inflammation have been observed in various autoimmune and inflammatory diseases. In EAE, Treg isolated from target organ at the peak of the disease has been shown to be poor suppressors, possibly because of the inflammatory microenvironment (Kerr *et al*., 2008a). In rheumatoid arthritis and multiple sclerosis, increased frequency of activated Treg has been reported in the synovium and CSF

respectively (van Amelsfort *et al*., 2004; Cao *et al*., 2004; Feger *et al*., 2007; Korn *et al*., 2007).

In the case of uveitis, presence of regulatory T cells among the ocular infiltrating T cells has been observed in animal models. CD4+ T cells that express FoxP3 have been shown to be present among retinal infiltrates relatively early in the course of EAU. However it was not clear whether they become functional in limiting inflammation (Kerr *et al*., 2008b). In addition, several AqH factors such as TGF-β and α-MSH have been shown to induce the generation of Treg cells (Nishida and Taylor, 1999; Taylor *et al*., 1997). In Lewis rats immunised with retina specific antigens, accumulation of Treg within the eye was observed with the resolution of first attack. Moreover, the Treg from monophasic EAU were more potent *in vitro* in their suppressive function compared to those from recurrent EAU (Ke *et al*., 2008).

In human uveitis, studies on regulatory T cells have only been conducted in peripheral blood, but not in AqH due to the difficulty in obtaining the sample and to the very small sample size. The aim of the experiments detailed in this chapter is to analyse whether Treg cells are present in AqH from uveitis patients and if present, to characterise their phenotype. I also aim to analyse the effect of inflammatory microenvironment on the phenotype and function of Treg and see if the AqH Treg are functionally active.

5.2 Identification and phenotypic analysis of AqH Treg

AqH and peripheral blood were collected from patients presenting themselves to the A&E with an acute inflammation in the eye characterised by redness, pain and photophobia. The AqH as well as PBMC isolated from peripheral blood were stained with Treg specific markers to identify CD4+CD25highCD127low Treg cells (Fig: 5.1A). In the previous chapter, I have shown an increased frequency of Treg cells in the peripheral blood from acute patients compared to healthy controls. However it is not possible to compare AqH from acute uveitis patients and healthy controls, as AqH from a healthy eye is generally acellular. Of the 20 AqH samples collected, 3 were acute pan uveitis samples and the rest were acute anterior uveitis (AAU) samples. None of the acute anterior uveitis patients were on systemic glucocorticoid treatment and 2 out of 3 pan uveitis patients were on systemic glucocorticoid treatment. The clinical details of these patients are explained in Table: 5.1.

Similar to published literature (Deschenes *et al*., 1988; Calder *et al*., 1999), the AqH infiltrates from the uveitis eye contained large number of CD4+ lymphocytes (Fig: 5.1B). CD4+CD25highCD127low Treg cells were also observed in the ocular infiltrates. Interestingly, the frequencies (of CD4+ T cells) of Treg in the AqH from acute anterior uveitis patients (median±SD: 14.57±3.2) were significantly higher compared to their peripheral blood counterpart (median±SD: 8.5±2.9) (Fig: 5.1B). There appeared to be no difference in the frequency of Treg in the AqH of pan uveitis patients. However, due to the small sample size and also due to the fact that 2 out of 3 patients in this group were on systemic glucocorticoid therapy (which could affect the

Treg frequency in peripheral blood), it was impossible to determine if there were any significant difference in frequency of Treg from the AqH of these patients (Fig: 5.1B). Interestingly AAU patients undergoing topical glucocorticoid treatment showed increased frequencies of Treg in their AqH compared to untreated patients (Fig: 5.1C).

5.3 AqH Treg have memory phenotype

T lymphocytes infiltrating the AqH have been shown to be primed (Curnow *et al*., 2004b). AqH cells were stained with CD45RO, a memory T cell marker (Fig: 5.2A). It was observed that the CD4+ Treg as well as Tconv population from AqH were composed almost entirely of antigen experienced cells expressing CD45RO (memory/primed), as compared to the peripheral blood T cells that contained both primed and naive (CD45RO-) cells (Fig:5.2B&C). T cells from AqH of acute uveitis patients have been reported to have increased CD69 (Dick *et al*., 1999) expression indicating their activated phenotype. However, Treg from peripheral blood and AqH of patients analysed in this study expressed very little CD69 and there was no significant difference in the CD69 expression between Treg from AqH and peripheral blood (Fig: 5.2D&E)). There was also no difference in the CD69 expression between Tconv cells from peripheral blood and AqH of the patients (Fig: 5.2D&F).

Patient no:/ uveitis/ sex	No: AqH cells	Laterality	AqH Cells	Fist/ Recurrent Episode	Treatment
1/a/M	6300	U	3	Recurrent	None
2/a/M	5100	U	3	first episode	None
3/a/M	8800	U	3	Recurrent	dex1%, cyclo 1 %
4/a/M	2660	U	1	Recurrent	None
5/a/M	5370	U	3	Recurrent	Pred 1%, cyclo 1%
6/a/M	1440	U	2	Recurrent	None
7/a/M	840	U	3	first episode	None
8/a/F	1580	U	3	Recurrent	G Predforte, G Cyclo 1%
9/a/F	3630	U	3	Recurrent	None
10/a/F	1940	U	3	Recurrent	None
11/a/F	3630	U	3	Recurrent	None
12/a/F	660	U	2	Recurrent	None
13/a/F	2300	U	2	Recurrent	Predforte
14/a/M	2450	U	2	Recurrent	Predforte
15/a/M	1940	B	2	Recurrent	None
16/a/M	2300	U	3	Recurrent	None
17/a/M	1130	U	3	Recurrent	None
18/p/M	8250	U	3	Recurrent	None
19/p/M	14300	B	3	Recurrent	IV methylpred, rimexolone
20/p/F	670	U	3	Recurrent	prednisolone 10mg

Table: 5.1 Clinical features and phenotype of Treg from peripheral blood and AqH of acute uveitis samples analyzed by flow cytometry.

Anterior (a) or pan (p) uveitis samples, no: of AqH cells per sample, laterality of the disease (unilateral/U or bilateral/B), anterior chamber activity recorded at the time of sampling, first/recurrent episode, treatment etc shown above.

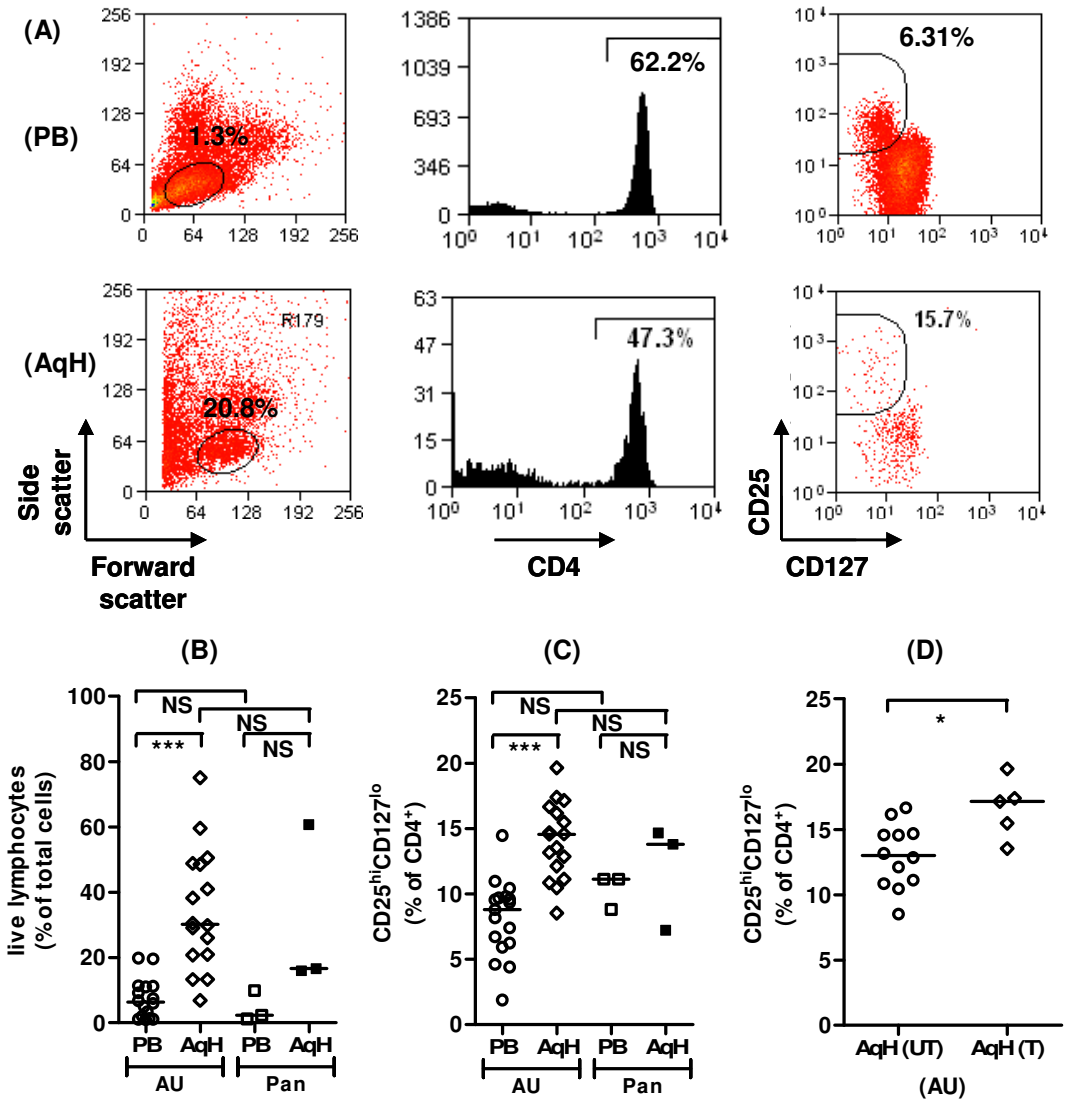


Fig: 5.1 Increased Frequency of regulatory T cells in AqH of AAU patients

(A) Staining of PBMCs from peripheral blood (PB) and aqueous humour (AqH) cells from acute uveitis patients with Treg markers to identify CD4+CD25high CD127low Treg. (B) Majority of the AqH cells were lymphocytic in nature. (C) Frequency of Treg in the AqH of AAU patients was significantly higher compared to their own peripheral blood. No difference in the frequency of Treg between PB and AqH of APU patients was observed. (D) Increased frequency of Treg in the AqH of AAU patients undergoing topical glucocorticoid treatment (T) compared to untreated patients (U). Horizontal bars represent median values. (Statistical tests used- Mann-Whitney test, Kruskall Wallis test, NS-not significant, *- $p<0.05$, ***- $p<0.001$).

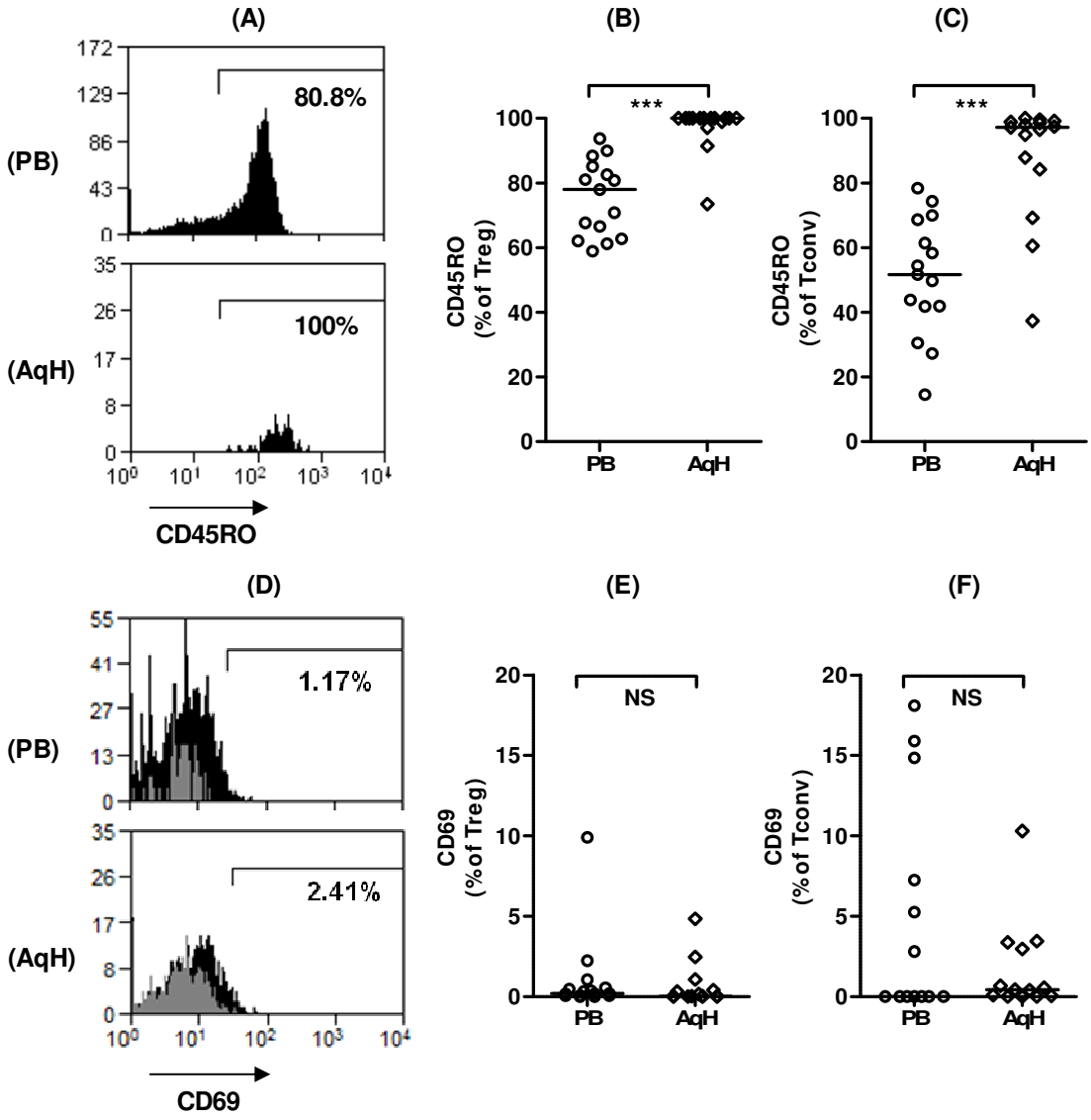


Fig: 5.2 AqH Treg have memory phenotype

(A) CD4+CD25highCD127low Treg cells from peripheral blood (PB) and aqueous humour (AqH) of AAU patients were analysed for CD45RO expression. Almost all the (B) Treg and (C) Tconv cells in the AqH had a memory phenotype (CD45RO+). (D) Treg from peripheral blood and AqH of acute uveitis patients did not express significant amount of CD69 (black curve-CD69 antibody and grey curve-isotype control). (E) No significant difference in the frequency of CD69+ Treg or (F) Tconv from peripheral blood and AqH of acute patients. Horizontal bars represent median values. (Statistical test used- Mann-Whitney test, ns-not significant, ***- $p<0.001$).

5.4 Increased FoxP3 expression of AqH Treg

Expression of intracellular FoxP3, the most important Treg marker was analysed on AqH Treg. Almost all the cells classified here as Treg expressed FoxP3. As almost all the AqH T cells had a memory phenotype (CD45RO+), only the memory Treg and Tconv cells from AqH were analysed for FoxP3 (Fig: 5.3A). It was observed that the CD45RO+ AqH Treg expressed significantly higher FoxP3 (4-5 fold increase) compared to peripheral blood Treg in acute patients (Fig: 5.3B). Of the 8 patients where the AqH was analysed for FoxP3 expression, there were 6 anterior uveitis and 2 pan uveitis samples (of which one was on systemic glucocorticoid treatment). Of the AAU AqH samples, 3 of them showed a clearly higher FoxP3 expression compared to others. However this was not due to the difference in their treatment status or sex of the patients, or laterality (unilateral/bilateral) of the disease. On the other hand, the conventional T cells from AqH did not express any significant increase in FoxP3 as compared to those from peripheral blood (Fig: 5.3C&D).

5.5 Increased CTLA-4 expression of AqH Treg

Similar to FoxP3, intracellular CTLA-4 expression was also analysed by flow cytometry. Even though the peripheral blood Treg from AAU patients showed no difference in the CTLA-4 expression compared to healthy controls, primed Treg from AqH expressed significantly higher levels of CTLA-4 compared to their peripheral blood counterparts (Fig:5.4A&B). Interestingly, Tconv cells from AqH also expressed increased levels of CTLA-4 compared to peripheral blood Treg (Fig: 5.4C&D). Of the 6 patients analysed here, only one patient was on topical glucocorticoid therapy and

the other 5 were not on any treatment and hence any effect of glucocorticoid therapy on CTLA-4 expression could not be analysed.

5.6 Increased frequency of CD39+ Tconv from AqH

Decreased frequency of CD39+ Treg and their defective function in controlling IL-17 production has been implicated in MS patients (Fletcher *et al*., 2009). However, CD39 expression of Treg was not different between Treg from AqH and peripheral blood of AAU patient (Fig: 5.5A&B). Interestingly, conventional T cells from AqH expressed increased levels of CD39 compared to their peripheral blood counterparts (Fig: 5.5C&D). There was only one pan uveitis sample available for this analysis and was undergoing systemic glucocorticoid treatment at the time of sampling. Of the anterior uveitis patients, only 2 were on topical glucocorticoid treatment and hence any effect of glucocorticoid therapy on the CD39 expression could not be analysed.

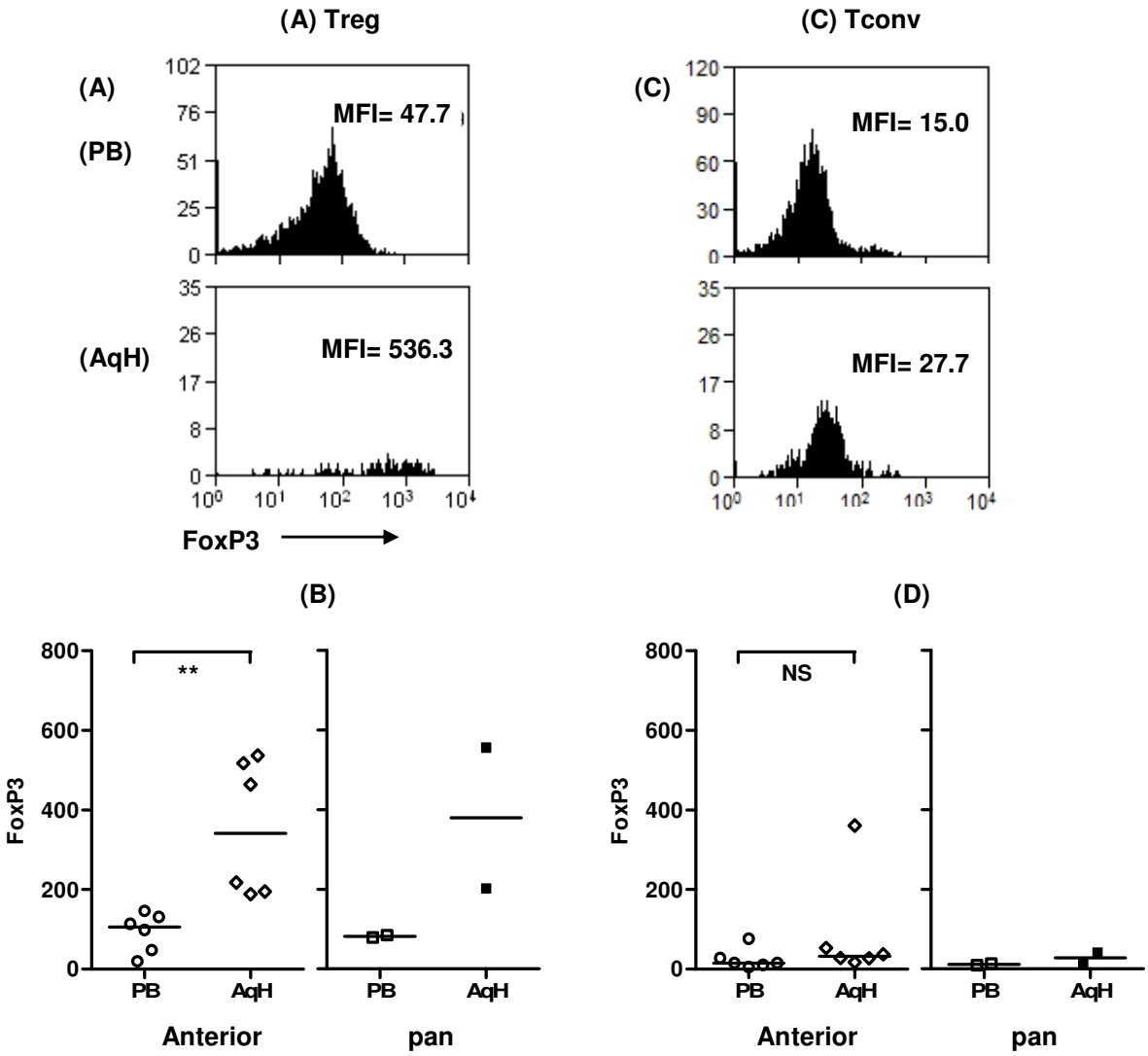


Fig: 5.3 Increased FoxP3 expression on Treg from uveitis AqH

(A) Representative histogram showing FoxP3 expression of CD45RO+ Treg from peripheral blood and AqH of AAU patients. (Median fluorescent intensity-MFI of FoxP3 for individual samples shown on the histograms). (B) Increased FoxP3 expression of Treg from AqH of AAU patients. Only 2 pan uveitis patients were available for FoxP3 analysis and hence any significant difference could not be observed. (C) Representative histogram showing FoxP3 expression of memory Tconv from peripheral blood and AqH of AAU patients. (D) No significant difference between the FoxP3 expressions of Tconv cells from PB and AqH of acute uveitis patients. Horizontal bars represent median values. (Statistical test used- Mann Whitney test, NS-not significant, ** - $p<0.01$).

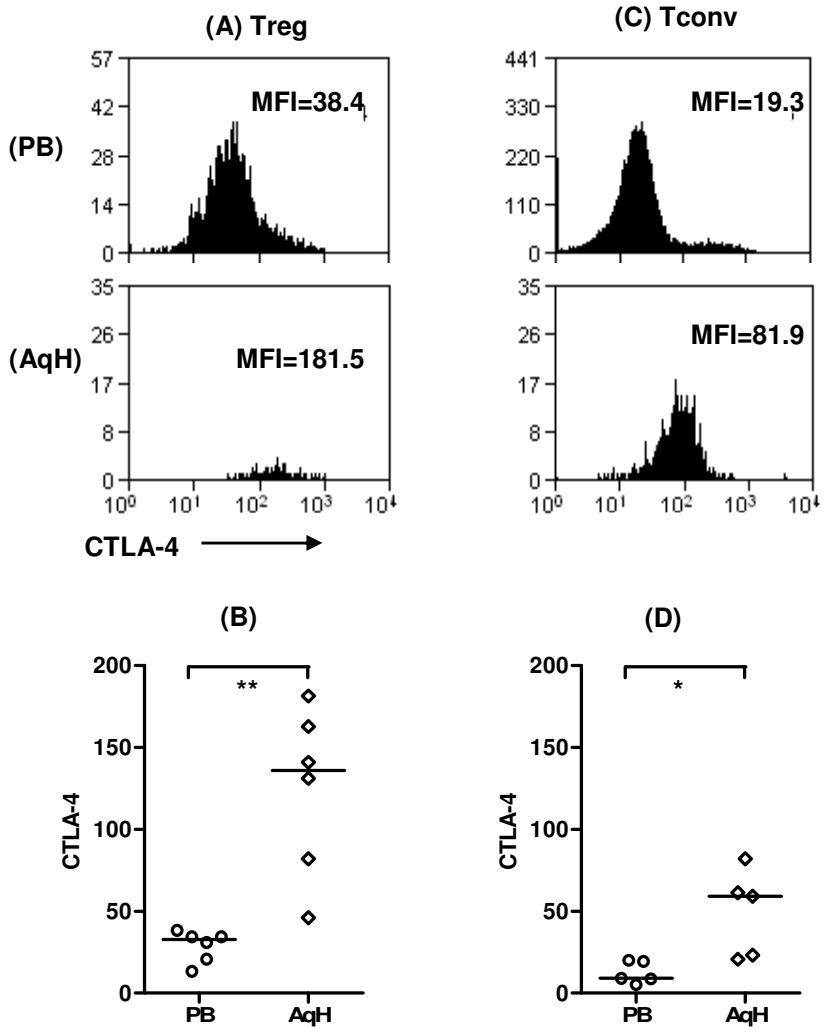


Fig: 5.4 Increased CTLA-4 expression on T cells from uveitis AqH

(A) Representative histogram showing CTLA-4 expression of CD45RO+ Treg from peripheral blood (PB) and aqueous humor (AqH) of AAU patients. (Median fluorescent intensity (MFI) of CTLA-4 for individual sample shown on the histograms). (B) Increased CTLA-4 expression of Treg from AqH of AAU patients. (C) Representative histogram showing CTLA-4 expression of CD45RO+ Tconv cells from peripheral blood and AqH of AAU patients. (D) Increased CTLA-4 expression of Tconv cells from AqH of AAU patients. Horizontal bars represent median values. (Statistical test used- Mann Whitney test, NS-not significant, *- $p<0.05$, ** - $p<0.01$).

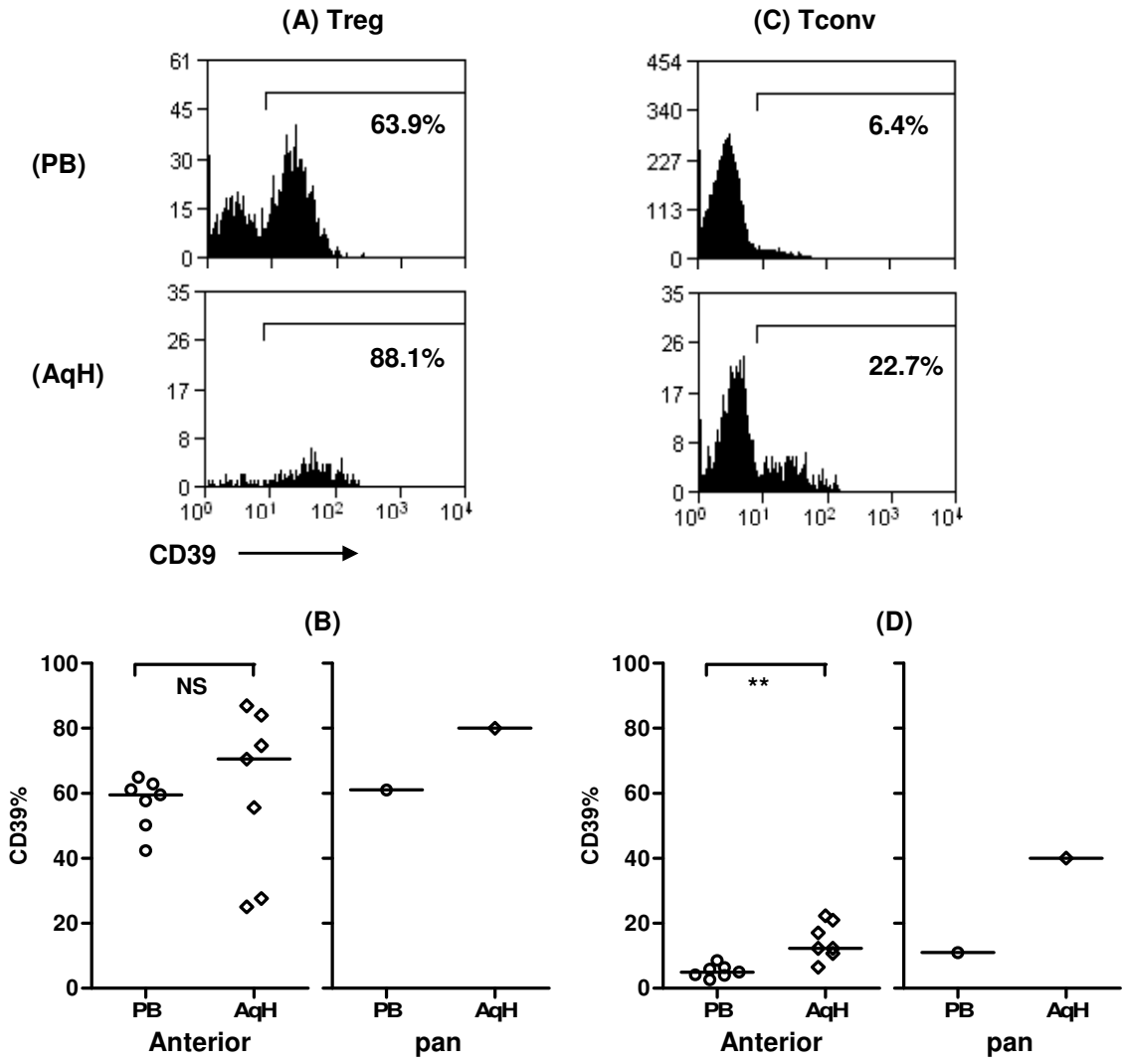


Fig: 5.5 Increased frequency of CD39+ Tconv cells from uveitis AqH

(A) Representative histogram showing CD39 expression of CD45RO+ Treg from peripheral blood and AqH of acute uveitis patients. (Percentage of CD39+ cells for individual samples shown in the histograms). (B) No difference in the frequencies of CD39+ Treg from AqH and PB of AAU patients. (C) Representative histogram showing CD39 expression of CD45RO+ Tconv cells from peripheral blood and AqH of AAU patients. (D) Increased frequencies of CD39+ Tconv cells from AqH of AAU patients. Only one pan uveitis patients was available for CD39 analysis and hence any significant difference could not be observed. Horizontal bars represent median values. (Statistical test used- Mann Whitney test, NS-not significant, ** - $p<0.01$).

5.7 Activation up regulated FoxP3 expression

Activated Tconv cells could express FoxP3 transiently, and also activated Treg cells could up regulate their FoxP3 expression (Wang *et al*., 2007). It is possible that the increased FoxP3 expression observed in AqH Treg could be due to their activated state. To test this hypothesis, Treg and Tconv cells from healthy controls were isolated and activated *in vitro* in the presence of anti-CD3/CD28 coated beads for different time periods (Fig:5.6A&B).

As has been reported in the literature (Walker *et al*., 2003b; Wang *et al*., 2007), human CD4+CD25lowCD127high Tconv cells upregulated their FoxP3 expression upon activation (Fig:5.6C). Treg expressed higher FoxP3 levels before activation and similar to Tconv cells, upregulated their Foxp3 expression upon activation which reached its peak by day 2 and was similar to what was seen in AqH Treg population (median±SD: 1104.4+59.7) (Fig: 5.6C). However, by day 3 (72 hours), FoxP3 expressions of both Treg and conventional T cells started coming back to resting levels (Fig: 5.6C). It has to be noted that during this transient upregulation of FoxP3 expression, Treg maintained the higher levels of FoxP3 expression compared to Tconv cells. The Tconv cells did not at any time point express similar FoxP3 expression to Treg, indicating that the AqH Treg might be recently activated Treg and not activated Tconv cells.

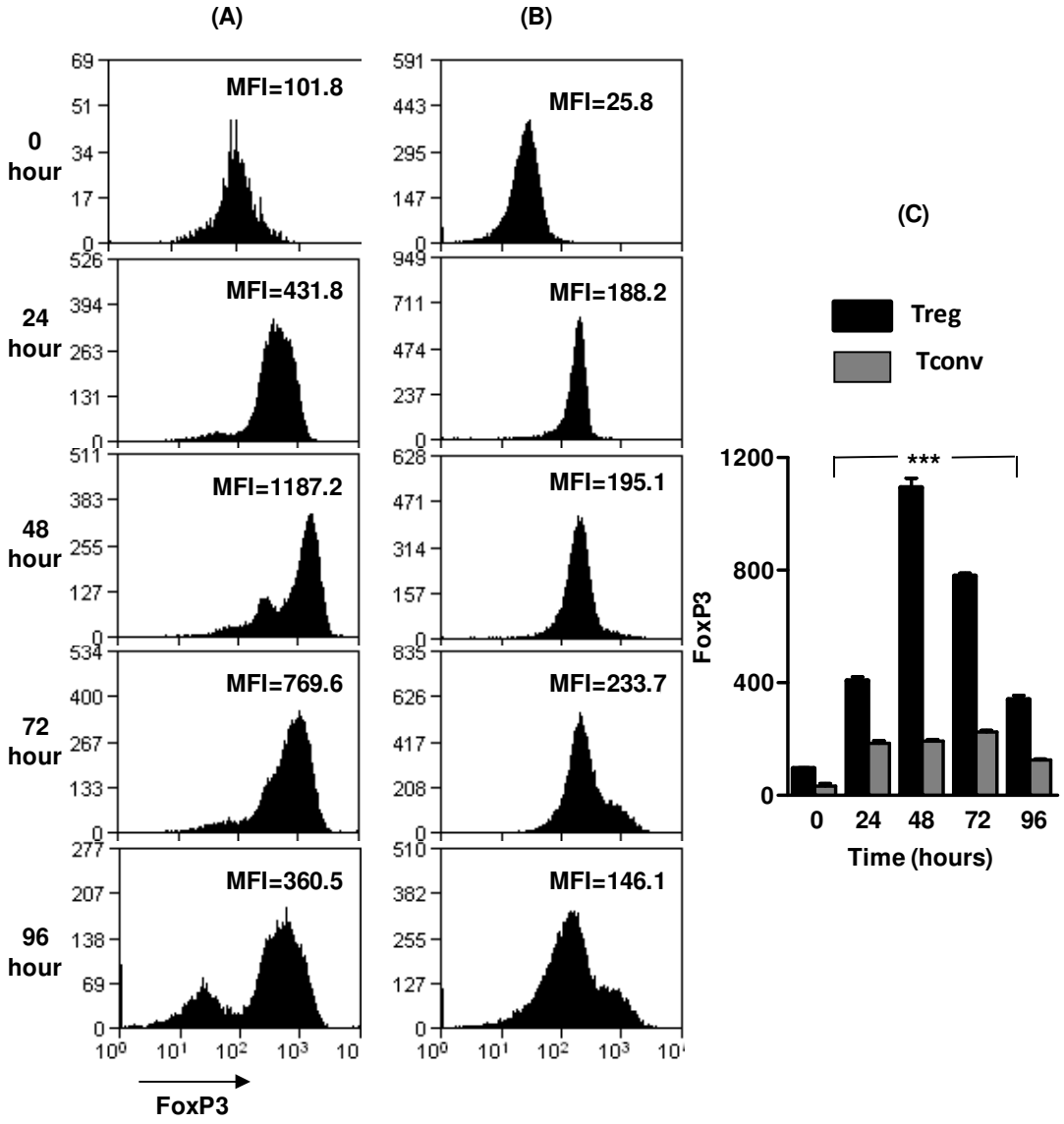


Fig: 5.6 Activation upregulated FoxP3 expressions of both Treg and Tconv

FoxP3 expression of activated T cells in culture. (A) Treg and (B) Tconv cells from peripheral blood of healthy controls activated using anti CD3/CD28 coated dynabeads for 4 days and FoxP3 expression of both populations analysed at different time intervals. (C) Activation upregulated FoxP3 expression of both Treg (black bars) and Tconv (grey bars). FoxP3 expression reached its peak by day 2 for Treg following which it started coming down to resting levels. FoxP3 expression of Tconv was upregulated, but never as much as Treg. (Statistical test used- Kruskal Wallis test, ***- $p \leq 0.001$)

5.8 Inflammatory cytokines and dexamethasone did not increase the FoxP3 expression *in vitro*

Cytokines in an inflammatory environment could affect the phenotype and function of Treg. Here, isolated peripheral blood Treg were cultured in serum free conditions (mimicking the AqH microenvironment) in 72 well micro well plates in the presence of cytokines including TNF-α, IL-6, IL-8, IL-1β and TGF-β2 which have been shown to be present in uveitis AqH (Fig:5.7A). The cytokines were added at concentrations similar to or greater than their biological concentrations found in the AqH. Treg were also cultured in the presence of dexamethasone, a topical glucocorticoid used in the treatment of uveitis (Fig: 5.7A.4). The aim of this experiment was to see if the increased FoxP3 expression observed in AqH Treg was in fact due to the effect of any of the above cytokines or topical glucocorticoid which comprise the AqH microenvironment. However, none of the cytokines or the glucocorticoid upregulated the FoxP3 expression of Treg *in vitro* (Fig: 5.7B). Any significant effect on FoxP3 expression was observed by the addition of IL-1β and there too the FoxP3 expression was decreased, not increased (Fig: 5.7B).

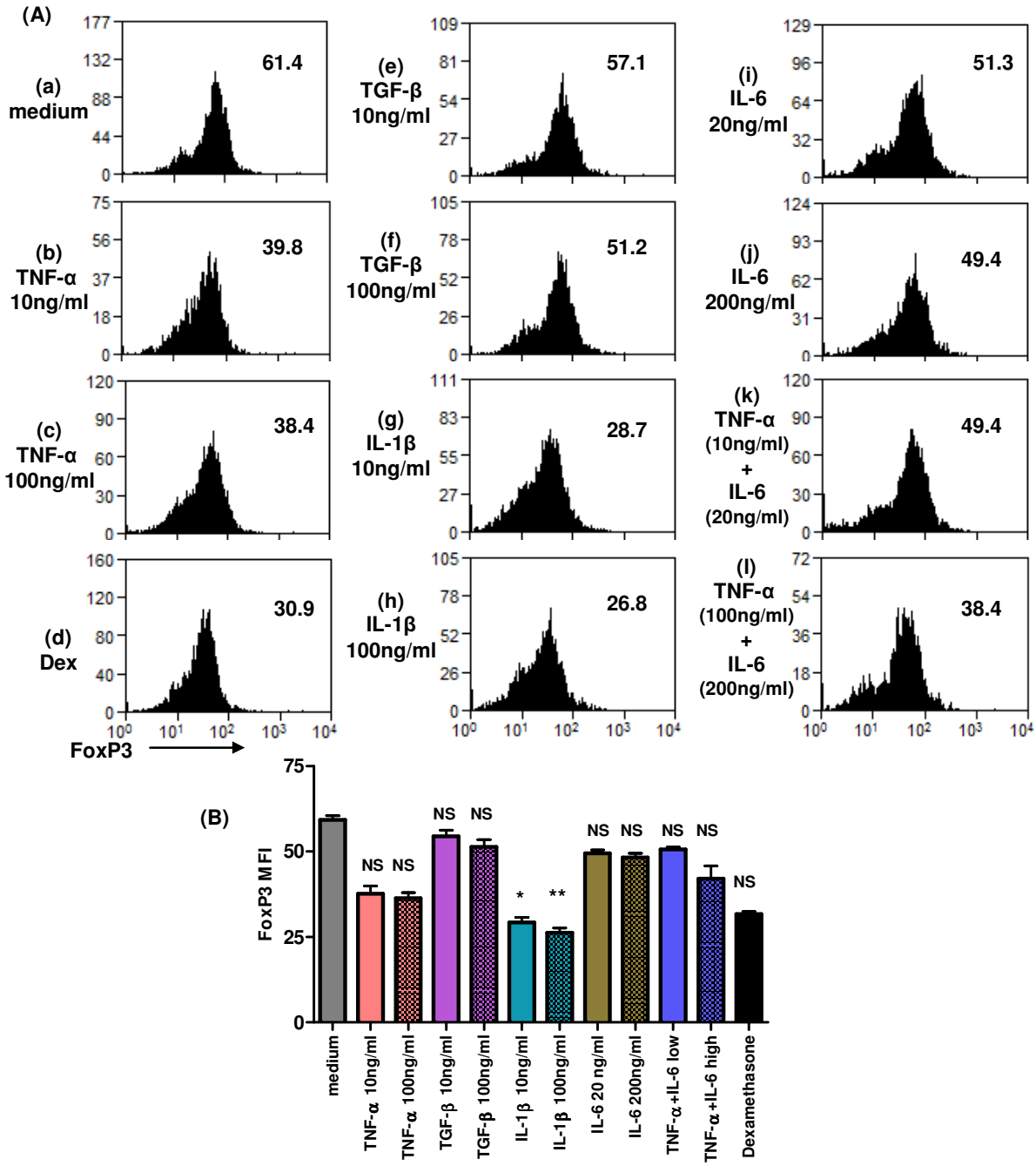


Fig: 5.7 Inflammatory cytokines and dexamethasone did not increase the FoxP3 expression of Treg *in vitro*

(A) Foxp3 expression of Treg cultured for 24 hours in serum free medium with or without Dexamethasone or with different concentrations of TNF-α, TGF-β2, IL-1β, IL-6, TNF-α or combinations of TNF-α and IL-6. (B) Pro-inflammatory cytokines at their physiological range did not increase the FoxP3 expression of Treg *in vitro*. Treg cultured with IL-1β showed decreased Foxp3 expression. (Statistical test used- Kruskal Wallis test, *- $p \leq 0.05$, **- $p \leq 0.01$, NS- not significant)

5.9 Uveitis AqH did not affect the FoxP3 expression of Treg

To analyse the effect of uveitis AqH microenvironment on FoxP3 expression, Treg cells were cultured in the presence of 6 AqH samples separately for 24 hours in serum free medium and the FoxP3 expression was analysed by flow cytometry (Fig:5.8A). The AqH samples were of varying disease activity and consisted of two 1+ samples, two 2+ samples and two 3+ samples (classified based on anterior chamber activity). There was no significant difference in the FoxP3 expression of Treg (Fig: 5.8B) or in the frequency of FoxP3+ Treg after culture with uveitis AqH (Fig: 5.8C). As Treg cells are more prone to apoptosis in vitro, the culture of these cells in serum free conditions are more likely to induce their apoptosis. This may be the reason for the biphasic expression of FoxP3 by Treg in this assay with the apoptotic Treg expressing lower FoxP3 levels.

5.10 Effect of non inflammatory AqH on T cells

To determine whether Treg can function normally in the presence of AqH, suppressive function of normal Treg cells in the presence of pooled control (non inflammatory) AqH was analysed. For this, CFSE labelled conventional T cells were co-cultured with unlabelled Treg with or without control AqH in 72 well micro plates for 4 days and the proliferation of CFSE labelled cells analysed by flow cytometry (Fig:5.9A). However, T cells could not survive in the presence of control AqH. In fact almost all the cells underwent cell death in the presence of control AqH with or without Treg cells as is evident from the very low number of live cells in the culture.

This is in concordance with the literature where non inflammatory AqH has been shown to have cytotoxic potential (D'Orazio *et al*., 1999).

5.11 Treg cells maintained their suppressive function in the presence of uveitis AqH *in vitro*

CFSE labelled conventional T cells were cultured in the presence or absence of AqH from 12 acute anterior uveitis patients (Fig: 5.10A). The AqH samples consisted of 6 samples with disease activity of 2+ and 6 samples with disease activity of 3+. All the samples were from patients who were not on any kind of treatment. Treg cells expressed normal suppressive function in micro-well plates in a final volume of 20µl (Fig: 5.10B). However unlike in control AqH, T cells survived better in uveitis AqH and the Treg cells expressed normal suppressive function in the presence of uveitis AqH.

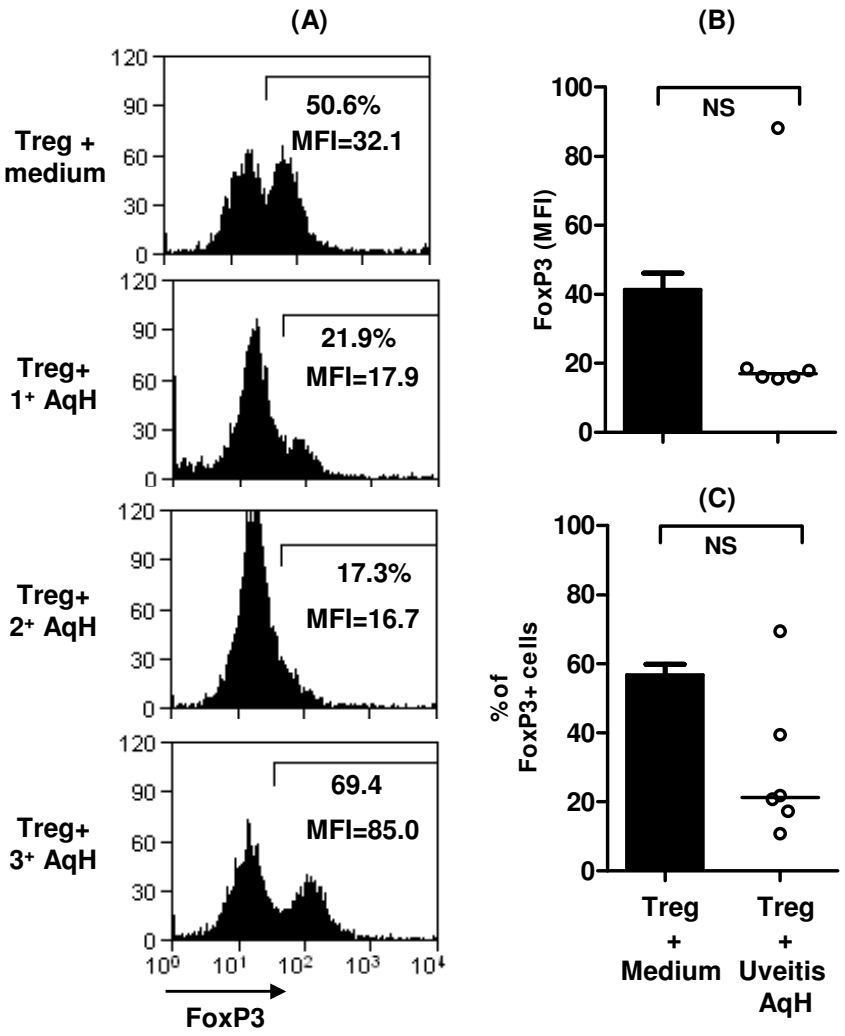


Fig: 5.8 Uveitis AqH did not affect the FoxP3 expression

(A) FoxP3 expression of CD4+CD25highCD127low Treg cultured in 72 well mini-tray for 24 hour in serum free medium in the presence of AqH with disease activities ranging from 1-3. (B) Uveitis AqH did not affect the FoxP3 expression of regulatory T cells. (C) Uveitis AqH did not affect the frequency of FoxP3 expressing Treg in the culture. Horizontal bars represent median values. (Statistical test used- Mann Whitney test, NS-not significant)

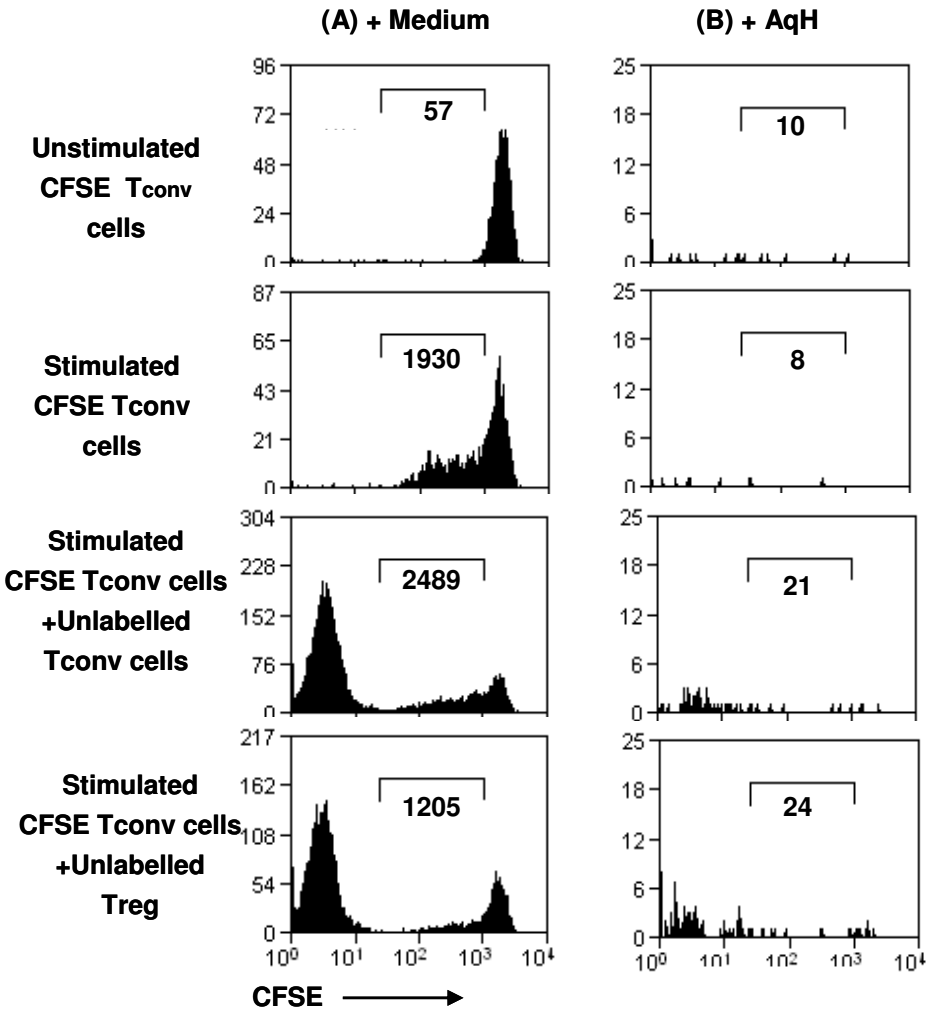
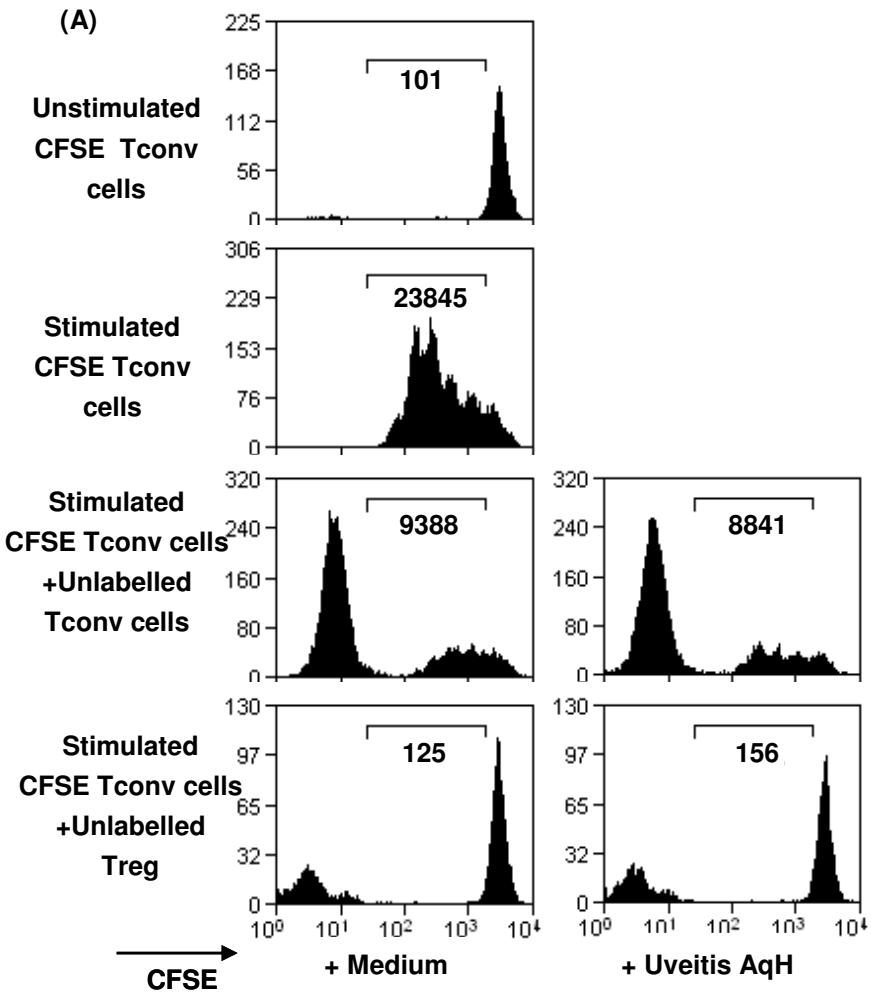


Fig: 5.9 Non-inflammatory AqH has cytotoxic activity on T cells

Representative data showing suppression assay in the presence of non inflammatory AqH. CFSE labelled Tconv were cultured in 72 well mini-trays with / without unlabelled Tconv cells or Treg cells either in the (A) absence or (B) presence of pooled control AqH. Number of proliferated CFSE labelled cells (analysed using counting beads) marked on the histogram. Non inflammatory AqH killed off CFSE labelled Tconv cells. Representative of n=2 experiments.



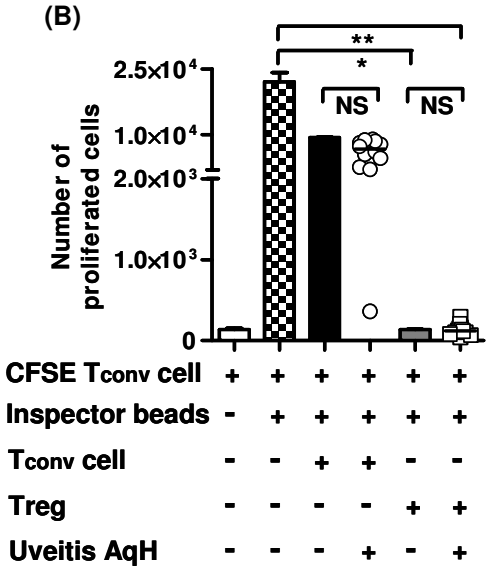


Fig: 5.10 Uveitis AqH did not affect Treg function

Polyclonal suppression assay carried out in the presence or absence of uveitis AqH. (A) Histogram representing the proliferation of CFSE-Tconv cells co-cultured with Treg or Tconv cells in the presence or absence of uveitis AqH. (B) Treg from a healthy control suppressed the proliferation of autologous Tconv cells. Uveitis AqH did not affect either the proliferation of Tconv cells or the suppressive function of regulatory T cells *in vitro*. Representative of n=2 experiments. (Statistical test used- Kruskal Wallis test, NS- not significant, *-$p \leq 0.05$. **-$p \leq 0.01$).

5.12 Analysis of suppressive function of AqH Treg

5.12.1 Optimisation of Treg depletion/ mock depletion

Due to the very small volume of AqH that can be obtained from each patient and the small number of cells present, it was not possible to isolate the Treg from AqH sample to analyse their suppressive function in a CFSE assay. Hence Treg cells were depleted from AqH samples and the proliferative response of the remaining cells were analysed. To optimise this assay, CD4+ T cells from healthy controls were isolated and depleted of CD25high Treg cells by incubating with anti CD25 antibody coated dynabeads (as explained in chapter 2). To optimise the number of beads that should be used to deplete Treg, different numbers (0, $1.6x10^4$, $8x10^4$, $16x10^4$, $32x10^4$ and $80x10^4$) of anti CD25 coated magnetic beads were added to $5x10^3$ T cells in a total volume of 50µl (Fig:5.11A). The cells were incubated at 4ºC with rolling and tilting for 30 minutes. The bead bound CD25+ cells were then separated out on a magnet. The remaining cells were then stained with CD4, CD25 and CD127 antibodies to determine the percentage of undepleted Treg left (Fig: 5.11A). The optimum depletion (with the least number of leftover Treg) was obtained by the addition of $8x10^4$ beads coated with anti CD25. As experiment control, similar number of cells were also subjected to a mock depletion process where cells were incubated with anti IgG coated beads at the same concentration as anti CD25 coated beads (Fig:5.11B). It was observed that mock depletion with anti IgG antibody did not deplete the Treg population.

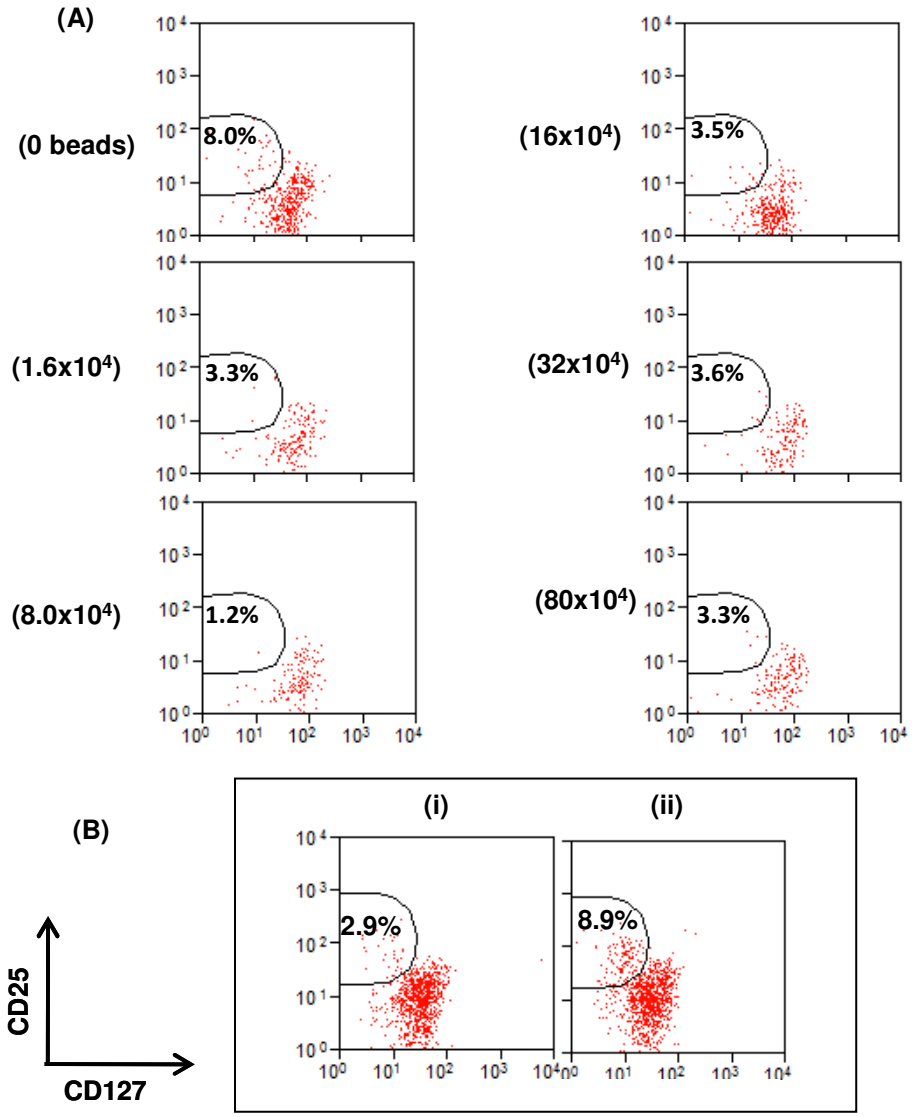


Fig: 5.11 Optimisation of Treg depletion / mock depletion from small number of cells

(A) Representative data showing Treg depletion using different number of anti CD25 coated beads. Cells were gated on CD4+ cells and the percentage of leftover CD4+CD25highCD127low Treg marked on histograms. The optimum depletion was observed (least number of leftover Treg present) when $8x10^4$ beads where used to deplete $5x10^3$ T cells off Treg.

Mock depletion was carried out as control using same number of sheep anti mouse IgG coated beads under similar conditions. (B) Histogram showing the percentage of leftover Treg cells following (i) Treg depletion and (ii) mock depletion.

5.12.2 Optimisation of time period for culturing depleted cells with Click-iT EdU

A normal CFSE based suppression assay was not possible with AqH Treg cells due to the small number of cells obtained. The proliferation of Treg depleted cells were analysed by culturing with Click-iT EdU (a less harsh BrdU alternative) and analysing its incorporation by flow cytometry. Due to the fact that AqH cells were prone to apoptosis and that EdU could be harmful to cells in culture, it was important to optimise the minimum incubation time required for culturing small number of cells with EdU. For this, CD4+ T cells were cultured with 10μM (company recommended concentration) click-iT EdU for different time periods (24, 36, 48 and 72 hours) with or without CD3/CD28 coated bead stimulation. The cells were then harvested, fixed, permeabilised and stained with azide to detect EdU. The detection was based on a click reaction, a copper catalyzed covalent reaction between an azide and an alkyne (EdU). The cells were also stained with a cell cycle stain, 7-AAD. Proliferating cells incorporated EdU into their DNA and the double positive cells for EdU and 7-AAD provided the percentage of cells in S-phase of DNA synthesis (Fig: 5.12). In this experiment, the unstimulated cells did not proliferate and the minimum time in which a proliferative burst could be seen among the stimulated cells was observed as 48 hours.

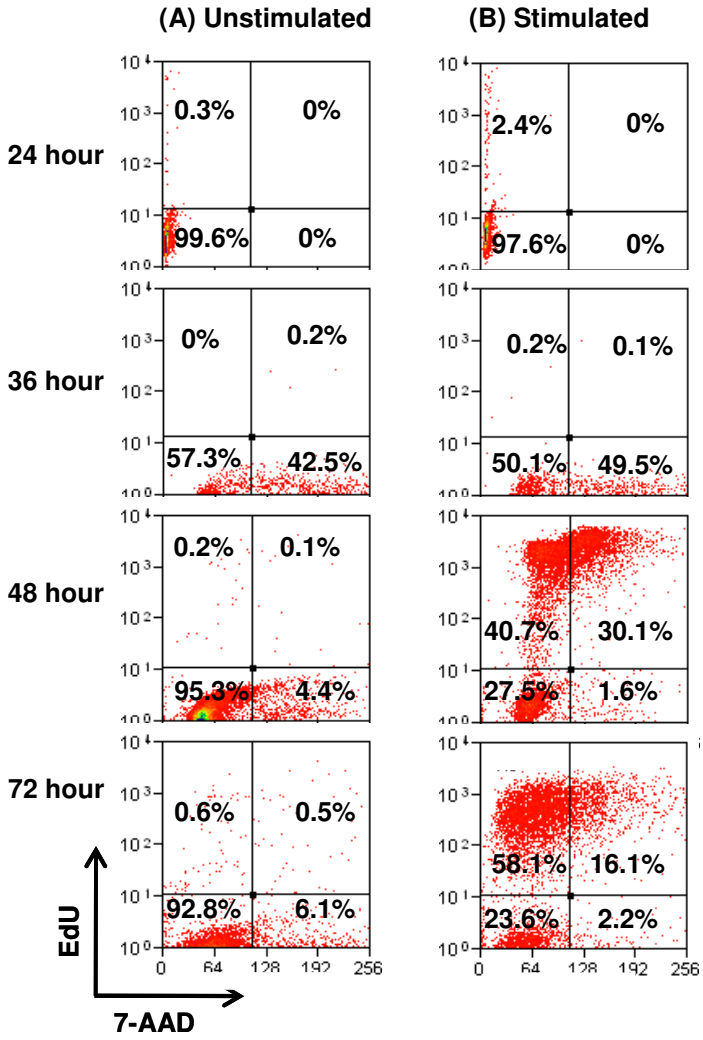


Fig: 5.12 Optimisation of time course to culture cells with click-iT EdU

CD4+ T cells from healthy controls were cultured with click-iT EdU for the duration of 24 hours, 36 hours, 48 hours and 72 hours and stained with azide (that recognizes EdU using a click-iT reaction) either (A) without stimulation or (B) stimulated with anti CD3/CD28 coated beads. Proliferating cells incorporated EdU during DNA synthesis and were positive for EdU. Cells entering S-phase of cell cycle were also identified using cell cycle staining dye, 7-AAD. The minimum period required to identify proliferating cells was found to be 48 hours. No proliferation was observed without stimulation.

5.12.3 Optimisation of Click-iT EdU concentration

As growth medium, cell density, cell type variations, and other factors could influence EdU labelling, it was also important to determine the optimum concentration of EdU required for *in vitro* culture with small number of cells under specific culture condition. Hence $5x10^3$ CD4+ T cells were cultured with different concentrations of EdU (1µM, 2µM, 5µM and 10µM) for 48 hours and stained with azide and cell cycle stain (Fig:5.13). Clear separation of proliferating cells which have incorporated EdU and non proliferating cells was observed at a concentration as low as 2µM EdU (Fig: 5.13).

5.12.4 Optimisation of culture conditions for depleted cells from AqH

Peripheral blood and AqH from acute anterior uveitis patients were collected. AqH cells were cultured with 2µM click-iT EdU with CD3/CD28 coated bead stimulation for 48 hours in micro well plates. EdU labelled CD4+ T cells from the peripheral blood of the same patients were also cultured alongside with CD3/CD28 stimulation as experiment control (Fig: 5.14). Since AqH cells were highly apoptotic *in vitro* and also due to the very small number of cells obtained from AqH, hanging drop method of culture was tested to ensure maximum contact between the cells in culture. Here the AqH cells were divided into two portions. One half was cultured in micro well plates up right and the other half was cultured in micro well plate which was then inverted to make a hanging drop culture. The plates were placed in a moist chamber to reduce evaporation of the medium. Peripheral blood T cells were also cultured under similar conditions. The peripheral CD4+ T cells proliferated well in normal and

hanging drop culture (Fig: 5.14A). However it was observed that AqH cells survived better and proliferated more in hanging drop culture (Fig:5.14B).

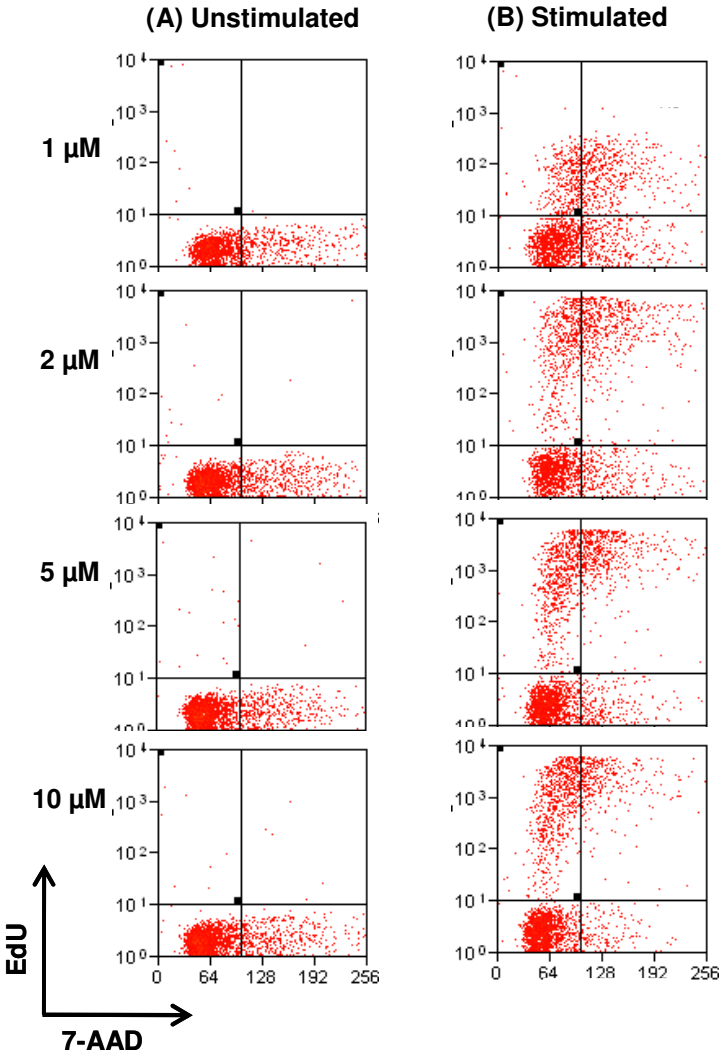


Fig: 5.13 Optimisation of EdU concentration

CD4+ T cells were cultured for 48 hours with different concentrations of click-iT EdU such as 1µm, 2µm, 5µm and 10µm either (A) without stimulation or (B) with stimulation with anti CD3/CD28 stimulation. The cells were then stained with pacific blue azide (that recognizes EdU using a click-iT reaction). Proliferating cells incorporated EdU during DNA synthesis and were positive for EdU. Cells entering S-phase of cell cycle were also identified using cell cycle staining dye, 7-AAD. Even at concentration as low as 2µM concentration of EdU, proliferating cells started becoming clearly visible following stimulation and subsequent staining. No proliferation was observed without stimulation.

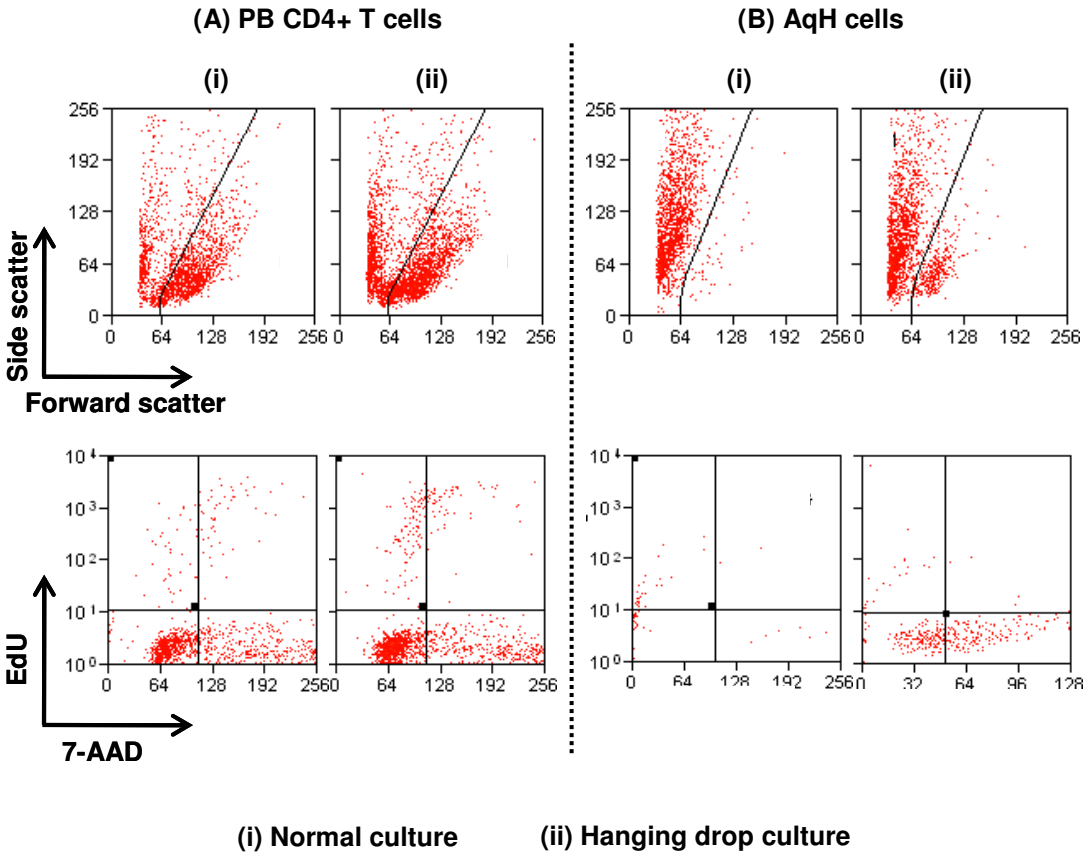


Fig: 5.14 Normal vs. hanging drop culture of depleted cells from AqH

CD4+ T cells from peripheral blood and AqH cells were cultured with stimulation in 72 well mini-trays in normal suspension cultures or as inverted hanging drop cultures in a moist chamber for 48 hours and stained with EdU and 7-AAD. (A) Culture of CD4+ T cells from peripheral blood as (i) normal culture or as (ii) hanging drop culture (B) Cells from AqH of AAU patient cultured in 72 well mini-trays as (i) normal culture or as (ii) hanging drop culture. Peripheral blood CD4+ T cells proliferated well in both normal and hanging drop cultures. However, AqH cells proliferated well in hanging drop culture compared to normal culture.

5.13 Depletion of Treg from AqH of AAU patients

Peripheral blood and AqH samples were collected from acute uveitis patients. As experiment control, CD4+ T cells from peripheral blood of the same patients were cultured in the presence of click-iT EdU with or without stimulation (Fig:5.15A&B). AqH cells were divided into two portions. One portion of AqH was depleted of Treg using anti CD25 coated beads and the other portion was mock depleted with anti IgG coated beads under similar conditions. The cells were then cultured with click-iT EdU for 48 hours as hanging drop cultures in moist chamber and then stained with azide and 7-AAD (Fig:5.15C&D). 4 AAU patients were recruited for this study. These patients had disease activities in the range of 2+ to 4+ and none of them were on systemic glucocorticoid treatment. However, no significant difference in the proliferation of depleted cells could be observed (Fig: 5.15E). This could probably be due to the very small number of cells in each sample. It is interesting to note that there was a tendency towards increased proliferation of Treg depleted cells in 3 out of 4 patient samples. However, it is also possible that this could be an experimental anomaly due to the small number of cells involved.

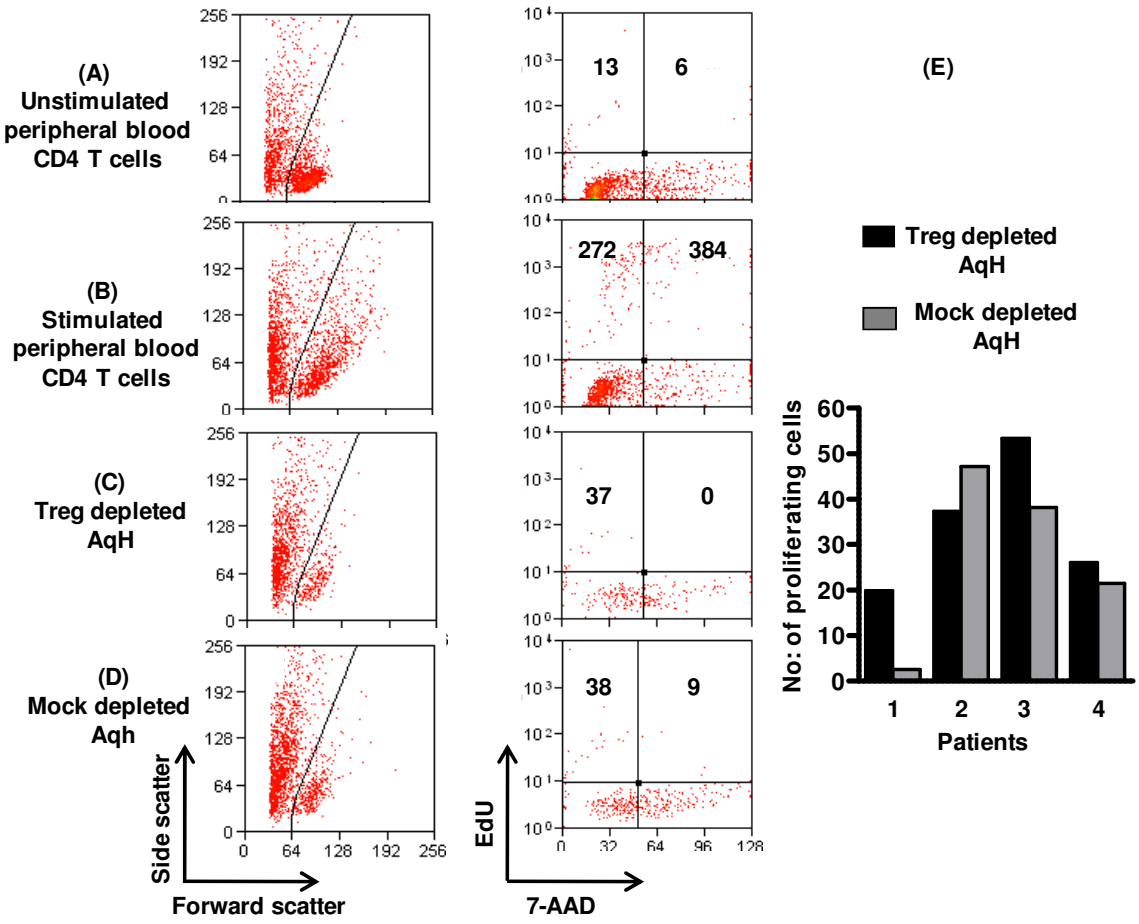


Fig: 5.15 Functional capacity of AqH regulatory T cells

CD4+ peripheral blood T cells were cultured in 72 well mini-trays for 48 hours with EdU (A) without or (B) with stimulation in hanging drop cultures. Proliferating cells were identified by staining with EdU and 7-AAD staining. T cells proliferated well with stimulation. AqH from the same patients were subjected to either (C) Treg depletion or (D) mock depletion and cultured for 48 hours with EdU. Proliferating cells were identified by staining with EdU and 7-AAD. (E) Result of AqH depletion assay from 4 patients. In 3 out of 4 patients, Treg depleted cells from AqH showed a tendency toward increased proliferation, though not significant.

5.14 Discussion

The ocular microenvironment, especially the aqueous humor (AqH), has the ability to suppress immune effector responses and inflammation which is believed to be important for the existence of ocular immune privilege (Mochizuki *et al*., 2000). AqH from non inflamed eye displays many immunosuppressive and anti–inflammatory properties *in vitro*. Various immunomodulatory factors in AqH like TGF-β and α-MSH have been shown to induce Treg generation *in vitro* (Cousins *et al*., 1991; Taylor *et al*., 1997; Taylor *et al*., 1992).

Here I have, for the first time observed an accumulation of Treg cells with a functional phenotype in the AqH of patients with non infectious acute anterior uveitis (Fig: 5.1). Studies in animal models of ocular inflammation had revealed similar accumulation of Treg in the inflamed eyes. In a rat model of uveitis, Ke *et al.* showed that following immunisation with retinal antigen, antigen specific Treg accumulated in the draining lymph nodes and the eye (Ke *et al*., 2008). Existence of a negative feedback system has been suggested in other autoimmune diseases where regulatory T cells are generated and triggered due to ongoing inflammation and accumulate at the site of inflammation (Korn *et al*., 2007; Cao *et al*., 2004).

Another interesting finding was the increased frequency of Treg in AqH of patients undergoing topical glucocorticoid therapy. Glucocorticoid therapy has been shown to upregulate the frequency and FoxP3 expression of Treg (Karagiannidis *et al*., 2004). Topical glucocorticoid therapy has been reported to directly induce CXCR4 upregulation of primed T cells in uveitis AqH (Curnow *et al*., 2004b). Zou *et al.* reported that CXCR4/CXCL12 signalling induced Treg trafficking to bone marrow

(Zou *et al*., 2004). CXCR4 has also been shown to be involved in the retention of leukocytes at the sites of chronic inflammation (Curnow *et al*., 2004b). Thus it is interesting to speculate that the increased CXCR4 induced by topical glucocorticoid therapy may be responsible for the increased retention of Treg within the inflamed eye and is one of the pathways by which glucocorticoid therapy resolves inflammation. In the rat model of EAU proposed by Ke *et al.*, accumulation of Treg in the eye correlated with the resolution of acute attack of the disease (Ke *et al*., 2008). However a longitudinal study of the patients will be required to confirm any such results in human uveitis.

Takase *et al.* established T cells clones (TCC) from ocular infiltrated T cells in non infectious uveitis patients and revealed that these TCCs were all memory activated Th1-like CD4+ cells (Takase *et al*., 2006b). In this study, I have shown similar results where AqH Treg cells from AAU patients were all found to be primed (CD45RO+) with a functional phenotype (Fig:5.2A&B).

One of the reasons for the increased FoxP3 expression of AqH Treg may be the effect of pro inflammatory milieu within the inflamed eye. Increased levels of pro-inflammatory cytokines like IFN-γ, IL-6, IL-8, TNF-α etc in the uveitis AqH have already been reported (Curnow *et al*., 2005; Lacomba *et al*., 2000; Takase *et al*., 2006a). Most studies reported that the inflammatory milieu have an inhibitory effect on the phenotype and function of Treg. TNF-α has been shown to down modulate Treg cells in rheumatoid arthritis (Valencia *et al*., 2006). IL-6, another pro inflammatory cytokine present in uveitis AqH, has also been shown to suppress TGF-β induced generation of Treg cells (Ohta *et al*., 2000). In contrast, Chen *et al.* and

Grinberg-Bleyer *et al.* reported that TNF, in concert with IL-2 could selectively activate Treg upregulating their FoxP3 expression (Chen and Oppenheim, 2010; Grinberg-Bleyer *et al*., 2010). In this study, I tested the effect of pro inflammatory cytokines (IL-6, IL-1β and TNF-α) on the FoxP3 expression of Treg *in vitro* in a serum free environment and found no such increase of FoxP3 *in vitro*. TGF-β2, an immunomodulatory cytokine which has been shown to be present at a lower level in uveitis AqH (Fu *et al*., 2004) also had no effect on the FoxP3 expression of Treg *in vitro*.

Culture of Treg cells in the presence of uveitis AqH also did not increase their FoxP3 expression. Interestingly, with the exception of one uveitis AqH sample, all other samples appeared to decrease the FoxP3 expression of Treg cells in vitro in serum free condition. However it has to be noted that this experiment was conducted using 3 test medium controls and 6 individual AqH samples. This preliminary data could be used to perform power analysis to identify the number of samples to be tested to get potentially significant results. The power of a statistical test determines the sensitivity of the test and is defined as the probability that the test will reject a null hypothesis when the null hypothesis is false. Power analysis can be done before (priory) or after (post hoc) the research study. A post hoc analysis of the above experiments with the given sample size gave a power of only 2%. A priori power analysis carried out using *Java applets for power and sample size* revealed that, we need to analyse the effect of at least 27 medium controls and 50 AqH samples on the FoxP3 expression of Treg in order to get a statistical power of 80% (which by convention is an acceptable level of power). In any case, our preliminary data indicate a decrease of FoxP3 expression of Treg in the presence of uveitis AqH, suggesting

that the higher levels of FoxP3 expressed by ocular Treg cells are not due to the effect of AqH.

Glucocorticoid treatment has also been shown to increase the frequency and FoxP3 expression of peripheral blood Treg cells (Karagiannidis *et al*., 2004). In the *in vitro* assay, I found no effect of dexamethasone, a glucocorticoid used in the topical treatment of uveitis (even at concentration more than what is used for topical treatment) on the FoxP3 expression of Treg *in vitro*. However, it has to be noted that even though I used serum free conditions in my *in vitro* cultures, it was impossible to recreate the exact microenvironment as in uveitis AqH *ex vivo*. I also observed that uveitis AqH itself did not change the FoxP3 expression or the frequency of FoxP3+ Treg cells *in vitro*.

Another possible reason for the increased FoxP3 on AqH Treg could be their recent activation status. Upon activation, even the conventional T cells could also transiently upregulate their FoxP3 expression. It has to be noted that the T cells in the AqH cells were all activated. In my *in vitro* activation assay I observed that both Treg and conventional T cells upregulated their FoxP3 expression, with Treg expressing FoxP3 in the range as seen in AqH Treg cells by day 3. However the FoxP3 expressions of activated conventional T cells were much less than activated Treg cells (Fig: 5.6). This is similar to a recent report that suggests that T cells activated *ex vivo* express low levels of FOXP3, at levels a log lower than true Treg (Miyara *et al*., 2009). This points to the fact that the high FoxP3 expressing cells in uveitis AqH are not activated conventional T cells but recently activated Treg cells.

Another interesting finding was the increased frequency of CD39+ conventional T cells in the AqH of acute uveitis patients (Fig: 5.5C&D). Moncrieffe *et al.* recently reported the presence of increased numbers of CD39+ T cells at the inflammatory site in human JIA (Moncrieffe *et al*., 2010). These CD39+ T cells had ATPase activity, but no regulatory capacity. They proposed that the link between CD39 and regulatory activity might be less tightly coupled in inflammation than in healthy T cells (Moncrieffe *et al*., 2010). It would be interesting to analyse the ATPase activity of CD39+ T cells from uveitis AqH and see if they have any regulatory properties.

Despite the presence of Treg with a functional phenotype in the AqH, inflammation is ongoing. One possible explanation for this is that the local pro-inflammatory environment might not be favourable to Treg mediated suppression of inflammatory cells. The ability of non inflammatory AqH to induce apoptosis of inflammatory cells (one of the mechanisms of immune privilege within the eye) has already been reported (D'Orazio *et al*., 1999; Dick *et al*., 1999). Not surprisingly, conventional T cells and Treg co-cultured in the presence of control/ non inflammatory AqH underwent apoptosis as shown in Fig: 5.9. Uveitis AqH on the other hand, has been shown to inhibit the apoptosis of T cells through IL-6 trans signalling (Curnow *et al*., 2004a). In this study, I have shown that CFSE labelled T cells survived well in uveitis AqH. The presence of uveitis AqH did not affect the suppressive function of normal Treg as shown in Fig: 5.10, suggesting that an inflammatory microenvironment may not prevent the accumulated Treg from functioning properly. However it has to be noted that this experiment had been carried out using resting peripheral blood T cells. As we know that the T cells entering the eye have a highly activated phenotype, it is also possible that the activation status of the T cells entering the eye might be too high

for the ocular Treg to control. As activated Treg are more prone to apoptosis (Ohara *et al*., 2002; Taams *et al*., 2001), the possibility that ocular Treg (with an activated phenotype) undergo apoptosis within the uveitic eye can also not be ignored.

Why, despite this, chronic inflammation persists is unclear. Although suppression assays using cells isolated from AqH might be helpful to address this issue, it was not possible due to the difficulty to obtaining enough cells from AqH. Results from Treg depletion and subsequent analysis of the proliferation of AqH T cells using click-iT EdU were inconclusive because of the very low number of cells. Nevertheless, suppressive capacity of Treg on conventional T cells within inflamed tissues has been demonstrated in a variety of settings. In rheumatoid arthritis, Treg from synovial fluid has been shown to be functional in suppressing conventional T cells from peripheral blood (Mottonen *et al*., 2005; van Amelsfort *et al*., 2004). Korn *et al.* recently reported that during experimental autoimmune encephalomyelitis, antigen specific Treg accumulate in CNS and that these Treg could suppress peripheral conventional T cells *in vitro* (Korn *et al*., 2007). However, the same Treg were unable to suppress activated conventional T cells isolated from CNS at the peak of the disease which has been shown to produce higher amounts of IL-6 and TNF than cells from the spleen. Interestingly, the addition of IL-6 and TNF to the *in vitro* co-cultures prevented the suppression of spleen-derived T cells by Treg cells, indicating that the cytokine milieu at the site of inflammation determine the ability of Treg to control autoimmunity at the peak of disease (Korn *et al*., 2007). Pro-inflammatory cytokines such as IL-6, IL-1 and TNF-α which are present in the inflamed tissue can also render conventional T cells less sensitive to Treg-mediated suppression (Korn *et al*., 2007; Pasare and Medzhitov, 2003).

Hence there are several possible reasons for the persisting inflammation in the AqH even in the presence of Treg cells. It is possible that the presence of Treg cells in the eye actually prevent the progression to an erosive inflammation there by maintaining ocular tissue damage to minimum and without them the inflammation might get worse. Another possibility is that the Treg suppress the immune system, thereby preventing the inflammation to naturally resolve. As Treg cells are more prone to apoptosis, it is possible that despite the enrichment, Treg may also be outnumbered to combat the ongoing inflammation. Similar to what was shown in CNS (Korn *et al*., 2007), increased resistance of AqH T cells to Treg mediated suppression could also not be ignored.

A number of ocular antigens have been proposed to be uveitogenic. In animal models, immunization with retinal-S antigen and interphotoreceptor retinoid binding protein (IRBP), among others, has been shown to induce uveitis. In humans, idiopathic non infectious uveitis is considered to be an autoimmune disease, mediated by autoreactive T cells. However, the specific auto-antigen responsible for human AAU is still not known. Uveitis patients have been reported to show lymphoproliferative response to various retinal antigens, especially to retinal S antigen *in vitro* (Tripathi *et al*., 2004; de Smet *et al*., 2001). In addition, lymphocyte responses could be generated against few peptide determinants derived from both the bovine and human S-Ag sequences (de Smet *et al*., 2001). This is in contrast to animal models where cells respond only to the antigen against which they are immunized. In some of the uveitis patients analysed, with each disease recurrence, the immune response shifted to a new epitope (epitope spreading) (Tripathi *et al*., 2004). Also antigenic mimicry between a peptide from retinal S-Ag (PDSAg) and other environmental peptides (eg: cas protein

from bovine mils, surface protein vp4 of rotavirus etc) has been reported (Wildner and Diedrichs-Mohring, 2003). It has been postulated that autoreactive T cells (including Treg) are primed extraocularly through environmental peptides which could then transgress the blood ocular barrier and mediate inflammation following recognition of their cognate antigen (Wildner and Diedrichs-Mohring, 2004; Wildner and Diedrichs-Mohring, 2003). Treg, once activated, can regulate immune response in an antigen specific or antigen non specific bystander fashion. The antigen specificity of T cells in the eye has not been analysed in the present study. It would be worthwhile to find out the antigen specificity of Treg and Tconv cells in the AqH of AAU patients for future therapeutic applications.

Whether spontaneous idiopathic uveitis is an autoimmune disease or whether it is caused by non specific systemic immune response is still a matter of debate. It has been demonstrated that activated T cells of any specificity can pass through the blood ocular barrier (Xu *et al*., 2003; Thurau *et al*., 2004). A non-specific systemic inflammatory response, can lead to systemic release of pro inflammatory cytokines such as IL-1β, IL-6 and TNF-α, which in turn can result in generalised activation of endothelial barriers, and may affect the blood-ocular barrier as well. This in turn would lead to recruitment of non specific memory T cells into the eye. However these cells would not be reactivated within the eye, but could cause bystander damage to surrounding tissues. Hence it is interesting to speculate as to whether a rapidly resolving uveitis represents non-specific breakdown in the blood-ocular barrier as a result of systemic inflammation but without the extra-ocular priming of ocular antigen specific T cells or their reactivation within the eye, whereas persistent (and possibly recurrent) disease represents a true autoimmune phenomenon mediated by ocular or

extra ocular priming. In this context, the function of ocular Treg will be of more importance in the recurrent disease. It is intriguing that almost all the patients in this study had recurrent disease. It need to be analysed whether the increased recruitment of Treg to the AqH is a feature of recurrent uveitis and is required for the resolution of the disease.

6 FINAL DISCUSSION

6.1 Introduction

During the course of this thesis, I have observed an increased frequency of Treg in the peripheral blood of non infectious AAU patients. In contrast these Treg were defective in their suppressive capacity to inhibit the proliferation of Tconv cells *in vitro*. Treg expressed a more prominent defect in chronic anterior uveitis patients as compared to acute anterior uveitis patients. I have also shown that CD4+ Tconv cells and Treg cells accumulate in the AqH during non infectious uveitis and there was an increased frequency of Treg in the AqH of the patients compared to their peripheral blood.

In the previous chapters, I have identified and analysed the phenotype and function of Treg from peripheral blood as well as AqH of uveitis patients. In this final discussion I wish to discuss the broader implications of Treg cells in uveitis, first with regard to the concept of central tolerance and peripheral tolerance and second with regard to the activation and migration of inflammatory as well as regulatory cells to the eye and the role of Treg in local immunosuppression at the site of inflammation.

6.2 Central tolerance

Central T cell tolerance is the mechanisms by which newly developing T cells in the thymus are made non reactive to self antigens. Thymocytes with high affinity to self antigens, which are presented by endogenous MHC molecules and activated thymocytes through interactions with T-cell receptors (TCRs), are eliminated via

apoptosis. Surviving thymocytes (with lower affinity to self antigens) undergo an additional process of positive selection and emerge from the thymus as mature T cells.

Thymic expression of peripheral tissue antigens is required for central tolerance which has been reported in humans (Takase *et al*., 2005). Several retinal Ags, including IRBP, have been shown to be expressed in the thymus and are under the control of the AIRE transcription factor (Anderson *et al*., 2002). Thymic expression of IRBP in mice eliminated many T cells with high affinity to IRBP and reduced the autoreactive uveitogenic T cell repertoire (Avichezer *et al*., 2003). Using highly sensitive detection methods and thymic transplantation, Avichezer *et al.* demonstrated that EAU-susceptible WT mice expressed IRBP in the thymus and displayed functionally significant levels of tolerance to this antigen (Avichezer *et al*., 2003). Mice lacking the transcriptional regulator AIRE had a limited autoreactive repertoire to eye antigens such as IRBP and arrestin and developed antibody and T cell responses directed at IRBP that resulted in uveitis (Anderson *et al*., 2002; DeVoss *et al*., 2006). Egwuagu *et al.* showed that the amount of IRBP expressed in the thymus correlated inversely with susceptibility to EAU as IRBP was expressed only by IRBP-resistant, but not by susceptible, mouse strains (Egwuagu *et al*., 1997). Thymic expression of retinal antigens among individual humans and mouse strains is variable (Takase *et al*., 2005; Egwuagu *et al*., 1997).

The thymus also generates natural T regulatory cells (nTreg), which arise from thymocytes whose TCRs have an affinity that is relatively high, but not quite high enough to trigger deletion. nTreg also has been shown to control the threshold of susceptibility to EAU, and their depletion by use of monoclonal antibodies

exaggerated the disease (Avichezer *et al*., 2003; Grajewski *et al*., 2006). Immunocompetent WT mice, deprived of thymus-derived regulatory cells, developed enhanced EAU scores. It has been shown that thymic expression of IRBP negatively selects effector T cells and positively selects natural Treg (Avichezer *et al*., 2003; Grajewski *et al*., 2006). These findings support the notion that an individual whose T cell repertoire contains retinal antigen–specific T cells with higher affinity and/or higher frequency due to the reduced expression of retinal antigens and /or reduced selection of antigen specific nTreg in the thymus may have a greater likelihood of developing uveitis (Takase *et al*., 2005; Sugita *et al*., 2007; Egwuagu *et al*., 1997).

Haas *et al.* has reported a decrease in the naive or recent thymic emigrant Treg in multiple sclerosis patients (Haas *et al*., 2007; Venken *et al*., 2008a). They defined naive $CD4^+CD25^+FoxP3^+$ T cells that co express CD31 (PECAM-1) as recent thymic emigrants (RTEs), and found reduced numbers of RTEs in the blood of patients with MS. This was compensated for by increased amounts of memory Treg, resulting in a stable cell count of the total Treg population (Haas *et al*., 2007). Interestingly, increased frequency of Treg (with a memory phenotype) was shown in the peripheral blood of non infectious AAU patients in my study. It is not clear whether this represents an increase in the thymic output of nTreg cells. The total peripheral Treg count in the chronic patients on the other hand remained similar to that in healthy controls. Even though there was no difference in the frequency of naïve (CD45RO-) Treg cells from the peripheral blood of both chronic and acute patients, it would be interesting to identify and analyse the recent thymic emigrant Treg in these patients (based on CD31 expression) and see if similar defects (as seen in MS) could be identified in these patients.

6.3 Peripheral tolerance

Negative selection in the thymus does not eliminate all autoreactive T cells. Normally, autoreactive thymic emigrants that have escaped negative selection are subjected to peripheral tolerance which induces T cells to become non-responsive (tolerant) to their specific antigen when they encounter that antigen under non inflammatory conditions. Peripheral tolerance is designed both to control responses to foreign antigens encountered in the periphery and to maintain tolerance to self antigens.

Treg can also be induced in the periphery (iTreg) by the activation of naive T cells under appropriate conditions (Wraith, 2004). Using IRBP knockout mice, Grajewski *et al.* demonstrated that although generation of IRBP specific Treg require endogenous expression of IRBP, Treg of other specificities that are activated by the mycobacterial component of CFA used for vaccination, may also participate in controlling EAU (Grajewski *et al*., 2006).

The peripheral pool of Treg includes both nTreg and iTreg. It is not clear whether the increased frequency of peripheral blood Treg in AAU patient in my study is due to an increased thymic output of Treg (nTreg) in these patients or an increased production of induced Treg (iTreg) in the periphery. Methylation status of the FoxP3 locus in Treg has been shown as a marker to differentiate between nTreg and iTreg (Baron *et al*., 2007; Janson *et al*., 2008; Lal *et al*., 2009). It has been demonstrated that the FoxP3 proximal promoter region was fully demethylated in nTreg whereas it was fully or partially methylated in recently activated Tconv cells and iTreg (Baron *et al*., 2007; Floess *et al*., 2007; Janson *et al*., 2008). Three conserved non-coding DNA sequence (CNS 1-3) elements at the FoxP3 locus have been identified recently in

mouse, which determine the size, composition and stability of Treg population (Zheng *et al*., 2010). CNS3 controls the *de novo* FoxP3 expression by thymic and peripheral Treg. CNS1 on the other hand plays an important role in the peripheral induction of Foxp3 and iTreg generation whereas CNS2 controls the heritable maintenance of Foxp3 expression in dividing mature Treg cells (Zheng *et al*., 2010). Hence a detailed methylation analysis and mapping of conserved sequences in the Foxp3 locus would be required to find out whether the peripheral blood Treg in AAU patients are thymic nTreg or in fact induced in the periphery.

Interestingly, it was recently reported in type 1 diabetes that the frequency of IFN-γ+ Treg was significantly increased in patients with type 1 diabetes compared to healthy controls. These IFN-γ+ Treg were CD4+CD25highCD127lowFOXP3+ and were predominately methylated at the TSDR, characteristics of iTreg (McClymont *et al*., 2011). Similar results were observed in relapsing remitting multiple sclerosis patients where an increased frequency of IFN-γ producing Foxp3+ Treg was found in untreated patients (Dominguez-Villar *et al*., 2011). It would be interesting to analyse the IFN-γ production by Treg from AAU patients and see if similar results could be obtained which may account for the defective function of Treg observed in these patients.

The hormone Leptin, which can also act as a pro-inflammatory cytokine, plays a potent role in the control of autoimmune diseases. It has been shown by De Rosa et al that leptin had a negative impact on Treg proliferation and survival. They showed that leptin could bind to receptors on Treg and that in vitro neutralization with leptin monoclonal antibody combined with TCR signalling reversed anergy and

hyporesponsiveness of Treg. Increased leptin is associated with ulcerative colitis (Tuzun et al., 2004) and the onset of multiple sclerosis (Matarese et al., 2005). Interestingly, increased serum levels of leptins were also reported in patients with VKH (Liu et al., 2008a) and Behcet's disease (Yalcindag et al., 2007). Moreover, increased expression of leptin has been observed in the retina, choroid, sclera and episclera of guinea pigs during experimental uveitis (Kukner et al., 2006). The levels of serum leptin in uveitis patients have not been analysed. Given the increased levels of serum leptins in other ocular diseases, it is interesting to speculate that the increased levels of serum leptins may account for the defective suppressive function of Treg in uveitis patients. As the normal suppression assays in this project were carried out in serum containing medium, it is possible that the overall lower suppressive function in our suppression assays may be due to the effect of leptins in the serum.

Treg have been shown to control various manifestations of autoimmunity (Sakaguchi *et al*., 2006b). It could be assumed that in individuals who develop uveitis, the threshold of susceptibility set by the peripheral Treg has been passed. Reduced Treg function has been shown to be associated with active uveitis in VKH patients (Chen *et al*., 2008). Other autoimmune diseases where a defective function of Treg has been reported include rheumatoid arthritis, multiple sclerosis, SLE and type 1 diabetes (Ehrenstein *et al*., 2004; Viglietta *et al*., 2004; Valencia *et al*., 2007; Lindley *et al*., 2005). However, identifying functional defects in Treg cells is made difficult both by the multiple mechanisms used by Treg cells to suppress inflammation (See chapter 1) and by the way in which they are isolated and the suppression is measured. Using stringent methods of isolation and selection of Treg cells, I have observed in my study

that Treg from the peripheral blood of both acute and chronic anterior uveitis patients express diminished suppressive function *in vitro* in suppressing the proliferation of autologous Tconv cells. Interestingly, Treg from chronic patients expressed a more prominent defect than acute patients, suggesting that there may be a progressive diminishing in the functional capacity of Treg as patients progress from acute to chronic stage. However, I was not able to confirm this, as it would require longitudinal study of patients as they progress from acute to chronic stage of the disease. It is particularly interesting in the context of the heterogeneity observed among AAU patients in my study, where some patients showed a clearer defect in their Treg suppressive capacity than others at almost all Tconv: Treg ratios. Such a longitudinal study would also help us to determine whether the AAU patients with a more prominent defect are more prone to develop a chronic disease.

It has to be noted that the *in vitro* assays of human Treg function may fail to mimic the *in vivo* milieu and that this is only one of the pathways in which Treg exert their suppressive function *in vivo*. Even though the suppressive function of Treg on proliferation was diminished *in vitro*, it is possible that the other pathways of Treg mediated suppression of inflammation may be functionally active *in vivo*.

6.4 Activation of antigen specific T cells in the periphery

The triggers which are responsible for activating antigen specific T cells to retinal antigens in the periphery, so that they could escape the control of Treg, are largely unknown in humans. Although Tissue antigen specific T cells become tolerant when

they encounter their specific antigen in healthy tissues, it is not the case for retinal antigens as they are sequestered behind the blood-ocular barrier and are relatively inaccessible. Thus, it has been suggested that circulating retinal antigen–specific T cells are likely to be ignorant rather than tolerant of their cognate antigen. However, they can be activated by a chance encounter with a microbial component that is immunologically similar in structure to their cognate retinal antigen (antigenic mimicry) (Wildner and Diedrichs-Mohring, 2004; Wildner and Diedrichs-Mohring, 2003).

Wildner *et al.* described antigenic mimicry between a peptide from retinal S-Ag and 'Cas' peptide from αs2casein, a major component of bovine milk, and a peptide from surface protein vp4 of rotavirus ('Rota'), a common gastrointestinal pathogen. Retinal S-antigen has been shown to be immunogenic in man and uveitogenic in rats. Peptides Rota and Cas and even the complete αs2casein protein were shown to be uveitogenic in rats by subcutaneous immunization with complete Freund's adjuvant (Wildner and Diedrichs-Mohring, 2003). Uveitis patients expressed increased humoral and cellular immune responses to S-antigen as well as casein/-peptide and rotavirus peptide both of which expressed antigenic mimicry with the retinal S-antigen (Wildner and Diedrichs-Mohring, 2003).

In idiopathic AAU, it is generally suggested that an antigen specific immune response to an ocular antigen arises through molecular mimicry to a pathogenic antigen. Autoreactive T cells may be generated via molecular mimicry or epitope/antigen spreading in which priming to self-antigens released during tissue damage may occur. This happens mostly in the context of persistent immune response to the original

antigen such as during chronic infection. One of the speculations is that autoreactive T cells to an ocular antigen can be generated by intra ocular inflammation thereby inducing an autoimmune uveitis which would continue after clearing of the pathogen.

Cross reactivity with environmental antigens has been implicated in the physiological induction of autoantigen specific Treg cells (von Herrath and Harrison, 2003). Silver *et al.* showed that hydrodynamic injection of plasmid expressing the retinal antigen IRBP, induced tolerance in mice that acted at least in part through the induction of IRBP specific Treg cells (Silver *et al*., 2007).

The antigen specificities of Treg from uveitis patients were not analysed in this study. De Smet *et al* showed that in patients with certain forms of uveitis, there were specific immunodominant determinants to human S-Ag. However, in individual patients, response was not limited to these determinants but spread over many determinants (epitope/determinant spreading) (de Smet *et al*., 2001). Deeg *et al.* examined long-term immune response of uveitic horses to various epitopes of S-Ag and IRBP for a period of 22 months. They found inter and intramolecular epitope spreading in equine recurrent uveitis, a model of spontaneous uveitis (Deeg *et al*., 2006). Here the authors postulated that the remission and relapse of uveitic episodes could be explained by determinant spreading where the reaction to a new epitope could lead to a new uveitic episode. As Treg cells take control, the inflammation ceases. The next episode of uveitis would then be generated by a shift of response to another epitope of the same autoantigen (intramolecular spreading) or another autoantigen (intermolecular spreading) (Deeg *et al*., 2006).

Treg can act in an antigen specific or non specific manner. Once activated through their cognate TCR, the Treg can also act to suppress T cells of other specificities (bystander suppression), thereby broadening their effectiveness especially where multiple antigens in the same tissue may be targeted (Sakaguchi *et al*., 2006a). Nevertheless, it would be very interesting to analyse the antigen specificity of Treg cells from uveitis patients, which would greatly help in designing future therapeutic targets.

6.5 Migration of T cells to the inflamed tissue

The blood ocular barrier was once thought to be a tight junction which does not allow the passage of any cells through it. However, using OVA-specific GFP+ T cells, Thurau *et al*. showed that activated T cells of any antigen specificity can migrate into ocular tissue within 30 min after injection into the tail vein (Thurau *et al*., 2004). Xu *et al.* demonstrated using intravital scanning laser microscopy and retinal whole mounts that adoptive transfer of non-Ag-specific activated T cells (concanavalin-A stimulated) into normal B10.RIII mouse could cause a transient local breakdown in the blood ocular barrier with passage of activated T cells across the endothelium. This mechanism was associated with the up regulation of adhesion molecule ICAM-1 on the endothelium and its interaction with LFA-1 on the T cell (Xu *et al*., 2003). This was supported by the observation by Prendergast *et al.* that activated T cells of any specificity, crossed the blood-ocular barrier within 24h and that this preceded any visible signs of inflammation (Prendergast *et al*., 1998).

In an adoptive transfer model of uveitis, Prendergast *et al.* showed that S antigen specific T cells enter the retina within hours following intravenous injection

(Prendergast *et al*., 1998). These cells exhibited a biphasic accumulation in the retina with the first peak at 24 hours post injection. The number of cells decreased until 72 hours and then again peaked at 96 and 120 hours concurrent with the onset of destructive intraocular inflammation (Prendergast *et al*., 1998). They also reported that anterior segment inflammation preceded retinal inflammation and postulated that this could be due to a delay in antigen presentation at the retina (Prendergast *et al*., 1998). Various factors such as infection, trauma and immunological response can cause anterior segment inflammation. By inducing anterior uveitis in rabbits, Bolliger *et al.* observed an increase in the vascular permeability of anterior uveal tract, causing the exudation of protein and migration of leukocytes into the anterior chamber (Bolliger *et al*., 1980).

The secretion of multiple cytokines, chemokines and upregulation of adhesion molecules by the activated T cells in the eye, could then promote the invasion of other T cells (Treg, Th17 etc) and other inflammatory cells (Takase *et al*., 2006a; Takase *et al*., 2006b; Hill *et al*., 2005).

Recruitment of regulatory T cells along with the inflammatory T cells has been reported at various inflammatory sites (Korn *et al*., 2007; Lange *et al*., 2011; Cao *et al*., 2004; Cao *et al*., 2003). The specific localization of human Treg is required for their ability to control ongoing inflammatory conditions (Siegmund *et al*., 2005; Wei *et al*., 2006; Cao *et al*., 2003; Cao *et al*., 2008). Similar to other leukocytes, the migration and infiltration of Treg into inflamed tissues is mainly governed by the expression of specific chemokine receptors (CCRs). Lee *et al.* studied the Treg homing program in primary and secondary lymphoid tissues, a process that is

important for migration of Treg to target tissue sites (Lee *et al*., 2007). They showed that Treg generated in the thymus emigrated primarily to secondary lymphoid tissues where they encountered the antigen and underwent a trafficking receptor switch. This involved down-regulation of CCR7 and CXCR4 and up-regulation of a number of memory/effector type homing receptors and enabled them to migrate to non lymphoid and inflamed tissues (Lee *et al*., 2007). This was supported by the data from Sather *et al.* that antigen-specific Treg cells up-regulated CCR4, CD103, and other skin-homing receptors when stimulated by their cognate antigen within subcutaneous lymph nodes under pro-inflammatory conditions (Sather *et al*., 2007).

Human Treg have been shown to specifically express chemokine receptors CCR4 and CCR8 (Iellem *et al*., 2001). CCR5, another inflammatory chemokine receptor, has also been shown to be to be expressed on Treg which preferentially infiltrate extra-lymphoid sites and sites of inflammation (Yurchenko *et al*., 2006). Several groups have analysed the AqH from uveitis patients and identified elevated levels of chemokines, including ligands for CCR4 and CCR5 such as CCL2 (MCP-1), CCL3 (MIP1α), CCL4 (MIP1β) and CCL5 (RANTES) (Curnow *et al*., 2005; Wallace *et al*., 2004; Sijssens *et al*., 2007; Verma *et al*., 1997). Ocular infiltrating T cells have also been shown to have the capacity to produce chemokines such as IL-8, MIP-1 and RANTES (Takase *et al*., 2006b). In this context, one could speculate that the elevated levels of the chemokines stated above in uveitis AqH could lead to enhanced migration of Treg that express CCR4 and CCR5 resulting in their accumulation in the eye. Similar results were shown in rheumatoid arthritis where Foxp3+ Treg expressing high levels of CCR4, CCR5, and CXCR4 has been shown to accumulate in synovial fluid in rheumatoid arthritis patients (Jiao *et al*., 2007). Once the activated

Treg reach the inflamed site (in this case, anterior chamber), they could control inflammation in an Ag specific or bystander fashion.

CXCR4 is a chemokine receptor that has shown to be required for the recirculation of naïve lymphocytes to lymphoid tissue as well as to enhance the active retention of highly differentiated primed T cells at sites of chronic inflammation. Curnow *et al.* reported that topical glucocorticoid therapy could directly induce CXCR4 upregulation on primed T lymphocytes in the AqH of uveitis patients (Curnow *et al*., 2004b). In my study, I have shown that the ocular infiltrating T cells also include primed Treg cells. Intriguingly, elevated frequency of Treg was observed in the AqH from patients undergoing topical glucocorticoid treatment as compared to those from untreated patients. Hence it could be assumed that glucocorticoid induced up regulation of CXCR4 on ocular Treg and there subsequent retention in the eye could be a possible pathway in which glucocorticoid therapy resolves ocular inflammation.

Schneider-Hohendorf *et al.* recently showed that human and murine Treg cells have increased migratory capacity as compared to conventional T cells both *in vitro* and *in vivo* (Schneider-Hohendorf *et al*., 2010). In contrast, Treg of patients with relapsing remitting multiple sclerosis (RR-MS) exhibited significantly impaired migratory capabilities under non-inflammatory conditions (Schneider-Hohendorf *et al*., 2010). They postulated that the presumed 'regulatory deficiency/defect' of Treg in MS patients could at least be partially due to impairment in Treg motility. Similar results were also reported in SLE patients where Treg from patients have been shown to have decreased migratory capacity to CCR4 ligands (Lee *et al*., 2008).

My study showed an increased frequency of Treg in the AqH of acute anterior uveitis patients. However, the role of ocular Treg cells in human chronic uveitis is not known. The AqH from chronic anterior uveitis was not analysed in this study. Whether there is an accumulation of Treg with a functional phenotype in AqH of chronic patients, is not known. However, it is possible that the more prominent defective function of Treg observed in chronic uveitis patients (with relapsing episodes of this disease), may be partially due to similar impairment in Treg migratory capacity and may be the reason for the persistent inflammation observed in these patients. Further analysis of Treg (peripheral and ocular) in patients with acute and chronic uveitis including the expression of chemokine receptor and chemokine receptor ligand-mediated chemotaxis capacity is required.

6.6 AqH local immunosuppression and ocular Treg

In their model of adoptively transferred EAU using GFP+ autoreactive T cells, Thurau *et al* showed that uveitis was induced only by those T cells that encountered their specific antigen in the eye and were reactivated (Thurau *et al*., 2004). The immune privilege of the eye usually prevents destructive intraocular immune reactions. The AqH itself has been shown to induce Fas mediated cell death on infiltrating lymphocytes (Griffith *et al*., 1995). Immunosuppressive microenvironment within the AqH has been well documented. Various AqH factors such as TGF-β, α-MSH, VIP and somatostatin contribute to this immunosuppressive microenvironment (Cousins *et al*., 1991; Taylor *et al*., 1997; Taylor *et al*., 1992; Taylor, 2007; Taylor *et al*., 1994b; Taylor and Yee, 2003). My own data, where T cells were cultured in the presence of AqH, showed that non inflammatory AqH prevented the proliferation of T cells by inducing cell death *in vitro* (chapter 5). Fas mediated apoptosis in AqH has

been shown to be functional even during idiopathic AAU (Dick *et al*., 1999). However under inflammatory conditions, AqH T cells were protected from survival factor deprivation induced apoptosis via IL-6/soluble IL-6 receptor trans signalling (Curnow *et al*., 2004a).

Accumulation of Treg in the AqH was reported in AAU patients in this thesis. One important question arising from this study is whether the increased numbers of Treg present in the AqH during AAU are due to sequestration of polyclonal Treg from the blood, or in situ activation and proliferation of Treg specifically within the eye. Using passive transfer of preformed effector cells to induce EAE (so that Treg are not primed by a peripheral Ag-adjuvant depot), O'Connor *et al.* suggested that Treg entered the CNS in response to inflammation and initiated a dramatic proliferative burst (O'Connor *et al*., 2007). In this study, they found an increased rate of proliferation in FoxP3+ Treg specifically within the inflamed CNS which remained high during the resolution phase of the disease (O'Connor *et al*., 2007). Although Treg are usually anergic *in vitro*, they can proliferate *in vivo* while maintaining their suppressive function (Walker *et al*., 2003a).

Various factors in the non inflammatory AqH including TGF-β and α-MSH have been shown to induce and promote Treg development and proliferation (Shevach *et al*., 2008; Taylor *et al*., 1997; Nishida and Taylor, 1999). However a pro-inflammatory milieu could prevent the induction of FoxP3+ Treg and enhance the induction of Th17 cells. Support for this hypothesis comes from data showing that whereas TGF-β can convert FoxP3- T cells into FoxP3+ Treg, addition of IL-6, an acute phase protein induced during inflammation, suppresses the TGF-β induced generation of Treg and

results in the induction of pathogenic Th17 cells (Veldhoen *et al*., 2006). Thus, it is more likely that the proinflammatory milieu present in the uveitis AqH with increased levels of TNF-α, IL-6 and IFN-γ and decreased levels of TGF-β2 (Curnow *et al*., 2005; de Boer *et al*., 1994; Hill *et al*., 2005; Yoshimura *et al*., 2009) could be more conducive for the induction of Th17 cells (Yoshimura *et al*., 2009) and is less likely to induce the production of Treg.

Accumulation of Treg with similar phenotypes to those seen in inflammatory AqH have been reported in other inflamed tissues (Cao *et al*., 2004; Feger *et al*., 2007; Grinberg-Bleyer *et al*., 2010) and in most cases the Treg from the target sites seem to be functional (de Kleer *et al*., 2004). Functionally active Treg have been shown to accumulate in rheumatoid synovium (van Amelsfort *et al*., 2004; Cao *et al*., 2003; de Kleer *et al*., 2004). In condylomata acuminata, a disease caused by infection with HPV virus, accumulation of functional Treg in large warts have been associated with an immune evasion mechanism (Cao *et al*., 2008). Zhang *et al.* showed that Treg accumulated in islet allograft and increased their suppressive function and that this education was required for optimal control of inflammation (Zhang *et al*., 2009).

The level of FoxP3 expression in Treg has been shown to reflect their potential to suppress T cell activation and prevent allograft rejection in transplantation (Chauhan *et al*., 2009b). Interestingly, the AqH Treg in AAU patients expressed significantly higher levels of FoxP3 compared to their peripheral blood counterparts. Hence it could be postulated that the AqH Treg could be functionally active and help control immunopathology and tissue destruction within the eye.

Korn *et al.* recently monitored Treg *in vivo* in an EAE model using a FoxP3/GFP.KI mice and showed that myelin specific Treg accumulated in CNS during the peak of the disease (Korn *et al*., 2007). They observed that even though CNS derived Treg at the peak of the disease could suppress the proliferation of spleen derived Tconv cells *in vitro*, they could not suppress the proliferation of CNS derived Tconv cells *in vitro* or *in vivo*. This was not due to the intrinsic defect of Treg but rather due to the increased resistance of CNS derived Tconv cells to Treg mediated suppression at the peak of the disease (Korn *et al*., 2007). This was largely attributed to the pro inflammatory cytokine milieu (especially IL-6 and TNF) present in the inflamed CNS. Reports show that even functionally competent Treg fail to control inflammation, if inflammatory mediators, such as IL-7, TNF or co-stimulatory molecules, are abundant (as it is the case in the inflamed synovium, inflamed CNS etc) (Korn *et al*., 2007; van Amelsfort *et al*., 2007)

Extrapolating this data to uveitis AqH, a similar inflammatory milieu is present in the uveitis AqH (Curnow *et al*., 2005). It has been shown that IL-6 and TNF-α can inhibit Treg function and render Tconv cells resistant to suppression and enable the initiation of an immune response in the presence of Treg (Ohta *et al*., 2000; Valencia *et al*., 2006). Thus, even if the AqH Treg in AAU patients are functionally active, it is possible that they could not exert their function on activated Tconv cells in the pro-inflammatory cytokine rich microenvironment. I have, in this thesis, shown that uveitis AqH did not affect the suppressive capacity of normal Treg (from healthy control) *in vitro*. However, it has to be noted that the activation status and functional capacities of Treg as well as Tconv cells within an inflamed eye may be different from that in an *in vitro* assay and hence the possibility that uveitis AqH might prevent

ocular Treg function or render the ocular Tconv cells resistant to suppression *in vivo* could not be ignored.

It has also been shown that Treg can only function in the resolution of the acute phase and could not control the chronic phase of the disease (Frey *et al*., 2010). In Lewis rats immunised with R16 (immunodominant peptide of IRBP), accumulation of Treg within the eye correlated with the resolution of first acute attack in both monophasic and recurrent EAU (Ke *et al*., 2008). The eye derived Treg cells from monophasic EAU (m-EAU) rats were more potent than those from recurrent EAU (r-EAU) and the transfer of ocular Treg from m-EAU converted recurrent form of EAU to monophasic form (Ke *et al*., 2008).

In the context of this thesis, it is interesting to speculate whether ocular Treg in AAU mediate the transition from acute to chronic phase of the disease, where functional ocular Treg mediate the rapidly resolving acute uveitis whilst persistent uveitis (usually recurrent) is caused by a defective function of the ocular Treg. However the main obstacle to analyse this is the absence of specific assays to isolate and analyse the function of the very small number of AqH Treg. One would also require longitudinal AqH and blood samples from a cohort of patients (ideally 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. But regular exposure to such an invasive technique would not be justified in humans.

6.7 Treg and Th17 in uveitis

Involvement of Th17 cells in human uveitis and scleritis has also been reported (Amadi-Obi *et al*., 2007). In the study by Amadi-Obi *et al.*, it was shown that in EAU model of uveitis, expression of IL-17 was correlated with the onset of EAU (Amadi-Obi *et al*., 2007). They demonstrated that Th17 cells are present in the peripheral blood of healthy controls and their numbers increased in active uveitis and scleritis patients and in EAU. T_H17 cells were abundant in the retina, especially at the peak of the disease which then subsequently declined with resolution of the disease. This study also proposed the induction of TNF-α in retinal cells by IL-17 as a mechanism by which these cells may contribute to uveitis pathogenesis (Amadi-Obi *et al*., 2007). In a study conducted by Yoshimura *et al*, IL-6 was found to be increased in the AqH of chronic uveitis patients. Using an animal model of EAU, they showed that IL-6 is responsible for ocular inflammation at least partially due to IL-6 dependant TH17 differentiation (Yoshimura *et al*., 2009).

Similar to that was shown in this thesis, increased frequency of Treg has been reported in EAU induced by IRBP in B10RIII mice (Sun *et al*., 2010a). These Treg cells were able to inhibit proliferation and IFN-γ production by CD4+CD25− target cells, and were associated with the regression phase of EAU (Sun *et al*., 2010a). Interestingly, these Treg could not inhibit IL-17 production by pathogenic T cells. In a mouse model for autoimmune dry eye disease, Chauhan *et al.* reported that CD4+CD25+FoxP3+ Treg were inefficient in suppressing pathogenic T cells which was attributed to the resistance of these T cells, especially Th17 cells to Treg mediated suppression (Chauhan *et al*., 2009a). *In vivo* blockade of IL-17 significantly

reduced the severity and progression of the disease as well as reversed Th17 frequency and Treg function in these mice (Chauhan *et al*., 2009a).

However, the role of Th17 cells in human acute or chronic uveitis was not analysed in this thesis. Increasing evidences show that chronically inflamed tissues are infiltrated with Th17 cells (Pene *et al*., 2008). Using an animal model of rheumatoid arthritis, Hirota *et al.* showed a preferential recruitment of CCR6-expressing Th17 cells to inflamed joints in rheumatoid arthritis (Hirota *et al*., 2007). The phenotype and function of Th17 cells in the peripheral blood and AqH of uveitis patients have to be analysed in order to understand the pathophysiology of human idiopathic uveitis. It would also be interesting to analyse whether the diminished suppressive capacity of Treg in uveitis patients was mainly confined to the Th17 compartment or not.

Plasticity of Treg cells is a newly emerging area of Treg cell biology that will need to be incorporated into these studies (Yang *et al*., 2008a). Under inflammatory conditions, especially in the absence of TGF-β and in the presence of IL-6, Treg have been shown to induce Tconv cells or could be self induced to become Th17 cells (Xu *et al*., 2007). Hans *et al.* showed that highly purified human memory Treg when stimulated with allergenic PBMC in the presence of exogenous r-IL2/ rIL-15, gave rise to a subset of IL-17–producing cells (Koenen *et al*., 2008). Similarly Voo *et al.* reported that human peripheral blood and lymphoid tissue contain a significant number of Treg cells that co-expressed FoxP3 and RORγt transcription factors and had the capacity to produce IL-17 upon activation (Voo *et al*., 2009). Hence it would be pertinent to analyse the peripheral blood and AqH Treg in uveitis patients for their capacity to be converted to Th17 cells. Further study of the relationship between Treg

cells and Th17 cells in these sites in human uveitis is important for possible Treg cell-mediated therapeutic applications.

As mentioned earlier, IFN-γ producing FoxP3+ Treg have been reported in autoimmune diseases (Dominguez-Villar *et al*., 2011; McClymont *et al*., 2011). In a recent study, Dominguez-Villar *et al*, showed that Treg cells cultured in the presence of IL-12, acquired the ability to produce IFN-γ and showed reduced suppressive capacity (Dominguez-Villar *et al*., 2011). Given the fact that IL-12 has been shown to be present in the AqH and vitreous of uveitis patients (el Shabrawi *et al*., 1998; Curnow *et al*., 2005), it would be very interesting to analyse whether ocular Treg have acquired the ability to produce IFN-γ and convert to a Th1 phenotype.

6.8 Treg therapy

Reports showing defective function of Treg in various autoimmune and inflammatory diseases (Ehrenstein *et al*., 2004; Korn *et al*., 2007; Venken *et al*., 2008b; Bonelli *et al*., 2008a) has generated the path for an attractive new therapy called regulatory T cell therapy for auto-immune diseases and transplantation. Here *ex vivo* activated Treg are re introduced into the system which could then potentially control inflammation in an Ag-specific or bystander fashion. However there are various issues challenging the successful use of Treg therapy in uveitis. Our knowledge of the Treg cell deficiency in uveitis as well as other autoimmune diseases is limited to analysis of cells isolated from peripheral blood and is mainly assessed by *in vitro* assays, which might or might not pertain to *in vivo* defects in suppression.

Several studies have shown that, Treg require antigenic stimulus to initiate suppressive activity, but the effector phase is mediated by an antigen non-specific mechanism. Polyclonally activated Treg have been shown to be efficient at blocking unwanted immunity in mouse models of graft-versus-host disease (GVHD) (Hanash and Levy, 2005). *In vitro* data show that suppressive functions of Treg require activation through their TCR (Tang *et al*., 2004), indicating that their *in vivo* activation and function is controlled by their antigen specificity. On the basis of this assumption, several groups have attempted to expand autoantigen- and alloantigen-specific Treg cells from the natural Treg cell repertoire. Tarbell *et al* showed that islet-antigen-specific Treg efficiently prevented diabetes caused by diabetogenic T cells in NOD mice (Tarbell *et al*., 2004). However, the antigenic determinant of human uveitis is not yet clear. This along with the heterogeneity among patient group will make it difficult to make an Ag specific Treg line for uveitis therapy.

It has also to be analysed whether the ocular conventional T cells are resistant to Treg mediated suppression under inflammatory conditions as in CNS during EAE. It has to be tested if polyclonal Treg, pre-activated *in vitro*, exert bystander suppressive effects when injected directly in the eye. In a graft-vs.-host disease model, in which IL-17 is the predominant cytokine, Treg have been shown to block the initial activation and expansion of T cells following recognition of the systemic antigen (Lohr *et al*., 2006). However, Treg were unable to ameliorate the disease when given late in the course indicating that the principal effect of these cells is to inhibit the initial T cell proliferation and generation of pathogenic effector cells (Lohr *et al*., 2006). Hence it is not yet clear whether Treg therapy may be successful in ameliorating established

immunological diseases. Thus Treg therapy in uveitis has a lot of hurdles to pass through before reaching successful a clinical trial.

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7 APPENDIX

Patient information sheet

See pages: I to V

Patient consent form

See page: VI

Study letter to GP

See page: VII

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

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 Academic Unit of Ophthalmology Birmingham & Midland Eye Centre Sandwell & West Birmingham NHS Trust Dudley Rd Birmingham B18 7QU	Miss Saaeha Rauz Academic Unit of Ophthalmology Birmingham & Midland Eye Centre Sandwell & West Birmingham NHS Trust Dudley Rd Birmingham 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

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
Centre Number: BMEC B18 7QH
Study Number: UKCRN 4654 Tel: 0121 554 3801
Patient Identification Number for this trial:
www.cityhospital.org.uk

CONSENT FORM: Immune mechanisms in the ocular microenvironment

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

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
Birmingham
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